STRUCTURAL AND MECHANISTIC ROLES OF NOVEL CHEMICAL LIGANDS ON QUORUM SENSING TRANSCRIPTION REGULATOR SDIA IN ENTEROHEMORRHAGIC ESCHERICHIA COLI

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DEDICATION

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even when I wanted to, my brother Khoa for motivating me to chase after my dream, and my brother Binh for supporting me even though he has no clue what I am doing.

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Microorganisms and their eukaryotic hosts have co-evolved for millions of years. How bacteria sense and adapt to different environments is still unclear. Most Gram-negative bacteria use the LuxR family of transcription factors to regulate gene expression to coordinate population behavior by sensing endogenously produced chemical signaling molecules, acyl-homoserine lactones (AHLs) [1]. However, some bacteria such as *Escherichia coli* (*E. coli*) do not produce AHLs and, therefore, their quorum sensing LuxR-type proteins are thought to be regulated by AHLs from other bacteria [2-4]. These sub-family of LuxR proteins known as LuxR solos can also regulate and detect non-AHL signals to regulate gene expression independently of AHLs

[5,6]. This AHL-dependent and –independent regulation of transcription is still unknown. Here we present several structures of one such solo LuxR-type protein, SdiA, from *E. coli*, in the presence and absence of AHL. Our study demonstrated that without AHL, SdiA is actually not in an apo-state, but regulated by a previously unknown endogenous ligand, 1-octanoyl-*rac*-glycerol (OCL), which is ubiquitously found throughout the tree of life, and serve as energy sources, signaling molecules, and substrates for membrane biogenesis. While exogenous AHL renders SdiA much higher stability and DNA binding affinity, we propose that OCL may function as a chemical chaperone placeholder in the absence of AHL and stabilizes SdiA as a dimer, allowing for some basal activity. Structural comparison between SdiA-AHL and SdiA-OCL complexes provides some crucial mechanistic insights into the ligand regulation of SdiA transcription activity.

Understanding the role of ligand binding on the function SdiA is important for elucidating how SdiA regulates expression of virulence genes in the human pathogen enterohemorrhagic *E. coli* (EHEC) O157:H7. Although EHEC causes foodborne infections worldwide that result in bloody diarrhea and hemolytic uremic syndrome (HUS), cattle is the major reservoir of EHEC. In cattle, EHEC colonizes predominately at the recto-anal junction (RAJ). Colonization at the RAJ poses a serious risk for fecal shedding and contamination of the environment. We previously demonstrated that EHEC senses AHLs produced by the microbiota in the rumen to activate the *gad* acid resistance genes necessary for survival through the acidic stomachs in cattle and to repress the locus of enterocyte effacement (LEE) genes important for colonization of the RAJ, but unnecessary in the rumen. Devoid of AHLs, the RAJ is the prominent site of colonization of EHEC in cattle. To determine whether the presence of AHLs in the RAJ could repress colonization at this site, we engineered EHEC to express the *Yersinia*

enterocolitica (*Y. enterocolitica*) AHL synthase gene *yenI*, which constitutively produces AHLs, to mimic a constant exposure of AHLs in the environment. The *yenI*$^{+}$ EHEC produces endogenous AHLs, and has a significant reduction in LEE expression, effector protein secretion, and attaching and effacing (A/E) lesion formation *in vitro* compared to the wild type (WT). The *yenI*$^{+}$ EHEC also activated expression of the *gad* genes. To assess whether AHL production, which decreases LEE expression, would decrease RAJ colonization by EHEC, cattle were challenged at the RAJ with WT or *yenI*$^{+}$ EHEC. Although the y*enI*$^{+}$ EHEC colonized the RAJ with equal efficiency to that of the WT, there was a trend for the cattle to shed the WT strain longer than the *yenI*$^{+}$ EHEC. The findings demonstrate that the regulation of EHEC in cattle is complex. Other factors such as fimbriae [161] may also contribute the colonization of EHEC in cattle. Identifying new factors and mechanisms of EHEC regulation is crucial for developing a better preventive approach against EHEC survival and colonization in cattle and subsequent EHEC contamination in the environment.

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3-oxo-C_6-HSL	N-(β-ketocaproyl)-L-homoserine lactone
3-oxo-C_8-HSL	N-β-oxo-octanoyl-L-homoserine lactone
86-24	Enterohemorrhagic *Escherichia coli* serotype O157:H7
Å	Angstrom
A/E	Attaching and effacing
AHL	Acyl-homoserine lactone
AR	Acid resistance
AR1	Acid resistance system 1
AR2	Acid resistance system 2
AR3	Acid resistance system 3
ATP	Adenosine triphosphate
Bp	Base pair
BSA	Bovine serum albumin
CDC	Centers for Disease Control and Prevention
CFU	Colony forming unit
CT	Critical threshold cycle
DBD	DNA binding domain
DMEM	Dulbeco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Dideoxy-nucleotide triphosphate

DTT	Dithiothreitol
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetracetic acid
EMSA	Electrophoretic mobility shift assay
$EspF_U$	*E. coli*- secreted protein F-like protein from prophage U
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
GABA	γ-amino butyric acid
GAD	Glutamate dependent acid resistance
GI	Gastrointestinal tract
Glu	Glutamate
HPLC	High performance liquid chromatography
HUS	Hemolytic uremic syndrome
IPTG	β-D-thiogalactopyranoside
IRSp53	Insulin receptor tyrosine kinase substrate p53
K_d	Dissociation constant
kDa	Kilo-Dalton
LB	Luria-Bertani broth
LDB	Ligand binding domain
LEE	Locus of enterocyte effacement
Ler	LEE-encoded regulator
MG	Monoacylglycerol

N-WASP	Nucleation-promoting factor Wiskott-Aldrich syndrome protein
Nle	Non-LEE encoded effector
NMR	Nuclear magnetic resonance
O.D.	Optical density
OCL	1-octanoyl-*rac*-glycerol
PAB	Plant associated bacteria
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PMSF	Phenylmethanesulfonylfluoride
PNK	Polynucleotide kinase
PON	Paraoxonases
qPCR	Quantitative PCR
qRT-PCR	Quantitative real-time PCR
QS	Quorum sensing
RAJ	Recto-anal junction
RAMS	Recto-anal mucosa swab
Rck	Resistance to complement killing
RNA	Ribonucleic acid
Rpm	Revolution per minute
RpoA	RNA polymerase subunit A
RpoS	RNA polymerase sigma S

SdiA	Suppressor of cell division inhibition
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEC-MALLS	Size-exclusion chromatography-multiangle laser light scattering
srgE	*sdiA*-regulated gene E
STM	Signature-tagged transposon mutagenesis
Stx	Shiga toxin
T3SS	Type 3-secretion system
Tir	Translocated intimin receptor
TLC	Thin layer chromatography
USDA	United States Department of Agriculture
UV	Ultraviolet
WT	Wild type
X-gal	5-bromo-4-chloro-3-indolyl β-galactopyranoside

CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

Microorganisms and their eukaryotic hosts have co-evolved for millions of years. Many microbes live harmoniously with their hosts in a symbiotic relationship. For example, the human gastrointestinal (GI) tract contains approximately 10^{14} bacteria from more than 1,000 different species [7]. The host provides nutrients needed for the bacteria to survive. In return, those bacteria, called the microbiota, perform crucial roles for the host, affecting metabolism and development of the innate immune system [7]. However, some microbes can cause disease in their host. These microbial pathogens are in a constant arms race with their hosts. Whether they are pathogenic or beneficial, microbes must adapt to changing environments. Mechanisms of how microbes sense the environment and communicate with their hosts for survival and colonization are still largely unknown. Elucidating these mechanisms is crucial for developing treatment against human diseases inflicted by microbial pathogens. The human pathogen EHEC serves as a model organism in this thesis project to explore the question of how a bacterium can sense and adapt to a changing environment. The findings from this thesis project may lead to important insights into the development of alternative preventive strategies to inhibit EHEC infection of humans.

Enterohemorrhagic *E. coli* pathogenesis

EHEC is a human pathogen that causes complications ranging from abdominal cramps and bloody diarrhea to the life-threatening condition known as hemolytic uremic syndrome (HUS) [8-10]. One dangerous attribute of EHEC is its low infectious dose; ingestion of 10-100 colony-forming units (CFU) can cause disease in humans [11]. Transmission through the fecal-oral route allows EHEC to travel through the intestines and attach to the epithelium within the large intestine, where EHEC releases Shiga toxin (stx) [12]. The toxin binds to globotriaosylceramide (Gb3) receptors on endothelial cells and is absorbed into the bloodstream, where the toxin can disseminate to other organs and cause disease [13]. For example, binding of the toxin to the renal glomerular endothelium, where in humans high levels of Gb3 receptors are expressed, results in acute renal failure, thrombocytopenia, and microangiopathic hemolytic anemia, all typical characteristics of HUS [9].

Currently no treatment is available for EHEC infection [14]. The use of conventional antibiotics exacerbates Shiga toxin-mediated cytotoxicity. In an epidemiological study conducted by the Centers for Disease Control and Prevention (CDC), patients treated with antibiotics for EHEC enteritis had a higher risk of developing HUS than untreated patients [15]. Additional studies support the controversy of treating EHEC infections with antibiotics; children on antibiotic therapy for hemorrhagic colitis associated with EHEC had an increased chance of developing HUS [16] [17] [18,19]. Antibiotics induce an SOS stress response and promote stx production by enhancing the expression of *stx* genes. Stx and the regulatory genes controlling *stx* transcription are encoded within a chromosomally integrated lambdoid prophage genome. Stx

induction also promotes phage-mediated lysis of the EHEC cell envelope, allowing for the release and dissemination of stx into the environment [20-22].

The natural reservoir of EHEC

Cattle are a major reservoir of EHEC, but unlike in humans, EHEC colonization in adult ruminants is asymptomatic [23-27]. The insensitivity to stx and differential preference in colonization sites renders cattle innocuous to EHEC-mediated diseases. Humans express Gb3 on their vascular endothelium that promotes much of the pathophysiology associated with stx; however, cattle lack vascular expression of Gb3 [28]. Although Gb3 receptors are detected in the kidney and brain of cattle, stx is unable to bind to the blood vessels in the cattle gastrointestinal (GI) tract [28]. As a result, stx cannot be endocytosed and transported to other organs to induce vascular damage in cattle. Another factor that prevents EHEC from causing disease in cattle is its site of colonization. EHEC colonizes the recto-anal junction (RAJ) of cattle (Figure 1) whereas it colonizes the colon in humans, where it causes electrolyte imbalance [29]. Cattle are EHEC's natural reservoir, which that contribute to the persistence and transmission of this human pathogen.

The shedding of this pathogen in ruminant feces facilitates transmission of EHEC from cattle to humans. Fecal shedding may be brief (≤ 10 days) or more extended (≤ 30 days) [24,30-34]. A proportion of positive animals shed more than 10^3 CFU per gram of feces [35]. These animals known as "super shedders" excrete more EHEC than others. Although the "super shedders" comprise about 8-9 % of cattle, it has been estimated that they may be responsible for over 95 % of all EHEC bacteria shed in the environment. Once shed into the environment,

humans acquire EHEC by consuming contaminated bovine-derived products such as meat, milk, and dairy products [36] or contaminated water, unpasteurized apple drinks, and vegetables that have become contaminated [37-39]. EHEC transmission also occurs through direct contact with infected ruminants at petting zoos, or infected people at their homes, daycare centers, and healthcare institutions [40-43]. Bovine manure can harbor viable EHEC for more than seven weeks [44], and the long-term environmental persistence of EHEC poses an increased risk for transmission of EHEC through the fecal-oral route through run-off to nearby farms or in contaminated forage consumed by other cattle. A better understanding of how EHEC colonizes cattle, can lead to novel strategies to limit fecal shedding of EHEC into the environment and limit environmental contamination, decreasing consequently human infection.

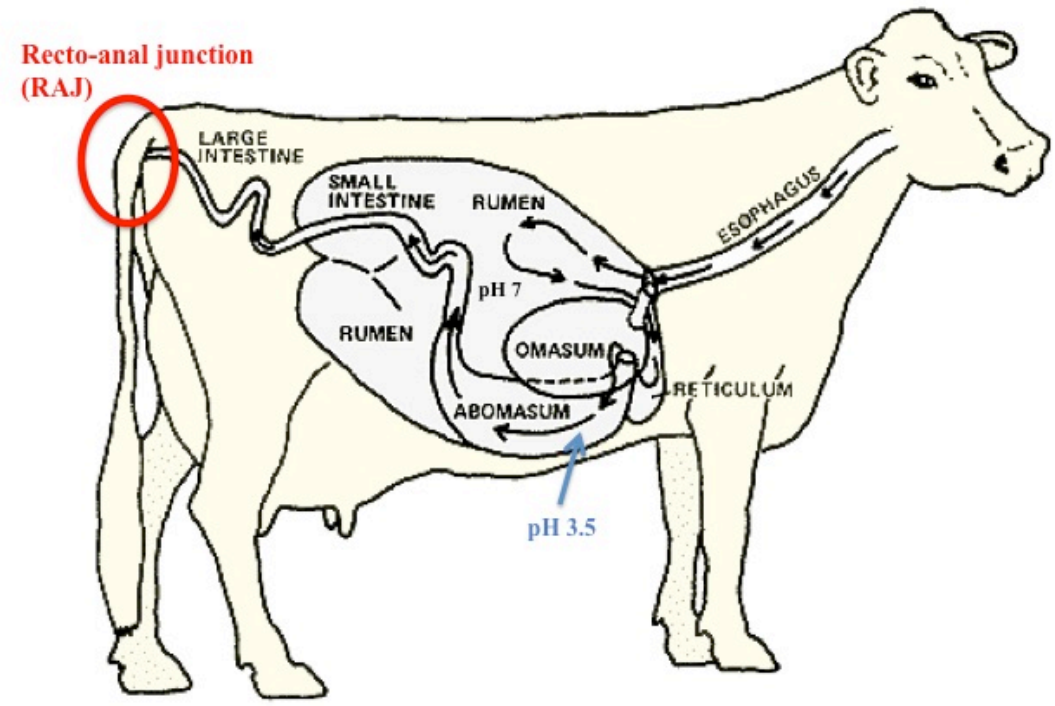


Figure 1. Cattle anatomy. Cattle have four stomachs consisting of the rumen, omasum, reticulum, and abomasum. RAJ is the prominent site of colonization of EHEC. (Figure adopted from http://www.extension.umn.edu/agriculture).

Important factors for EHEC survival and colonization in cattle

Glutamate acid resistance system

EHEC has a fecal-oral lifestyle in cattle and other ruminants. Upon ingestion, EHEC enters the rumen of cattle. Before EHEC reaches the RAJ for colonization, EHEC must first breach the acidic barrier of four stomachs (Figure 1). EHEC has an intricate acid resistance (AR) system, which enables it to survive the acidic stomach environment. Three important AR systems have been identified in *E. coli*: AR1 (glucose-repressed or oxidative), AR2 (glutamate dependent (GAD)), and AR3 (arginine dependent). RNA polymerase sigma S (RpoS) is required for activation of AR1 [45]. An *rpoS* mutant of EHEC is shed from calves less significantly than wild type [46]. RpoS is the central regulator of the general stress response. It is possible that the *rpoS* mutant is susceptible to other stresses present in the GI tract of cattle; therefore, it is still unclear whether the *rpoS* mutant's reduced survival in low gastric pH of cattle is due to a defective AR1 or to other pleiotropic effects. In another study, the EHEC AR2 mutant also has a reduced fitness in cattle compared to wild type, while AR3 does not affect the shedding of EHEC from cattle [47]. These studies demonstrate that of the three AR systems, AR2 plays a major role for passage and survival of EHEC through the acidic stomachs and subsequent colonization of the RAJ in cattle [47].

The glutamate-dependent AR system consists of the glutamate decarboxylases GadA and GadB that convert glutamate to γ-amino butyric acid (GABA) by displacing the α-carboxyl group of the amino acid with a proton that is transported from the environment into the cytoplasm. GABA is exchanged for new amino acids through the cognate antiporter GadC [48,49]. The conversion of glutamate to GABA increases the internal pH and helps maintain pH

homeostasis (Figure 2) *in vitro*. The induction of the glutamate-dependent AR system varies depending on culture conditions [50]. For example, regardless of the pH of the media, GAD is induced at stationary phase; however, if the cells are challenged with pH 2.5 or less, GAD is activated regardless of the growth phase [49,51,52]. The magnitude of GAD induction is dependent on the culture media. For instance, acid resistance by GAD is achieved when cells are grown at exponential logarithmic phase in low glucose media but not in complex media such as LB until the cells enter stationary phase [51]. Activation of GAD under a variety of different environmental conditions is a result of a complex regulation of the *gad* genes by multiple regulators including the important global regulators RpoD and RpoS [50]. These regulators primarily promote the expression of *gadE,* which encodes for the central activator of the *gadA* and *gadBC* loci that are crucial for acid resistance [53]. Of the AR systems in *E. coli*, GAD provides the most robust acid protection, allowing cells to survive in a pH as low as 2 for several hours [45,49].

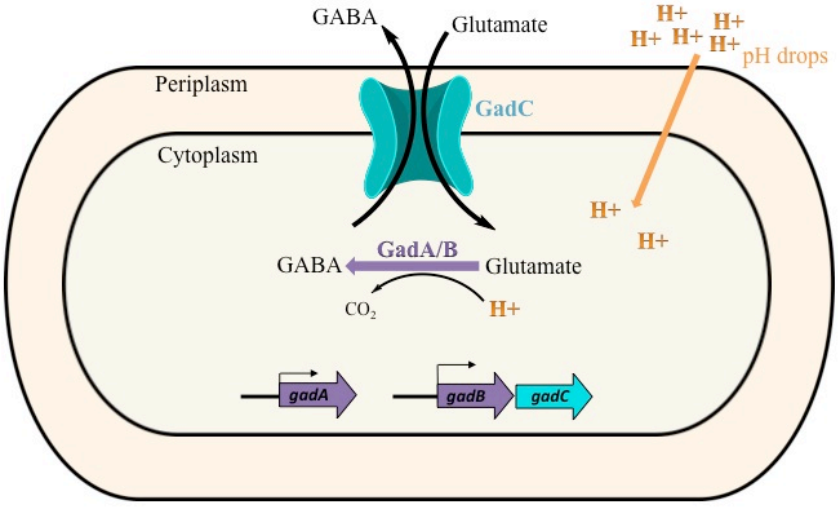


Figure 2. Schematic of the glutamate dependent acid resistant system. The *gad* genes *gadA* and *gadB* encodes for the glutamate decarboxylases that converts glutamate to γ-amino butyric acid (GABA) by displacing the α-carboxyl group of the amino acid with a proton. GABA is exchanged for new amino acids through the cognate antiporter GadC.

Locus of enterocyte effacement (LEE)

After passage through the acidic barrier, EHEC forms attaching and effacing lesions on the mucosal epithelium at the RAJ, allowing for its colonization of this site. A/E lesions are characterized by effacement of the microvilli, intimate attachment of the bacteria to the cell, and accumulation of polymerized actin beneath the site of bacterial attachment to form a pedestal-like structure cupping individual bacteria [54] (Figure 3A). The majority of the genes required for A/E lesion formation are encoded within a chromosomal pathogenicity island named the LEE [55,56]. The LEE consists of 41 genes, the majority of which are organized into five major operons (*LEE1-5*) that encode a type 3 secretion system (T3SS), regulators, chaperones, and effector proteins. The LEE-encoded regulator (Ler), the first gene encoded within *LEE1*, acts as the master transcription factor of this pathogenicity island, activating the expression of all the LEE genes [56,57] (Figure 3B).

EHEC injects effector proteins through its "molecular syringe-like" T3SS needle directly into the cytoplasm of target cells [58]. One important effector is the translocated intimin receptor (Tir) that acts as the receptor to the bacterial adhesin intimin, and allows for EHEC to adhere intimately to the epithelium. Once released into the host cytoplasm, Tir is directed to the host cytoplasmic membrane and is inserted as a hairpin-like structure, with its N- and C-terminus in the cytoplasm and its central domain exposed to the surface. The central domain of Tir interacts with the LEE- encoded surface protein intimin to form a tight attachment of the bacteria to the epithelium [59,60]. The non-LEE encoded effector protein, *E. coli* secreted F-like protein from prophage U ($EspF_U$), is secreted into the cell and works co-operatively with Tir to recruit host proteins leading to rearrangement of the host cytoskeleton and actin polymerization. $EspF_U$ recruits actin nucleation-promoting factor Wiskott-Aldrich syndrome protein (N-WASP) and the

insulin receptor tyrosine kinase substrate p53 (IRSp53), an important regulator for actin cytoskeleton reorganization. This results in accumulation of actin beneath attached bacteria, forming the characteristic pedestal-like structure [61,62] (Figure 3A).

In vitro studies demonstrated the crucial role that A/E lesion formation plays in EHEC attachment to cultured epithelial cells. Various reports have investigated whether A/E lesion formation is also required for EHEC to attach to bovine intestinal epithelial cells to promote colonization in cattle. Immunofluorescence staining of tissues reveals that EHEC adheres tightly predominately to the epithelial cells in the RAJ of cattle [29]. Dziva *et al.* performed signature-tagged transposon mutagenesis (STM) to identify EHEC genes required for colonization and survival in cattle. Transposon insertions in the genes encoding for the T3SS machinery resulted in reduced fecal shedding of EHEC [63]. Similarly, deletion of the *LEE4* operon, which encodes for essential structural components of the T3SS, resulted in reduced cattle colonization [64]. Tir and intimin have also been shown to play an important role in intestinal colonization in neonatal calves and piglets [65-68] and in adult cattle and sheep [69]. Together these data indicate that LEE-mediated adherence of EHEC to the intestinal epithelia is important for promoting cattle colonization.

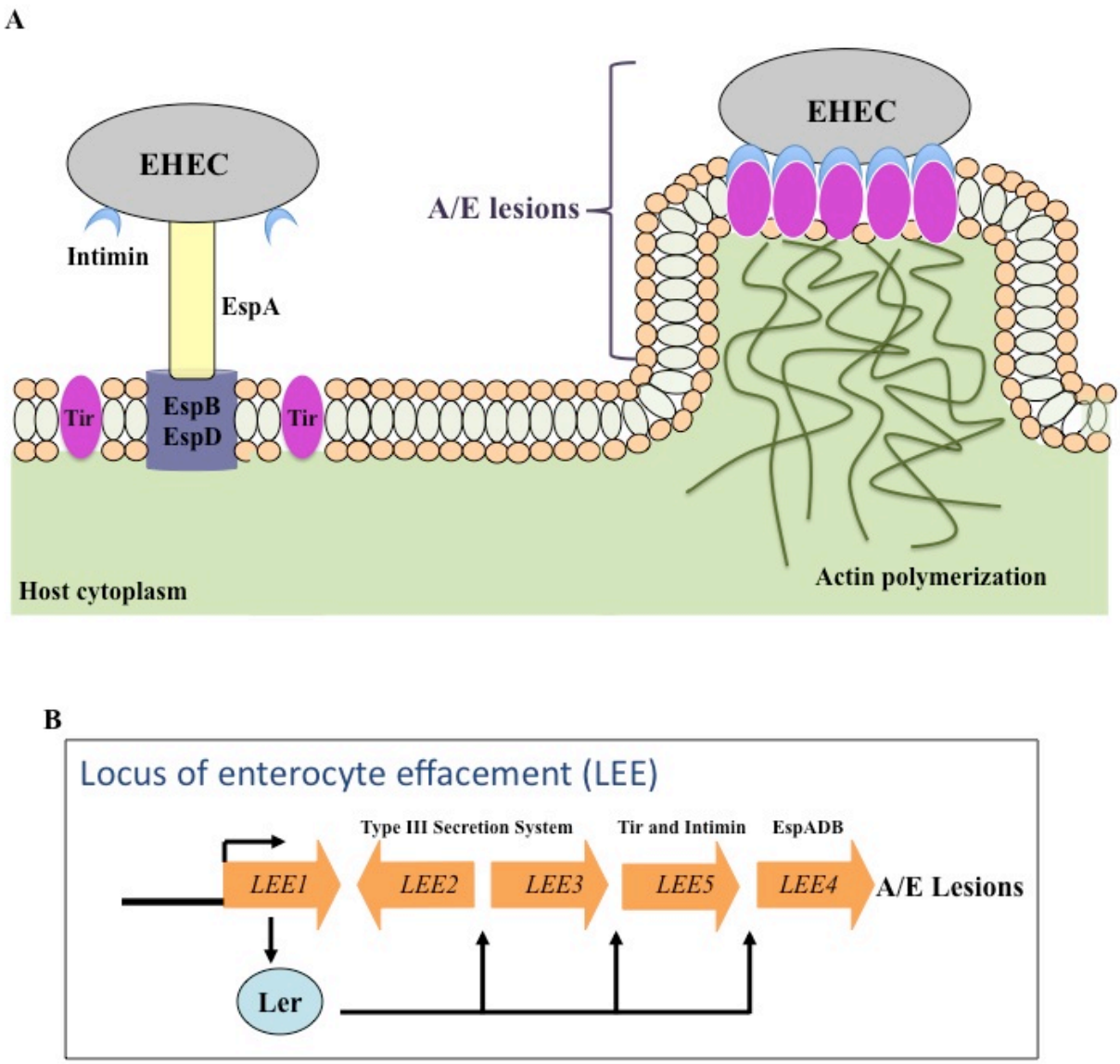


Figure 3. (A) Schematic diagram of attaching and effacing (A/E) lesion fromation. EHEC injects effector proteins such as Tir into the host cytoplasm through the T3SS. Tir localizes to the host membrane and binds to intimin to intimately attach the bacteria to the cell. Tir with $EspF_u$ recruit host factors to promote actin polymerization forming pedestal-like structures. (B) Most of the genes required for A/E lesions are encoded within the locus of enterocyte effacement (LEE). The LEE is constituted of 41 genes, with the majority of them being organized in 5 operons named *LEE1-5*. Encoded within the *LEE1* is the LEE-encoded regulator *ler* that activates transcription of all the LEE genes.

The SdiA transcription factor

In addition to the *gad* acid resistance system and the LEE, SdiA (suppressor of cell division inhibitor) is also necessary cattle colonization [2,63]. SdiA is a LuxR-like quorum sensing transcription factor [70] found in *E. coli, Klebsiella pneumoniae* (*K. pneumoniae*)*,* and *Salmonella enterica* (*S. enterica*) [71]. Overexpression of *sdiA* from a multicopy plasmid led to the discovery that SdiA suppresses the expression of a number of chromosomally encoded cell division inhibitors in *E. coli* [73]. However, chromosomal deletion of *sdiA* shows no defects on cell division in *E. coli*, suggesting that the role of SdiA on cell division is an artifact of overexpression [73]. In EHEC an intact *sdiA* gene is necessary for intestinal colonization of calves [63]. A recent study demonstrated that the absence of SdiA in EHEC results in significantly reduced survival under acidic conditions compared to wild type, both in cell culture and in the rumen of cattle. Additionally, wild type EHEC outcompetes the *sdiA* mutant for colonization at the RAJ [2].

In *Salmonella* SdiA regulates the expression of the *rck* (resistance to complement killing) operon located on the *S. enterica* serovar Typhimurium (*S.* Typhimurium) virulence plasmid [74], and the gene *srgE* (*sdiA*-regulated gene E) [71]. In addition to inducing *S.* Typhimurium resistance to bactericidal action of the host's complement system, Rck also allows *Salmonella* adherence and invasion of epithelial cells [74]. SrgE is a T3SS effector of unknown function [75]. Activation of the *rck* and *srgE* genes by SdiA increased *S.* Typhimurium fitness in mice [3].

LuxR/I quorum sensing (QS) systems

Prototypical LuxR/I QS system

SdiA is a member of the quorum sensing LuxR family of transcription factors. Quorum sensing is a mechanism by which bacteria coordinate their behavior through the production and sensing of chemical signals [76]. Many Gram-negative bacteria encode for the prototypical LuxR/I QS system, which was initially discovered in *Vibrio fischeri* (*V. fischeri*) [77,78]. Briefly, the bacteria encode for *luxR*/*luxI* pairs that are generally genetically linked. LuxR is a transcription factor and the LuxI is the cognate ligand synthase. The LuxI synthase produces the chemical signal AHLs, which diffuse freely across the bacterial membrane into the environment [79]. AHLs are composed of a conserved lactone ring linked to a variable acyl chain by an amide bond. The acyl chain varies by length and modification at a carbon at position 3, which can either be part of a methylene group or carry an oxo- or hydroxyl group. This variable acyl chain allows for specificity towards the cognate LuxR sensor [76] (Figure 4A). When the bacterial population reaches a sufficient density, AHLs accumulate in the environment and diffuse back into the bacterial cytoplasm, subsequently binding to the cognate transcriptional regulator LuxR. LuxR senses AHLs through an AHL-binding region at the amino terminus, enabling LuxR to dimerize and bind DNA through a helix-turn-helix at the carboxyl terminus to modulate expression of target genes [76,77,80]. Intensive structural and biochemical studies on TraR, another LuxR-like protein, suggested that binding to AHLs is required for stability and dimerization of LuxR-like proteins. Oligomerization of these regulators is crucial for binding to target genes and to regulate transcription [81] (Figure 4B). Since its initial discovery, more than 50 species have been shown to harbor the LuxR/I homologs, including an important human

pathogen *Pseudomonas aeruginosa* (*P. aeruginosa*), and the plant pathogens *Erwinia carotovora* (*E. carotovora*) and *Agrobacterium tumefaciens* (*A. tumefaciens*) [1]. These LuxR/I homologs regulate diverse biological processes [76].

SdiA-AHL signaling in cattle

Of note, a subset of Gram-negative bacteria such as *E. coli* and *S.* Typhimurium do not have a *luxI*-like gene, but encode for a LuxR-like homolog, SdiA [72,74]. SdiA binds to exogenous AHLs [82] to regulate gene expression [2,83] instead of responding to self-produced AHLs. For example, *S.* Typhimurium detects AHLs produced by *Aeromonas hydrophila* in the gut of turtles through SdiA to induce expression of the *srgE* gene [4]. AHLs are also detected in rumen of cattle and not in any other regions of the GI tract [2,84]. AHLs extracted from the rumen upregulated expression of the *gad* genes and downregulated expression of the LEE in EHEC [2]. In laboratory *E. coli* strain MG1655, exogenous AHLs also activate the *gad* genes via SdiA to enhance bacterial fitness at low pH [2]. These studies led to a model in which SdiA senses AHLs produced by the microbiota in the rumen to activate acid fitness genes that allow EHEC passage and survival through the acidic stomachs and repress the LEE genes to prevent colonization in a hostile environment. EHEC does not encounter AHLs beyond the rumen, alleviating the SdiA-mediated repression of the LEE and thus allowing EHEC to colonize the RAJ (Figure 5).

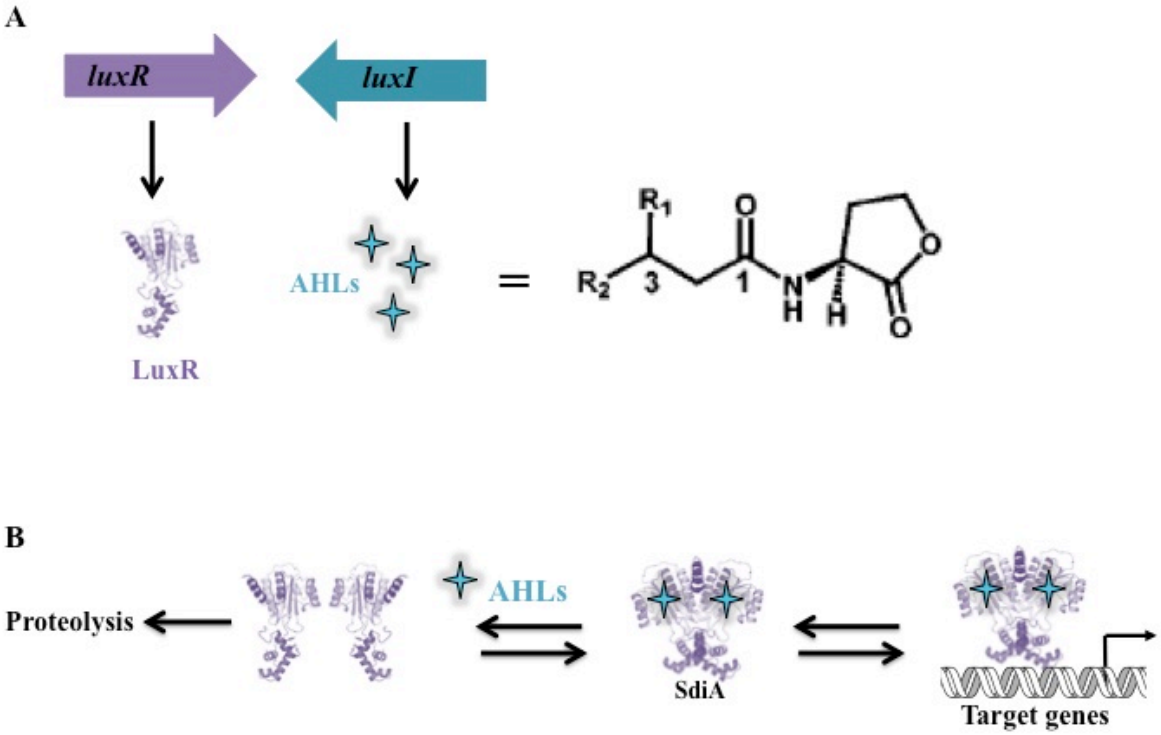


Figure 4. LuxR/I quorum sensing (QS). (A) Gram-negative bacteria contain the *luxR/I* pair that encodes for a LuxR transcription factor and a LuxI AHL-synthase, respectively. (B) The dogma of prototypical LuxR proteins. AHLs stabilize and promote dimerization of the LuxR transcription factors; thus, allowing these proteins to regulate target gene expression.

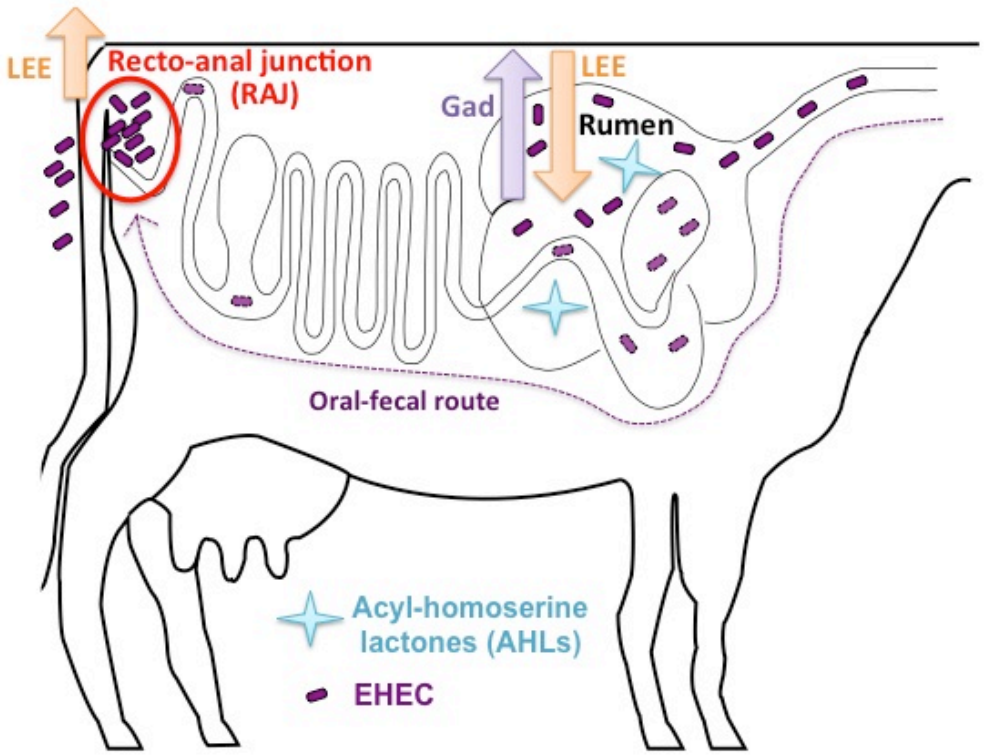


Figure 5. Proposed SdiA-AHL signaling in cattle. Once ingested, EHEC through SdiA senses AHLs produced by the microbiota in the rumen to activate the *gad* acid resistance system and to repress the LEE. After passage through the acidic barrier, EHEC reaches the RAJ. The lack of AHLs at the RAJ alleviates the SdiA-AHL repression of the LEE, allowing EHEC to colonize the host.

Nguyen Y and Sperandio V. (2012). Enterohemorrhagic *E. coli* (EHEC) pathogenesis. Mini Review. *Front Cell Infect Microbiol.* 2, 90.

LuxR solos

Although SdiA regulation of the LEE depends on AHLs, survival studies have suggested that SdiA can modulate transcription of the *gad* genes independently of AHLs [2]. Additionally, SdiA binds to the promoter of the *ftsQ* gene and modulates its expression in the absence of AHLs [85]. This deviates from the dogma of prototypical LuxR proteins. Extensive functional studies of TraR from *A. tumefaciens*, indicates that AHLs are necessary for the function of this LuxR protein and in the absence of AHLs, this protein is targeted for proteolysis [76].

A subset of LuxR-like proteins known as the LuxR solos further raise the dependency of LuxR proteins to AHLs into question [86]. Typically, the *luxI/R* pairs are genetically located adjacent to each other, but some Proteobacteria only harbor the *luxR* gene is encoded [87] (Figure 5). Hence, these proteins are termed LuxR solos or orphans [86]. A phylogenetic study of 265 proteobacterial genomes showed that the LuxR solos are found not only in bacteria lacking LuxI-like synthases, such as *E. coli* and *S.* Typhimurium, but also in AHL-producing bacteria with one or more LuxR/I pairs [87] (Table 1).

Well-characterized examples of LuxR solos in AHL producers are ExpR of *Sinorhizobium meliloti* (*S. meliloti*) and QscR of *P. aeruginosa*. ExpR can respond to endogenous AHLs synthesized by the SinI synthase, and regulate expression of its target genes separately from the SinI associated-LuxR homologue SinR [88,89]. Similarly to SdiA, ExpR can also target a different subset of genes when AHLs are absent. ExpR, unlike many LuxR proteins, are soluble and stable in the absence of AHLs [89,90]. *P. aeruginosa* has two complete AHL QS systems, LasI/R and RhII/R. Not associated with any LuxI homologues, QscR detects LasI-produced AHL and modulates distinct genes from the LasI/R and RhII/R circuits [91,92]. Unlike LasR and RhIR, which have high specificity towards AHLs produced by the cognate AHL

synthases, QscR can respond to several AHLs [93]. This suggests that *P. aeruginosa,* in addition to detecting self-produced AHL signals, also monitors its neighbors by sensing exogenous AHLs produced by other bacteria in the environment. This relaxed specificity is not limited to QscR since SdiA, ExpR, and several other LuxR solos are capable of binding to and responding to an array of AHLs [86] (Table 1). This portrays the important role of LuxR solos in interspecies communication.

In addition to AHLs, LuxR solos can detect non-AHLs signals (Figure 6). For example, SdiA can respond to an unidentified compound, which is endogenously produced by *E. coli* [83]. Most recently, the LuxR receptor PluR from the insect pathogen *Photorhabdus luminescens* was reported to detect endogenously produced α-pyrones [94] (Table 1). The LuxR solos XccR, OryR, PsoR, XagR, and NesR from the Plant Associated Bacteria (PAB) *Xanthomonas campestris* pv. *campestris*, *Xanthomonas oryzea* pv. *oryzae*, *Pseudomonas fluorescens*, *Xanthomonas axonopodis* pv. *glycines*, and *Sinorhizobium meliloti,* respectively, have evolved to no longer respond to AHLs. With the exception of NesR, these solos all have been demonstrated to respond to a yet unidentified low molecular weight plant compounds to regulate various biological processes and virulence in their plant hosts [95-100] (Table 1). Compared to other LuxR proteins, PAB LuxR solos lack conservation in the AHL-binding domain, which may allow these LuxR solos to bind to non-AHL plant compounds [97,101-103]. This shows that LuxR solos also involved in inter-kingdom signaling with eukaryotic hosts.

The AHL-dependent and –independent regulation of LuxR solos is still unclear. Understanding how AHLs induce conformational changes in the protein structure may shed light into the AHL-dependent and independent function of the LuxR solos. Although the functions of LuxR proteins have been studied extensively, only few structures are available. Thus far there

are no structural information of the same LuxR in the absence and presence of AHL. These structural and biochemical studies have been limited due to the instability of the majority LuxR sensors in the absence of AHL signals. One part of this thesis project is to determine the structure of SdiA; to gain better understanding of how bacteria uses these LuxR proteins to sense the environment and interact with the host. Our understanding of host-pathogen interaction will allow us to develop strategies to prevent pathogens from colonizing the host and spreading infections, which we will explore in the second part of this project.

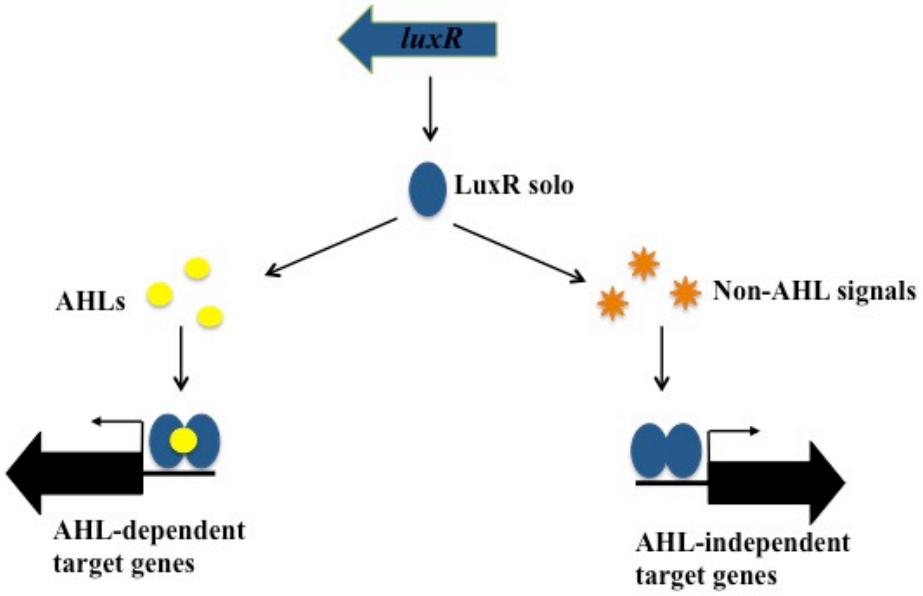


Figure 6. LuxR solos can respond to AHL and/or non-AHL signals to regulate AHL-dependent and –independent target genes.

Table 1. LuxR solos and their function

LuxR solo	Organism	LuxR/I system	Binding molecule (s)	Function	Ref
SdiA	*Escherichia coli; Salmonella enterica* serovar Typhimurium	None	3-oxo-C_8-HSL, 3-oxo-C_6-HSL, 3-oxo-C_4-HSL, 3-oxo-C_{10}-HSL, 3-oxo-C_{12}-HSL, C_6-HSL, C_8-HSL	GAD acid resistance; formation of A/E lesions; adhesion and resistance to complement killing	[70,71,82]
ExpR	*Sinorhizobium meliloti*	SinI/SinR	C_{14}-HSL, 3-oxo-C_{14}-HSL, C_{16}-HSL, 3-oxo-C_{16}-HSL, C_{18}-HSL	Synthesis of symbiotically active galactoglucan (EPSII) and succinoglycan	[89,90,104]
QscR	*Pseudomonas aeruginosa*	LasI/R, RhlI/R	3-oxo-C_{12}-HSL, 3-oxo-C_{10}-HSL	Fine tune expression of AHL production and virulence factors	[91,92]
OryR	*Xanthomonas oryzae* pv. *oryzae* (Xoo)	None	Rice plant signals	Regulate expression of proline imiopeptidase (*pip)* and motility genes;virulence in rice	[95,97]
XccR	*Xanthomonas campestris* pv. *Campestris* (Xcc)	None	Plant signals	Regulate neighboring *pip* genes; virulence in cabbage	[96]
PsoR	*Pseudomonas fluorescens*	None	Plant signals	Control the transcription of anti-microbial-related genes	[98]
XagR	*Xanthomonas axonopodis* pv. *Glycines* (Xag)	None	Plant signals	Virulence in soybean	[100]
NesR	*Sinorhizobium meliloti*	None	Unidentified	Survival under stress and low nutrient conditions	[99]
PluR	*Photorhabdus luminiscens*	None	α-pyrones	Regulate cell clumping	[94]

CHAPTER TWO

MATERIALS AND METHODS

Strains and plasmids

All strains and plasmids used in this study are listed in Table 2. Unless otherwise stated, *E. coli* strains were grown aerobically in Luria-Bertani (LB) broth at 37°C and 250 rpm. Where indicated strains were grown in high-glucose Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen). Media was supplemented where necessary with the selective antibiotics streptomycin, kanamycin, ampicillin and chloramphenicol, which were added to a final concentration of 100, 50, 100, or 30 μg/mL respectively.

Recombinant DNA methods

Standard methods used for PCR amplification, plasmid purification, and transformations [105]. Oligonucleotide primers (Table 3) were designed using Primer Express v1.5 (Applied Biosystems). Δ*stx2a*, *yenI*$^{+}$Δ*stx2a*, and vector Δ*stx2a* were constructed using λ Red mutagenesis [106]. Briefly, to generate Δ*stx2a* in wild type EHEC strain (86-24), PCR product was amplified using Phusion polymerase (Invitrogen), primers stx2AλRed F and stx2AλRed R, and pKD3 plasmid as the template. PCR product was digested with Dpn1 to remove the template DNA and then gel purified (Qiagen). Wild type EHEC strain transformed with the helper plasmid pKD46 were prepared for electroporation and transformed with the resulting *stx2a* PCR product. Colonies were screened for ampicillin sensitivity and chloramphenicol resistance. The chloramphenicol cassette was removed from Δ*stx2a* deletion candidates with the

resolvase plasmid pCP20. Final verification was performed by PCR amplification and sequencing to yield the resolved Δ*stx2a* EHEC strain (YNN01). *yenI* and the empty vector were chromosomally integrated into YNN01 background to yield *yenI*$^{+}$ Δ*stx2a* and vector^{+}Δ*stx2a* EHEC strains using the same λ Red mutagenesis method. Primers yenIλRed F and yenIλRed R were used to amplify the λP_R-*yenI*-FRT-kan-FRT or λP_R-FRT-kan-FRT cassettes from pJLD1600 and pJD500 [83], respectively, and the sequences homologous to *lacI* integration site in the chromosome of Δ*stx2a* EHEC.

To construct the wild type EHEC strain expressing chromosomally 3xFLAG-tagged SdiA, the PCR product was amplified using Phusion High-Fidelity DNA Polymerase (Thermo Scientific), sdiAFLAGF and sdiAFLAGR primers, and pSUB11 (Kn^R) plasmid as the template. PCR product was digested with Dpn1 to remove the template DNA and then gel purified (Qiagen). Wild type EHEC strain transformed with the helper plasmid pKD46 were prepared for electroporation and transformed with the resulting gel purified PCR products. Colonies were screened for ampicillin sensitivity and kanamycin resistance. Successful recombination of the FLAG sequence into the chromosomal *sdiA* gene of positive colonies was confirmed by PCR amplification of the integrated region using primers sdiAUP and sdiADOWN. The kanamycin cassette was removed with the resolvase plasmid pCP20. PCR amplification and DNA sequencing were performed for final verification of the resolved chromosomal 3xFLAG-tagged SdiA EHEC strain (YNN04).

Western blotting

Wild type and *yenI*$^{+}$ EHEC strains were grown in high glucose DMEM at 37°C in either the presence of 10 μM 3-oxo-C_6-HSL [107] or an equivalent amount of DMSO to an OD_{600} =

1.0 and secreted proteins were prepared as previously described [58]. Protein samples were electrophoresed in sodium dodecyl sulfate 12 % polyacrylamide gels (SDS-PAGE). Samples were subjected to western blotting as described previously [105]. Blots were probed with rabbit polyclonal antisera to EspA and EspB (Cocalico Biologicals) and visualized with enhanced chemiluminescence (BioRad).

To assess how AHLs affect endogenous levels of the SdiA protein, overnight cultures of YNN04 grown aerobically at 37°C in LB were diluted at 1:100 into low-glucose DMEM in the presence or absence of 10 μM 3-oxo-C_6-HSL or 10 μM 3-oxo-C_8-HSL. At late-log growth phase (OD_{600} of 1.0), cells were collected by centrifugation and lysed at room temperature in urea lysis buffer (100 mM NaH_2PO_4, 10 mM Tris-Cl, 8 M urea pH 8.0) for two hr. Cellular debris was removed by centrifugation and whole cell lysates were electrophoresed in 12 % SDS-PAGE. Samples were subjected to immunoblotting as described previously [105]. Blots were probed with a mouse monoclonal antibody to FLAG (Sigma) (1:5,000) and RpoA (Santa Cruz) (1:5,000) and visualized with enhanced chemiluminescence (GE Healthcare).

Fluorescent actin staining assay

To assess A/E lesion formation, fluorescent actin staining (FAS) assays were performed as previously described [108]. Briefly, HeLa cells were grown on coverslips in low glucose DMEM supplemented with 10 % fetal bovine serum (FBS) (Invitrogen) at 37°C in 5 % CO_2 and were infected with 1:100 dilution of overnight static bacterial cultures for 6 hr. The coverslips were washed, fixed, and permeabilized with 0.2 % Triton X-100. Fluorescein isothiocyanate (FITC)-labeled phalloidin (Sigma-Aldrich) was used to visualize actin accumulation and propidium iodide was added to stain bacteria and HeLa nuclei.

RNA extraction

To determine the effects of endogenous AHLs on the expression of the LEE or *gad* genes in the *yenI*$^{+}$, overnight cultures of YNN03 grown aerobically at 37°C in LB were diluted at 1:100 into high-glucose DMEM and grown in triplicate to late-exponential growth phase (OD_{600} = 1.0). For wild type strains that do not produce endogenous AHLs (YNN01 and YNN02), 10 μM of exogenous 3-oxo-C_6-HSL was dissolved in DMSO at a 10 mM concentration and added directly to DMEM at 1:1000 dilutions. For samples assessed without exogenous signals, the respective concentration of DMSO was used to ensure the solvent did not alter gene expression. RNA from these biological replicates was extracted using TRIzol (Invitrogen) and the RiboPure Bacteria RNA isolation kit (Ambion) according to the manufacturer's instructions.

To evaluate the endogenous SdiA at different growth stages, overnight cultures of YNN04 grown aerobically at 37°C in LB were diluted at 1:100 into low-glucose DMEM and grown in triplicate to early, mid, and late exponential growth phase in the absence or presence of 10 μM 3-oxo-C_6-HSL. Samples were split for western blotting and for RNA extraction using TRIzol (Invitrogen) and the RiboPure Bacteria RNA isolation kit (Ambion) according to the manufacturer's instructions.

Quantitative real-time PCR

The primers used for the real-time PCR assays were designed using Primer Express v1.5 (Applied Biosystems) (Table 3). Amplification efficiency and template specificity of each of the primers were validated, and reaction mixtures were prepared as previously described

[109]. Quantitative real-time reverse transcriptase PCR (qRT-PCR) was performed in a one-step reaction using an ABI 7500 sequence detection system (Applied Biosystems). Using the ABI sequence Detection 1.2 software (Applied Biosystems), data were collected and normalized to endogenous levels of *rpoZ*. Data was analyzed by using the comparative critical threshold cycle (C_T) method and presented as fold changes compared to WT levels. Error bars represent the standard deviations of the $\Delta\Delta C_T$ values. Statistical significance was determined by Student's *t*-test, and a *P value* of ≤ 0.05 was considered significant.

AHL detection

AHL extraction and detection were performed as previously described [110]. Briefly, wild type, *yenI*$^+$, and vector control strains were grown in high glucose DMEM to late exponential phase. AHLs were extracted three times with ethyl acetate and concentrated to 50 μL. For analytical thin layer chromatography (TLC), 1 μL of concentrated extracts from wild type and vector strains or 10 μL of concentrated extract diluted at 1:100 from *yenI*$^+$ were applied to C_{18} reverse-phase TLC plates (200 μm layer, Whatman). The chromatograms were developed with 70 % methanol, dried, and overlaid with a culture of the *A. tumefaciens traI:lacZ* [111] indicator strain and 60 μg/mL X-gal for 16 hr at 30°C. For positive control, 1 μL of 10 μM 3-oxo-C_6-HSL was also included.

Cattle experiments

Two groups of five mature Holstein steers were housed in quarantined facilities. All personnel followed strict biosafety procedures and all procedures were approved by the Institutional Animal Care and Use and Biosafety Committees. O157 cultures were adjusted to

the desired bacterial concentration by dilution in phosphate-buffered saline and confirmed by viable plate count. Cattle received a single rectal application of 10^9 CFU of wild type or *yenI*$^+$ EHEC, as previously described [112]. EHEC was cultured from recto-anal junction mucosa swabs (RAMS) as previously described [30] on the days indicated.

SdiA vector construction, expression, and protein purification

The plasmid pYN1 was constructed by PCR amplification of the *sdiA* gene from the 86-24 EHEC using Phusion High-Fidelity DNA Polymerase (Thermo Scientific) with the primers SdiAF-pET21 and SdiAR-pET21 and cloning the resulting PCR product into the EcoRI and SalI cloning sites of the pET-21a expression vector. The resulting plasmid pYN2 was transformed into BL21 cells. Transformed BL21 cells were cultured in LB broth with 100 μg/mL of ampicillin to an OD_{600} of 0.8 at 37°C and were induced with 400 μM isopropyl β-D-1-thiogalactopyranoside (IPTG) (Sigma) at 15°C overnight. Cells were harvested, suspended in lysis buffer (50 mM Tris-base buffer at pH 8.5, 300 mM NaCl, 1 mM EDTA, 5 mM imidazole, 5 mM 2-β-mercaptoethanol, and 5 % glycerol), and lysed by homogenization. The lysed cells were centrifuged, and the lysates were incubated with Ni^{+2}-NTA agarose beads (Qiagen) and loaded onto a gravity column (Qiagen). The column was washed with wash buffer 1 (50 mM Tris-base buffer pH 8.5, 300 mM NaCl, 0.1 mM EDTA, 10 mM imidazole, 5 mM 2-β-mercaptoethanol, and 5 % glycerol) and wash buffer 2 (50 mM Tris-base buffer pH 8.5, 300 mM NaCl, 0.1 mM EDTA, 50 mM imidazole, 5 mM 2-β-mercaptoethanol, and 5 % glycerol) and the protein was eluted in the elution buffer (50 mM Tris-base buffer pH 8.5, 300 mM NaCl, 0.1 mM EDTA, 250 mM imidazole, 5 mM 2-β-mercaptoethanol, and 10 % glycerol). SdiA was concentrated for further use.

Site-directed mutagenesis

To define the key residues that are important for ligand recognition by SdiA, site-directed mutagenesis was used to generate single-point mutations in *sdiA* gene expressed on the pBAD/Myc-HisA expression vector. The plasmid pYN2, which expresses wild type SdiA-6xHis protein, was constructed by PCR amplification of the *sdiA* gene from the 86-24 EHEC using Phusion High-Fidelity DNA Polymerase (Thermo Scientific) and the primers SdiAF-pBAD and SdiAR-pBAD. The resulting PCR product was cloned into the EcoRI and BglII cloning sites of the pBAD/Myc-HisA expression vector. Plasmids (pYN3-11) containing single-point mutations of different SdiA amino acid residues were generated using primers as listed in Table 3, pYN1 as the template, and the QuickChange II-XL Site-Directed Mutagenesis kit (Strategene) as instructed by the manufacturer. Verified plasmids were transformed into Δ*sdiA* EHEC strain DH1 to yield YNN06-15. To test the expression levels of these SdiA point mutants, YNN06-15 cells were grown in low-glucose DMEM supplemented with ampicillin, 0.2 % arabinose, and 10 μM 3-oxo-C_6-HSL, 10 μM 3-oxo-C_8-HSL (Sigma), or DMSO to late-log growth phase (OD_{600} of 1.0). Cells were pelleted by centrifugation, lysed with urea lysis buffer (100 mM NaH_2PO_4, 10 mM Tris-Cl, 8 M urea pH 8.0), and whole cell lysates were subjected to western blotting as described below. Blots were probed with mouse monoclonal antibody to His and RpoA as a loading control.

Electromobility shift assays (EMSAs)

To assess the effects of AHLs on SdiA binding to the *ler* promoter, EMSAs were performed with purified SdiA in the presence or absence of 3-oxo-C_6-HSL or 3-oxo-C_8-HSL

and labeled *ler* DNA probe. The *ler* probe was defined as +86 bp downstream and -218 upstream from the P2 start site. The promoter region was amplified from 86-24 genome using primers R2 and Ler-218F and Phusion High-Fidelity DNA Polymerase (Thermo Scientific). The *kan* promoter region, used as a negative control, was amplified from pRS551 plasmid with primers KanF and KanR. The resulting PCR products were gel extracted (Qiagen) and end-labeled with [32^P]-ATP (Perkin Elmer) using T4 polynucleotide kinases (NEB) and standard procedures [105]. The end-labeled DNA fragments were purified using the Qiagen PCR purification kit. EMSAs were performed by adding increasing amounts of purified SdiA protein (0–30 μM) to end-labeled probes in binding buffer (50 mM Tris-HCl pH 8.5, 50 mM NaCl, 2 mM magnesium acetate, 0.1 M EDTA, 0.1 mM DTT, 25 μg/mL of BSA, and 1 μg of poly(dI-dC) with 2X the concentration of 3-oxo-C_6-HSL or 3-oxo-C_8-HSL as SdiA or corresponding amounts of DMSO as a solvent control for 20 min at 22°C. Immediately before loading, a 5 % ficol solution was added to the mixtures. The reactions were electrophoresed for ~6 hr at 160 V on a 5 % polyacrylamide gel, dried, exposed, and analyzed using the Storm Phosphorimager.

Pulse-chase

To measure the stability of SdiA *in vivo*, pYN1 (expresses SdiA) and pJD410 (expresses T7 RNA polymerase) were transformed into wild type EHEC (86-24) and the resulting strain YNN05 was used for the pulse-chase experiments. YNN05 was cultured in M9 minimal media containing 100 μg/mL of ampicillin at 37°C to an OD_{600} = 0.7, then switched to 45°C for 20 min to induce T7 RNA polymerase expression. The temperature was changed back to 37°C and SdiA expression was induced with 400 μM IPTG for 20 min in either the presence of AHLs by adding 10 μM 3-oxo-C_6-HSL or 10 μM 3-oxo-C_8-HSL, or absence of AHLS by adding an

equivalent amount of DMSO. Cells were treated with a final concentration of 200 μg/mL of rifampicin to inhibit the host RNA polymerase for 10 min, and then labeled [35S] methionine was added at final concentration 5 μCi/mL. After 10 min 5 mM of non-labeled methionine were added to stop incorporation of the radiolabeled amino acid and 10 mL of the culture was collected at 0, 5, 10, 30, 60 min post-addition of the cold methionine. Samples were pelled and lysed with 250 μL of lysis buffer (50 mM Tris-base buffer at pH 8.5, 300 mM NaCl, 1 mM EDTA, 5 mM imidazole, 5 mM 2-β-mercaptoethanol, and 5 % glycerol, 1 mg/mL lysozyme, and 0.1 mg/mL PMSF) for 1 hr at 4°C. Cell lysates were collected by centrifugation (13,000g for 30 min) and 30 μL of the samples were electrophoresed in 12 % SDS-PAGE and analyzed using the Storm PhosphorImager.

Size-exclusion chromatography−multiangle laser light scattering (SEC−MALLS)

To determine the absolute molecular weight of SdiA purified in the absence of AHL, SEC-MALLS experiment was performed using the mini-DAWN Treos static light scattering instrument (Wyatt) equipped with an in-line refractive index detector. SdiA was expressed and purified as described above. 400 μL sample containing 60 μM of SdiA were injected onto a Superdex 75 (10/300) analytical gel filtration column to separate oligomeric species and protein aggregates. Molecular mass determinations were subsequently determined via in-line MALLS detection and calculated using Wyatt Astra software.

Intracellular concentration of SdiA

Wild type EHEC grown aerobically at 37°C in LB overnight were diluted at 1:100 in LB and grown to an OD_{600} of 0.5. Cells were pelleted for 10 min at 4,000 rpm at 4°C and lysed at

room temperature rocking in 250 μL of urea lysis buffer for 2 hr. Lysed cells were pelleted by centrifugation at maximum speed for 30 min at room temperature. A total of 25 μL of each sample was loaded in duplicates and electrophoresed on a 12 % SDS-PAGE gel along with a standard concentration of purified SdiA. Samples were subjected to immunoblotting as previously described [105]. Blots were probed with a custom rabbit polyclonal antibody to SdiA (1:1,500) generated by Cocalico Biologicals and visualized with SuperSignal West Femto Maxium Sensitivity Substrate (Thermo Scientific). The band intensities were quantified using Image J. For the standard, the calculated intensities were graphed on the x-axis and the amount of SdiA on the y-axis. A best-fit exponential curve was graphed and the equation derived from the standard was used to calculate the amount of SdiA present in each sample. The total amount of SdiA in a single cell was calculated by dividing the total amount of SdiA in the lysate by the total input cells. To get the molar concentration of SdiA in a cell, the total grams of SdiA per cell was divided by the approximate volume of an *E. coli* cell (0.6-0.7 mL).

Structure determination of SdiA

Full-length SdiA was expressed, purified, and crystallized in the absence of AHL. As judged by its elution volume on gel filtration chromatography, SdiA exists as a dimer in solution. Although SdiA can be purified at typical protein concentrations for crystallization, the best crystals of SdiA were obtained by hanging drop vapor diffusion method on SdiA (1.0-1.5 mg/mL) at 4°C; initial crystals appeared within two to three days and grew to their full size in about two weeks. These crystals consistently diffract X-ray between 3.5-3.1 Å using synchrotron radiation and belonged to space group $P6_522$. Experimental phase was obtained by SAD phasing using crystals of seleno-methionine derived SdiA. To obtain the SdiA-AHL

complex, purified protein was incubated with 5 mM of either 3-oxo-C_6-HSL or 3-oxo-C_8-HSL for one hr on ice prior to crystallization. These crystals diffracted X-ray to 2.8 Å and belonged to space group $P2_12_12$. The SdiA-AHL complex was determined by molecular replacement using the AHL-free SdiA structure as the search model. Diffraction data were processed using HKL2000 and the structures were built in Coot and refined in PHENIX; statistics for these structures are summarized in Table 4. The SdiA crystal without AHL contains only one subunit per asymmetric unit and therefore its molecular dyad coincides with a crystallographic 2-fold symmetry. The SdiA-AHL complexes contain three subunits in an asymmetric unit with one of the subunits forming a dimer with its crystallographic symmetry-related partner.

Endogenous ligand extraction/identification

SdiA was purified from six liters cultures of YNN07 as described above and eluted in 300 mL of elution buffer. Endogenous ligand was extracted from the purified SdiA three times with ethyl acetate at 1:1 volume ratio. The combined organic phase was concentrated under reduced pressure. The residue was then partitioned between H_2O and *n*-hexane. The aqueous phase was further extracted sequentially with $CHCl3_3$ and 1-butanol. The $CHCl_3$ soluble portion was further purified via reversed phase HPLC (Phenomenex, C_{18}, 250 x 10.0 mm, 2.5 mL/min, 5 mm, UV 240 nm) using a gradient solvent system from 10 % CH_3CN to 100 % CH_3CN (0.1 % FA) over 40 min. Eight fractions were collected across 5 min time integrals. Fraction four was further purified via reversed phase HPLC (same column as before) with a gradient solvent system from 20 % CH_3CN to 75 % CH_3CN (0.1 % FA) over 30 min to afford (2,3)-dihydroxypropyl octanoate as a colorless residue. ^{1}H NMR (600 MHz, CD_3OD): d 4.12 (dd, J = 11.4, 4.3 Hz, 1H), 4.07 (dd, J = 11.4, 4.3 Hz, 1H), 3.79 (m, 1H), 3.53 (dd, J = 2.9, 2.5

Hz, 1H), 2.32 (t, *J* = 7.4 Hz, 2H), 1.58 (m, 2H), 1.27 (m, 8H), 0.87 (t, *J* = 6.9 Hz, 3H). 13C NMR (125 MHz, CD_3OD): d 174.0, 71.2, 66.2, 64.0, 36.2, 34.9, 28.5, 28.1, 25.7, 23.6, 14.4

Table 2. Strains and plasmids

Strain or plasmid	Relative genotype or description	Ref
86-24	Wild type EHEC strain (serotype O157:H7)	
DH1	86-24 Δ*sdiA* mutant	[2]
YNN01	86-24 Δ*stx2a*	[107]
YNN02	YNN01 λP_R	[107]
YNN03	YNN01 λP_R-*yenI*	[107]
YNN04	86-24 chromosomal 3xFLAG-tagged SdiA	This study
YNN05	86-24 with pJZ410 and pYN1	This study
YNN06	DH1 with pYN2	This study
YNN07	DH1 with pYN3	This study
YNN08	DH1 with pYN4	This study
YNN09	DH1 with pYN5	This study
YNN10	DH1 with pYN6	This study
YNN11	DH1 with pYN7	This study
YNN12	DH1 with pYN8	This study
YNN13	DH1 with pYN9	This study
YNN14	DH1 with pYN10	This study
YNN15	DH1with pYN11	This study
pBAD/Myc-HisA	Used for expression of 6xHisMyc-tagged proteins	
pET21a	Used for expression of 6xHisMyc-tagged proteins	
pKD3	λRed template plasmid	3
pKD46	λRed helper plasmid (recombinase)	3
pJLD1600	λP_R-*yenI*-FRT-kan-FRT template plasmid	
pJLD500	λP_R-FRT-kan-FRT vector template plasmid	
pCP20	λRed resolvase plasmid	3
pJZ410	Plasmid with a gene encoding for T7 RNA polymerase	5
pSUB11	Template plasmid containing priming and 3xFLAG sequences for chromosomally epitope-tagging SdiA	2
pYN1	WT *sdiA* cloned in pET21	This study
pYN2	WT *sdiA* cloned in pBAD/Myc-HisA	This study
pYN3	S43A *sdiA* cloned in pBAD/Myc-HisA	This study
pYN4	Y63A *sdiA* cloned in pBAD/Myc-HisA	This study
pYN5	W67A *sdiA* cloned in pBAD/Myc-HisA	This study
pYN6	Y71A *sdiA* cloned in pBAD/Myc-HisA	This study
pYN7	D80A *sdiA* cloned in pBAD/Myc-HisA	This study
pYN8	W107A *sdiA* cloned in pBAD/Myc-HisA	This study
pYN9	F52A *sdiA* cloned in pBAD/Myc-HisA	This study
pYN10	F59A *sdiA* cloned in pBAD/Myc-HisA	This study
pYN11	L77A *sdiA* cloned in pBAD/Myc-HisA	This study

Table 3. Oligonucleotide primers used in this study

Name	Sequence	Description
stx2AλRedF	CTTTTTTATATCTGCGCCGGGTCTGGTGCTGATTACTTCAGCCAAAAGG AACACCTGTATGTGTAGGCTGGAGCTGCTTCG	To generate Δ*stx2A* in 86-24 EHEC
stx2AλRedR	CATTAACAGAAGCTAATGCAAATAAAACCGCCATAAACATCTTCTTCAT GCTTAACTCCTCATATGAATATCCTCCTTAG	To generate Δ*stx2A* in 86-24 EHEC
yenIλRed F	AGGGTTTTCCCAGTCACGACGTTGTAAAACGACGGCCAGTGAATCTGTA ATCATAGTCATGATACGACTCACTATAGGGCG	To generate chromosomal integration of the *yenI* gene into 86-24 EHEC
yenIλRed R	AAAACCTTTCGCGGTATGGCATGATAGCGCCCGGAAGAGAGTCAATTC AGGGTGGTGAATGTTGACCATGATTACGCCAAGC	To generate chromosomal integration of the *yenI* gene into 86-24 EHEC
stx2AUP	CTGCATTATGCGTTGTTAGCTCAG	Fwd check primer for Δ*stx2A*
stx2ADown	ATCCGCCGCCATTGCATTAAC	Rev check primer for Δ*stx2A*
lacZF	TGCAAGGCGATTAAGTTGGGTAACG	Fwd check primer for chromosomal integration of *yenI*
IRlacIR	TGTGACCTGGCGTCAGCATTTTAAATCT	Fwd check primer for chromosomal integration of *yenI*
SdiAF-pET21	ATGAATTCATGCAGGATACGGATTTTTTCAGCTGGC	To clone *sdiA* gene into pET21 expression vector
SdiAR-pET21	ATGTCGACAATTAAGCCAGTAGCGGCCG	To clone *sdiA* gene into pET21 expression vector
SdiAF-pBAD	ATATATAGATCTCAGGATACGGATTTTTTCAGCTGGC	To clone *sdiA* gene into pBAD/Myc-His expression vector
SdiAR-pBAD	TAGAATTCGCAATTAAGCCAGTAGCGGCCG	To clone *sdiA* gene into pBAD/Myc-His expression vector
R2	CGGGATCCGTATGGACTTGTTGTATGTG	To amplify *ler* promoter fragment from +86
Ler-218F	GCGAATTCCGCTTAACTAAATGGAAATGC	To amplify *ler* promoter fragment from -218
KanF	CCGCGATTAAATTCCAACATGGATGCTG	To amplify fragment of kan gene from pRS551
KanR	GACCATCTCATCTGTAACATCATTGGCAACG	To amplify fragment of kan gene from pRS551
sdiAFLAGF	CACCAAATAAGACCCAGGTTGCCTGTTACGCGGCCGCTACTGGCTTAAT TGACTACAAAGACCATGACGG	To FLAG-tag *sdiA* chromosomally at the C-term
sdiAFLAGR	CAGTATTTTCCAAAACGGTTAAGCAAATTAAACAAGCCTACCGTCAGTA ACATATGAATATCCTCCTTAG	To FLAG-tag *sdiA* chromosomally at the C-term
sdiAUP	CTGATCGAAGACGAAACCCAGGCT	Fwd check primer for chromosomal FLAG-tag *sdiA*
sdiADOWN	CGGAAGGGAAGACATCAGCAGAGATAG	Rev check primer for chromosomal FLAG-tag *sdiA*
sdiARTF	GGCCGCGTAACAGGCAACCT	Fwd qRT-PCR for *sdiA*
sdiARTR	CGCGCACATGGTTTACGCCG	Rev qRT-PCR for *sdiA*

Table 3. Oligonucleotide primers used in this study (continue)

Primer	Sequence	Description
S43A	CAGCTGGAGTACGATTACTATGCGTTATGTGTCCGC	To generate S43A *sdiA* point mutant
S43A_antisense	GCGGACACATAACGCATAGTAATCGTACTCCAGCTG	To generate S43A *sdiA* point mutant
Y63A	ACCTAAAGTGGCTTTCTACACCAATGCCCCTGAGGCGTGG	To generate Y63A *sdiA* point mutant
Y63_antisense	CCACGCCTCAGGGGCATTGGTGTAGAAAGCCACTTTAGGT	To generate Y63A *sdiA* point mutant
W67A	CACCAATTACCCTGAGGCGGCGGTTAGTTATTATCAGGCA	To generate W67A *sdiA* point mutant
W67A_antisense	TGCCTGATAATAACTAACCGCCGCCTCAGGGTAATTGGTG	To generate W67A *sdiA* point mutant
Y71A	CAATTACCCTGAGGCGTGGGTTAGTTATGCTCAGGCAAAAAACTTTC	To generate Y71A *sdiA* point mutant
Y71A_antisense	GAAAGTTTTTTGCCTGAGCATAACTAACCCACGCCTCAGGGTAATTG	To generate Y71A *sdiA* point mutant
D80A	AAAAAACTTTCTCGCAATTGCTCCGGTGCTGAACCC	To generate D80A *sdiA* point mutant
D80A_antisense	GGGTTCAGCACCGGAGCAATTGCGAGAAAGTTTTTT	To generate D80A *sdiA* point mutant
W107A	TTCAACGAAGCACAGCCGTTAGCAGAAGCCGCGCG	To generate W107A *sdiA* point mutant
W107A_antisense	CGCGCGGCTTCTGCTAACGGCTGTGCTTCGTTGAA	To generate W107A *sdiA* point mutant
F52A	CACTTTAGGTCGAGTGGCTGGTACCGGGTGGCGG	To generate F52A *sdiA* point mutant
F52A_antisense	CCGCCACCCGGTACCAGCCACTCGACCTAAAGTG	To generate W52A *sdiA* point mutant
F59A	CTCAGGGTAATTGGTGTAGGCAGCCACTTTAGGTCGAGTG	To generate F59A *sdiA* point mutant
F59_antisense	CACTCGACCTAAAGTGGCTGCCTACACCAATTACCCTGAG	To generate F59A *sdiA* point mutant
L77A	CACCGGATCAATTGCGGCAAAGTTTTTTGCCTGATAATAACTAACCCA	To generate L77A *sdiA* point mutant
L77A_antisense	TGGGTTAGTTATTATCAGGCAAAAAACTTTGCCGCAATTGATCCGGTG	To generate L77A *sdiA* point mutant

Table 4. Data collection and refinement statistics

Crystal ID	SdiA-SeMet	SdiA-OC6	SdiA-OC8
Data collection:			
Space group	$P6_522$	$P2_12_12$	$P2_12_12$
Molecules/ASU	1	3	3
Cell dimensions			
a, b, c (Å)	129.9, 129.9, 125.7	88.6, 92.7, 119.7	88.5, 92.4, 119.0
α, β, γ (°)	90, 90, 120	90, 90, 90	90, 90, 90
Wavelength (Å)	1.083	1.000	1.000
Resolution (Å)[a]	50.00-3.10 (3.15-3.10)	50.00-2.85 (2.90-2.85)	50.00-2.85 (2.90-2.85)
R_{merge} (%)	13.8	10.3	9.6
I/σI	44.7 (2.3)	20.2 (1.6)	22.4 (2.1)
Completeness (%)	100.0 (100.0)	99.5 (97.7)	99.9 (99.7)
Redundancy	28.4 (29.3)	5.0 (4.5)	5.0 (5.0)
Wilson B-factor	95.5	66.0	69.1
Refinement			
Resolution (Å)	45.16-3.10 (3.21-3.10)	41.55-2.84 (2.94-2.84)	41.47-2.84 (2.94-2.84)
No. reflections	11898 (1147)	23706 (2142)	23559 (2229)
R_{work}/R_{free} (%)[b]	19.70 / 23.91	21.97 / 25.13	23.44 / 27.12
No. atoms			
Protein	2012	5844	5873
Ligands	37	60	51
Water	5	7	8
B-factors			
Protein	92.4	74.3	77.8
Ligands	114.9	81.0	65.3
Water	87.0	50.8	63.9
R.m.s. deviations			
Bond lengths (Å)	0.006	0.009	0.004
Bond angles (°)	0.75	1.07	0.90
Ramachandran favored (%)	95.0	97.0	96.0
Ramachandran outliers (%)	0.0	0.14	0.14

[a] Highest resolution shell is shown in parenthesis.

[b] 5% of data wa set aside for calculation of R_{free}.

CHAPTER THREE

STRUCTURAL AND MECHANISTIC ROLES OF NOVEL CHEMICAL LIGANDS ON QUORUM SENSING TRANSCRIPTION REGULATORS

INTRODUCTION

Chemical signaling is an effective means for cells to communicate. Chemical communication amongst bacterial cells occurs through QS. The LuxR/I type QS systems are commonly seen in most Gram-negative Proteobacteria, where the signaling molecule is usually an endogenously produced AHL. These bacteria encode both the LuxI synthase that produces the AHL signal and the cognate LuxR transcription factor whose function is regulated by AHL [81,113,114]. AHLs have a conserved homoserine lactone ring connected through an amide bond to a variable acyl chain. Acyl-chains vary in length and modification of the third position, and variations in acyl chains ensure differential AHL recognition by specific LuxRs [1]. Since the first discovery of the prototypical LuxR/I system in *V. fischeri* [77], more than 50-species have been shown to contain LuxR/I homologs, regulating diverse biological processes [115]. However, some bacteria, such as *E. coli* and *Salmonella*, contain only the LuxR-type protein, SdiA, but not the LuxI-type synthase. While these orphan LuxR proteins can sense exogenous AHLs from other bacteria, they have also been shown to regulate gene transcription in the absence of AHLs [3,6]. This observation contradicts the conventional view that AHLs are necessary for stabilizing LuxR and regulating its function, and raises the possibility that the orphan LuxR sensors can detect and respond to other endogenous, non-AHL chemical signals.

Although extensive structural and functional studies have been performed on LuxR proteins, their AHL-dependent and -independent regulatory mechanisms remain largely unknown. It is also noteworthy that certain LuxR-type proteins are inhibited by AHLs [116]. One major reason can be attributed to the instability of LuxR proteins in the absence of AHLs. To date there are four full-length structures of the LuxR proteins [117-121] and two AHL-binding domains [82,122] that have been solved. There is no structural information on a LuxR protein both in the absence and presence of AHL, to assess the role of ligand-binding in the function of these proteins.

RESULTS

To reveal the structural basis of AHL-dependent and independent function of LuxRs, we performed structural studies on the EHEC orphan LuxR protein, SdiA, in the presence and absence of AHL. Previous studies suggested that AHL binding is essential for the stabilization and homodimerization of LuxR [76]. Purified SdiA, on the other hand, forms a stable dimer in solution even in the absence of AHLs (Figure 7). As dimerization is a requirement for LuxR family transcription factors to bind and regulate their target genes, this observation suggests that even in the absence of AHLs, SdiA is likely in a DNA-binding conformation, consistent with the observation that SdiA regulates subsets of genes in the absence of an AHL signal [2,83]. SdiA is known to have high sensitivity to AHL molecules with a keto modification at the third carbon and an acyl chain length of six to eight [70]; we therefore crystalized SdiA in complex with two AHLs: 3-oxo-C_6-HSL and 3-oxo-C_8-HSL, as well as in the absence of AHL, and determined their structures to 2.8 Å, 2.8 Å and 3.1 Å, respectively.

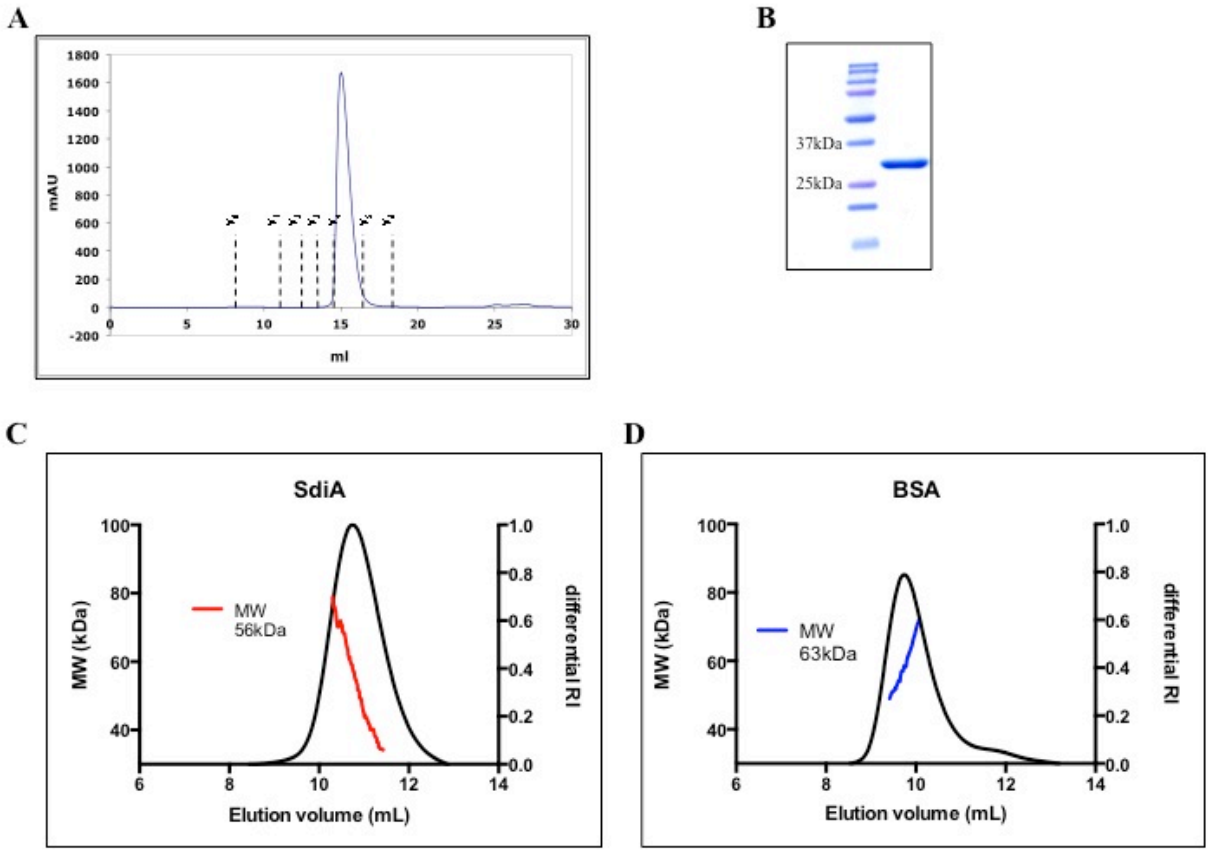


Figure 7. (A) Gel-filtration chromatography of SdiA showing that the protein is in a dimer form (56 kDa). Samples were ran on Superdex 200 10/300 GL column. The monomer of SdiA is 28 kDa. V_0 = volid volume, V_1-$_6$ are standards; V_1 = Apoferritin 443 kDa, V_2 = Amylase 200 kDa, V_3 = Alcohol dehydrogenase 15 0kDa, V_4 = Albumin 66 kDa, V_5 = Carbonic anhydrase 29 kDa, V_6 = Cytochrome C 12.4 kDa. (B) SDS-PAGE analysis of the eluted fraction. SEC-MALLS measurements of SdiA (C) and BSA control (D). In the absence of AHL, SdiA is predominately dimeric (calculated molecular mass of 56 kDa).

As seen in solution, SdiA forms a dimer in the crystals both with and without the AHL ligand, and shares similar overall structure that resembles that of QscR [120]. Here, we use the 3-oxo-C_6-HSL SdiA complex to describe the overall architecture of the dimer (Figure 8A). Each subunit contains an N-terminal ligand-binding domain (LBD), which forms a α-β-α sandwich structure, and a C-terminal 4-helix DNA-binding domain (DBD), which has the classical helix-turn-helix (HTH) DNA binding motif. There are two major dimer interfaces along the two-fold axis. The first one is between the two LBDs and involves residues mainly from the N-terminal ends of helix 1 and 8 (Figure 8B). The other more extensive interaction is predominantly between the helix 12 of each DBD (Figure 8C inset 1 and Figure 9). Some residues from the LBD β-turns also participate in dimerization interactions at this interface. One particularly interesting inter-subunit interaction occurs through the phenyl ring of F52 from the LBD of one subunit intercalating into a hydrophobic pocket consisting of residues from the same subunit (V50, Y233, and A236) and those from the neighboring subunit (A192, A235, and I240) (Figure 8C inset 2). This key-lock interaction is in a strategic position, inter-connecting LBD and DBD, and could relay a conformational change at the LBD to its DBD.

Intriguingly, the two SdiA DBDs in both AHL bound and unbound states align reasonably well with each other (Figure 10 and 11) as well as with those of the DNA-bound TraR dimer, another LuxR-type protein [117,118], indicating that the SdiA dimer adopts a similar DNA binding conformation with or without AHL. This is consistent with ours and others' reports that SdiA can bind to DNA and regulate transcription in the absence of AHLs [2,83,85]. To examine the effect of exogenous AHL on SdiA's function, we performed footprinting of SdiA on the *ler* gene in EHEC (Figure 12). The *ler* gene encodes for a master transcription activator of key EHEC virulence genes, as well as the genetic repertoire EHEC

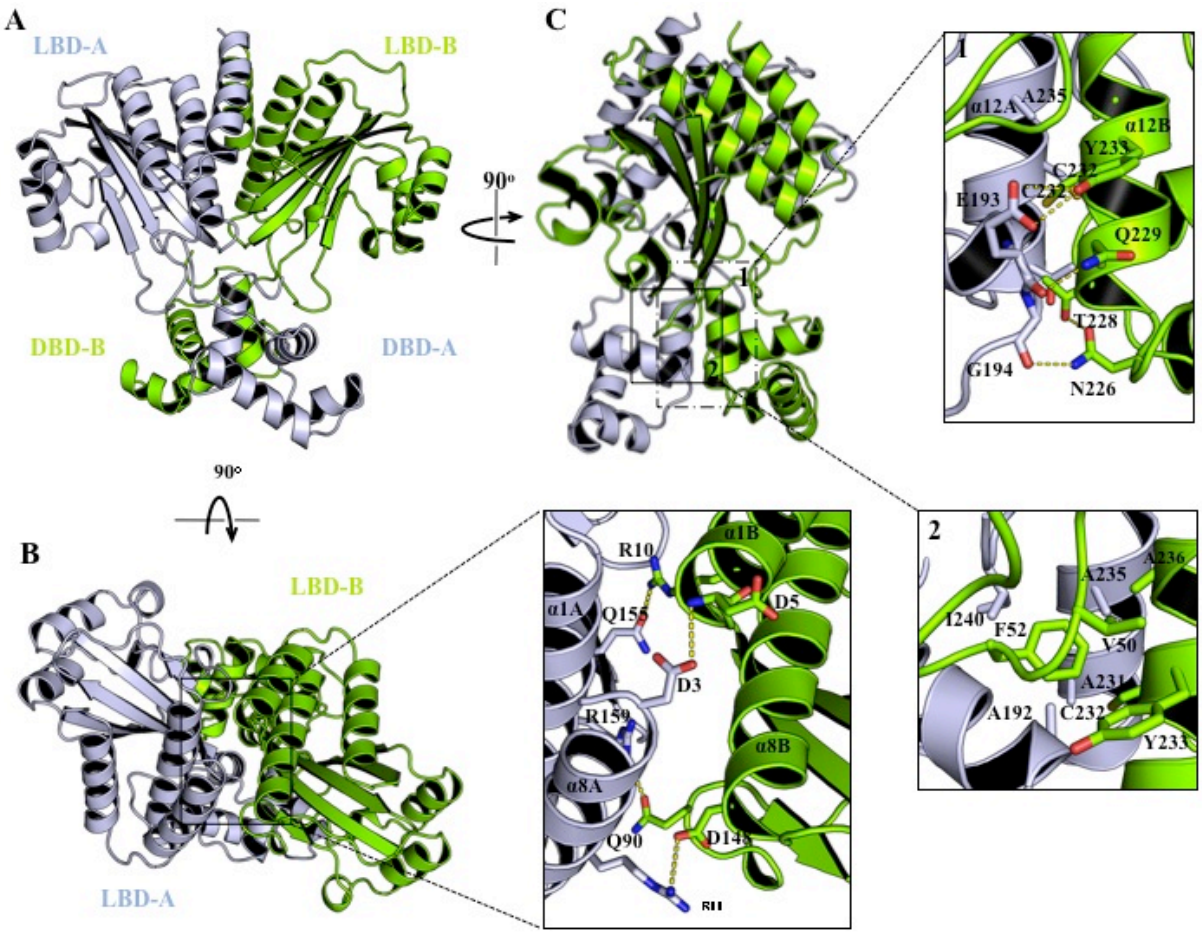


Figure 8. Analysis of the crystal structure of the SdiA dimer using SdiA-3-oxo-C_6-HSL (2.8 Å) for illustration and DNA binding of SdiA-AHL and no AHL. (A) Overall architecture of SdiA colored chartreuse and grey to represent each monomer. (B) View of the ligand binding domain (LBD) and detailed atomic interactions (inset). (C) View of the DNA-binding domain (DBD) and atomic interactions at the DBD interface (inset 1) and the atomic interaction between the LBD and DBD (inset 2).

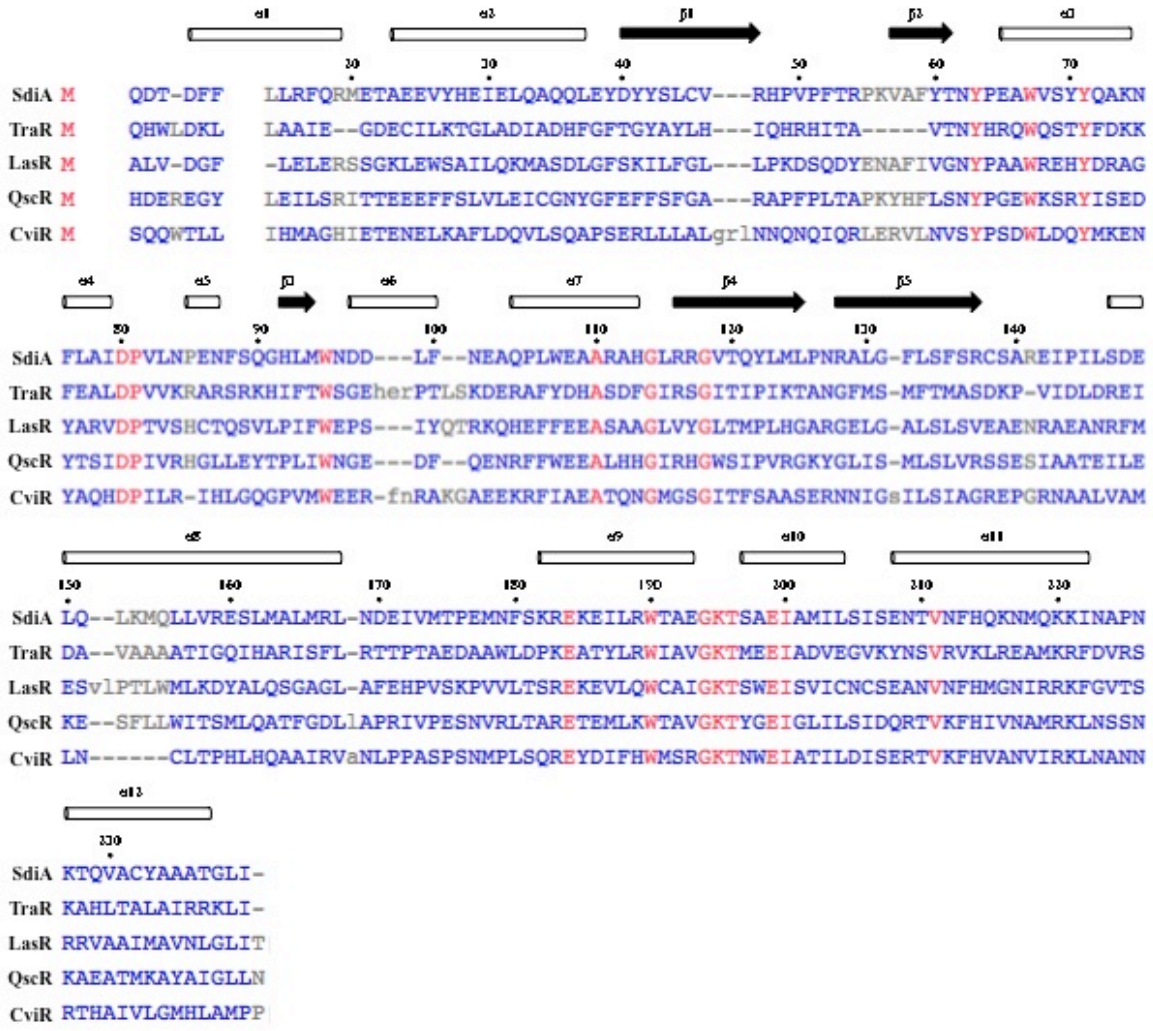


Figure 9**.** Structure-based sequence alignment of SdiA, TraR, LasR, QscR, and CviR. Shown above the aligments are elements of the secondary strucutre of SdiA. The numbering shown is from SdiA. Identical residues are in red. The organisms and accession codes for the sequences are as follows: *sdiA*, *E. coli* O157:H7 (gi:15802351), *traR*, *A. tumefaciens* (gi:3377775), *lasR* and *qscR*, *P. aeruginosa* (gi:15596627 and gi:15597095), and *cviR*, *Chromobacterium violaceum* (gi:288973234).

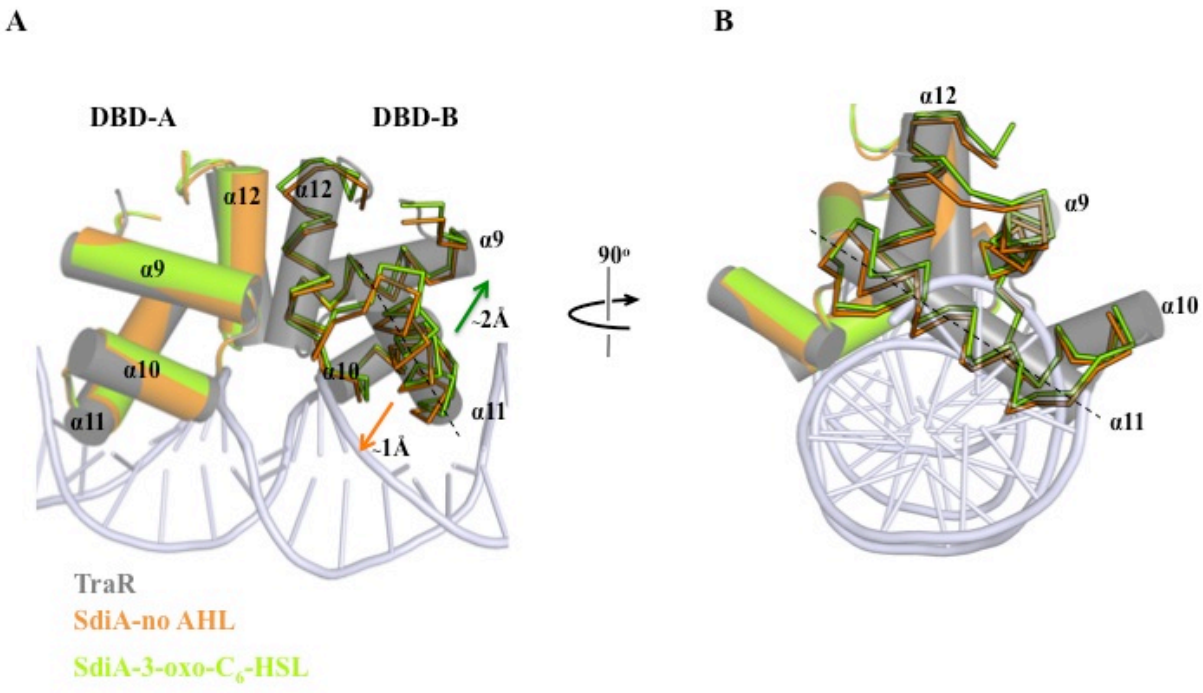


Figure 10. Analysis of the crystal structure of the SdiA dimer using SdiA-3-oxo-C_6-HSL (2.8 Å) for illustration and DNA binding of SdiA-AHL and no AHL. (A) The DBD of SdiA-no AHL (orange cylinders and ribbon) and SdiA-3-oxo-C_6-HSL (chartreuse cylinders and ribbon) are aligned to the DBD of TraR (grey cylinders) and its co-crystallized DNA. Helices α10 and α11 form the HTH motif on SdiA involved in DNA binding, with α11 positioned in the major groove of the modeled DNA. Relative to the DBD of TraR, the DBD of SdiA-OCL moves down toward the major groove of DNA by ~1 Å whereas the DBD of SdiA-3-oxo-C_6-HSL tilts up toward α9 by ~2 Å. (B) View of the DBD of TraR and SdiA through the vertical axis of the modeled DNA. (C) The DBD of SdiA-3-oxo-C_6-HSL (chartreuse cylinders and ribbon) and SdiA-3-oxo-C_8-HSL (yellow cylinders and ribbon) are aligned to the DBD of TraR (not shown) and its co-crystallized DNA.

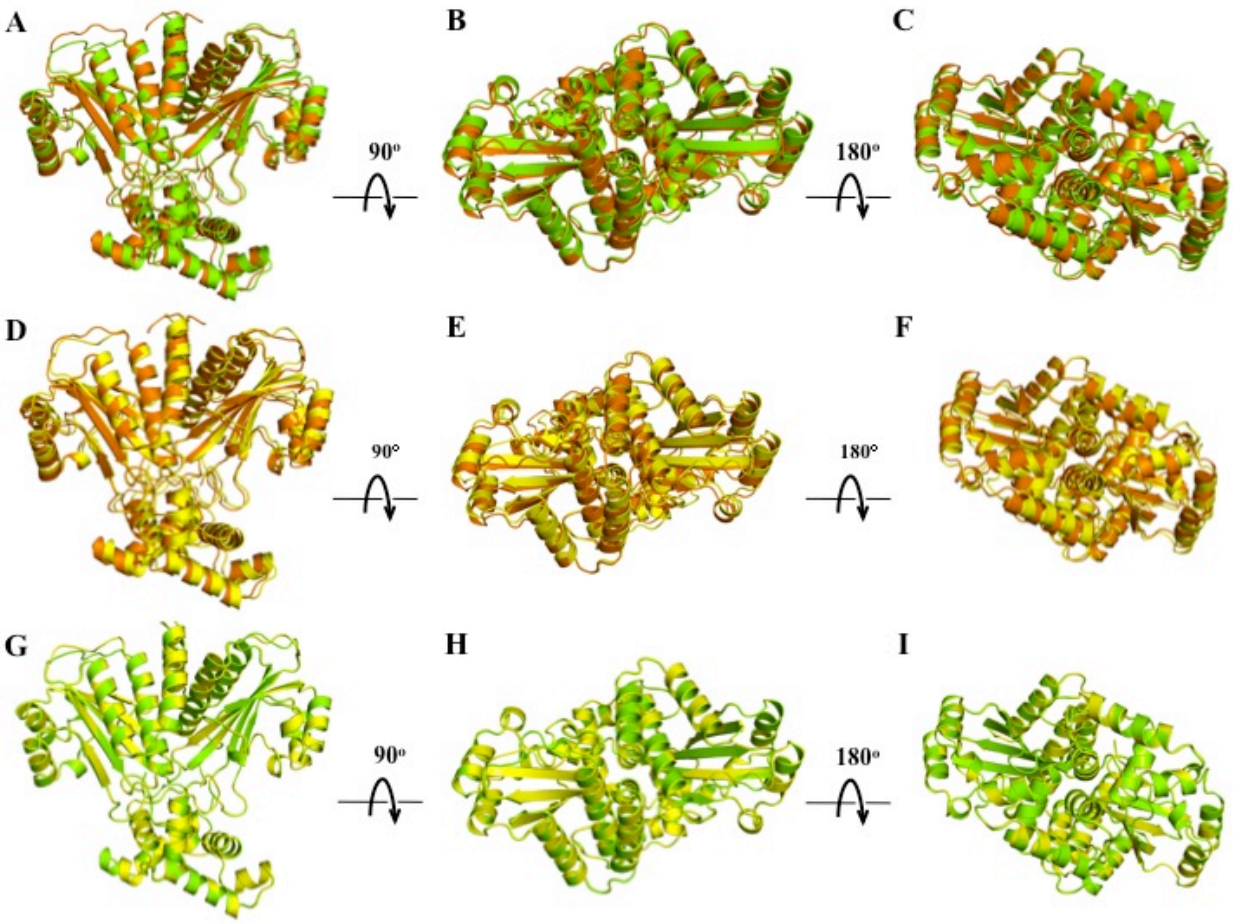


Figure 11. Structural alignment between SdiA dimers without AHL (orange) and (A) with 3-oxo-C_6-HSL (chartreuse), without AHL and (D) with 3-oxo-C_8-HSL (yellow), or (G) with 3-oxo-C_6-HSL and 3-oxo-C_8-HSL. (A), (D), and (G) shows full-length structural alignment. (B), (E), and (H) shows views from the ligand binding domain and (C), (F), and (I) shows views from the nucleotide binding domain.

utilizes to establish colonization in its natural reservoir, cattle [123]. The *ler* gene is transcribed from two promoters in EHEC, P1 and P2 [124], and SdiA-AHL has been previously shown to directly bind to and repress the transcription of this gene [2]. We mapped SdiA-AHL binding to two sites, site 1 is in between P1 and P2, and site 2 overlaps with the P2 promoter, thus preventing transcription of *ler*. The SdiA-no AHL only binds to site 2 (Figure 12). However, the addition of either 3-oxo-C_6-HSL or 3-oxo-C_8-HSL increased the binding affinity of SdiA to the *ler* promoter (Figure 13 A and B). SdiA-AHL had a K_d of 16.5 μM to *ler*, while SdiA without AHL had a K_d of 23.5 μM (Figure 13B). This enhancement of DNA-binding affinity by AHL may allow the protein to bind and regulate transcription of certain genes, which otherwise have much lower affinity for SdiA binding. SdiA-AHL readily binds to both sites in the *ler* promoter as evident by the supershifts of DNA probes with increasing concentration of SdiA-AHL proteins. However, SdiA without AHL is only able to bind to one of these sites, with the supershift being absent without AHL (Figure 13A and C) suggesting, together with the footprinting data (Figure 12), that the SdiA-AHL form can only bind one of these sites. These data are congruent with the small structural shifts in SdiA-AHL compared to SdiA absent of AHL as discussed below (Figure 8 and 11). Although SdiA is already in a DNA-binding conformation in the absence of AHLs, AHLs enhance this protein's affinity to DNA (Figure 11), allowing it to regulate transcription of genes that have lower affinity sites to this protein, whose SdiA regulation only occurs in the presence of AHLs [2].

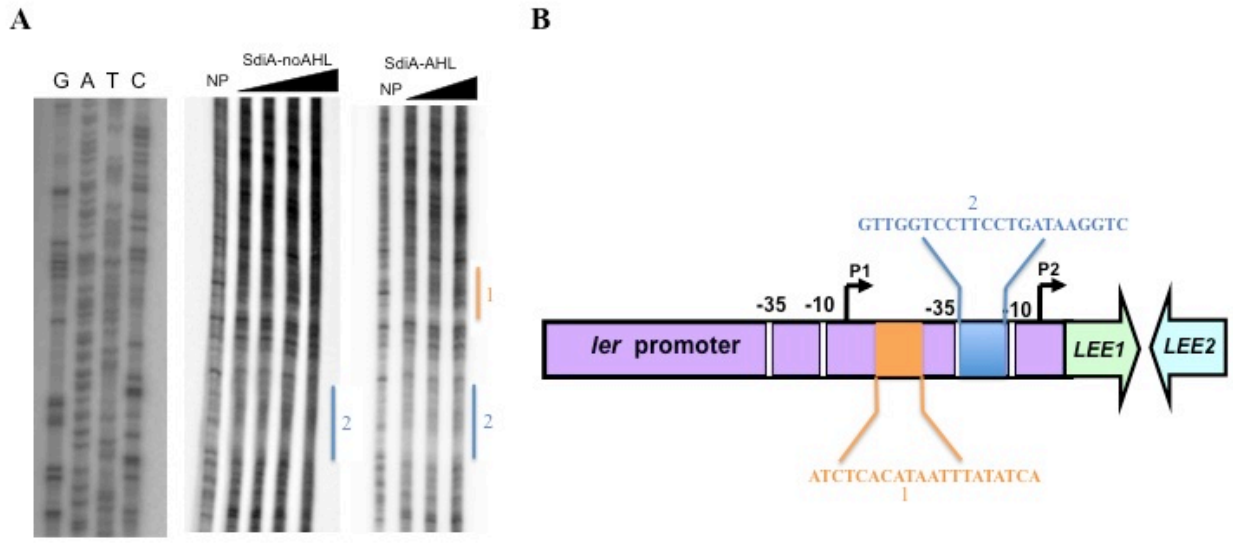


Figure 12. (A) DNase 1 footprinting of the *ler* promoter to predict the binding sites of SdiA. A 350 nt-DNA fragment was end-labeled with ^{32}P and incubated with no or increasing concentration of SdiA (10, 15, 20 μM) in the absence or presence of 3-oxo-C_6-HSL. (B) Illustration of the mapped potential binding sites of SdiA to the *ler* promoter.

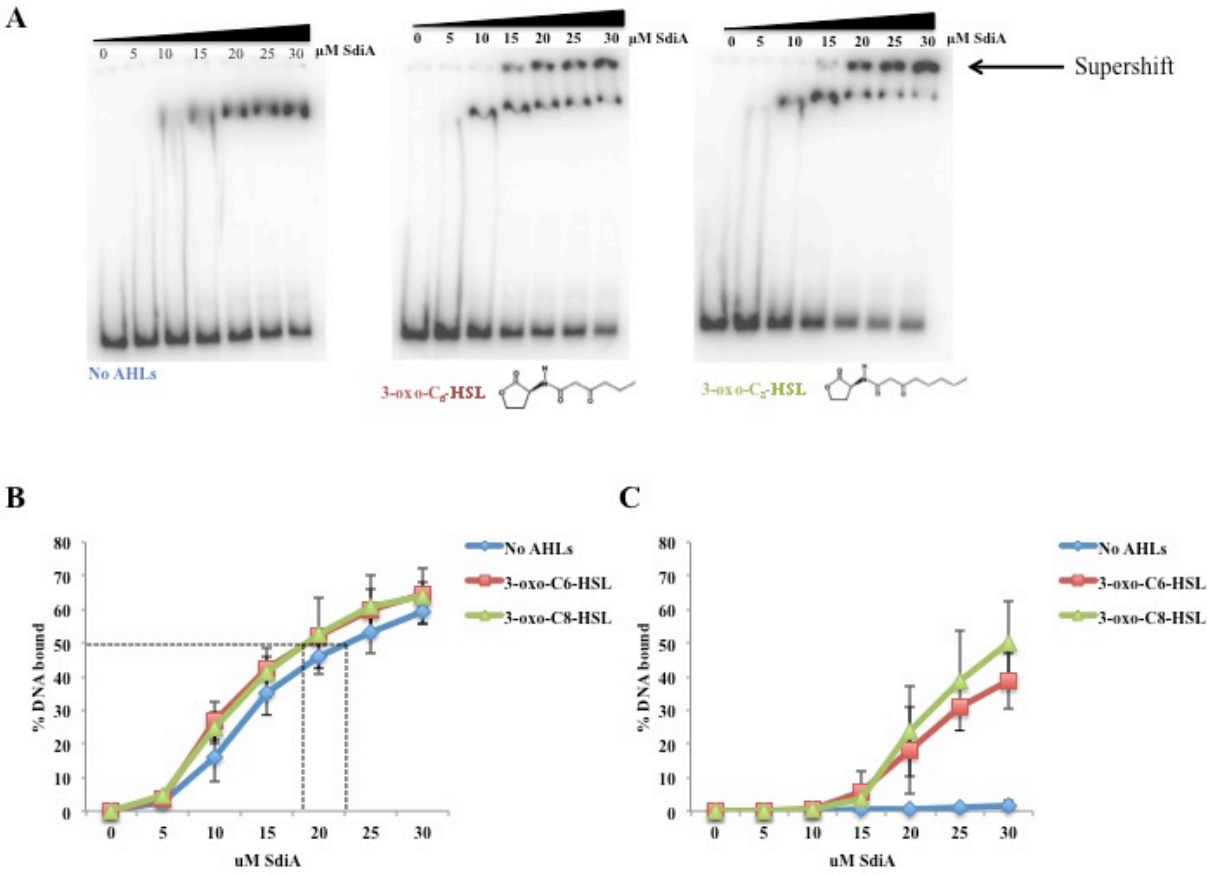


Figure 13. AHLs increase SdiA DNA-binding affinity and stability. (A) Electromobility shift assays to determine the binding affinities of SdiA in the absence or presence of AHLs. A 350 nt-DNA fragment was end-labeled with ^{32}P and incubated with no or increasing concentration of SdiA (0-30µM) in the absence or presence exogenous 3-oxo-C_6-HSL or 3-oxo-C_8-HSL and K_D were calculated in (B). (C) Measurement of the supershift band from SdiA EMSAs in the absence and presence of AHLs.

Consequently, how do we reconcile the observation that AHL has significant functional effects on SdiA but only introduces subtle structural changes as compared to its non-AHL state? In light of the above functional data, the subtle structural change is expected given that the two HTHs have to maintain a similar relative orientation in order to straddle the major grooves of its DNA target. However, the small structural change even at a sub-Å range could be sufficient to weaken the salt-bridges/H-bond between protein residues and DNA bases. Indeed, the two DNA-binding HTHs does show sub-Å conformational change upon AHL binding (Figure 10).

In addition to increasing DNA binding affinity, AHL can significantly stabilize SdiA and prolong its lifetime *in vivo* as was demonstrated in the following two experiments. First, we chromosomally Flag-tagged SdiA in wild type EHEC, and measured endogenous levels of SdiA in the absence (in the DMSO solvent) or presence of increasing concentrations of AHLs (diluted in DMSO). In the absence of exogenous AHLs, only a low amount of SdiA is detected, but is significantly increased when AHLs are present (Figure 14A). In the absence of AHLs the intracellular concentration of SdiA in EHEC is approximately 11,400 molecules per cell (Figure 15). AHLs regulate SdiA post-transcriptionally, as an increase in SdiA protein levels (Figure 14) does not correspond to an increase in *sdiA* mRNA, which is not affected by AHLs (Figure 16). Second, the pulse-chase experiments on SdiA in the presence or absence of AHLs demonstrated that the increased SdiA levels are the result of an enhanced stabilization of SdiA by AHLs (Figure 14B and C), hence decreasing the rate of protein degradation. The DNA binding and protein stability data suggest a double mode of action for AHLs on SdiA activity, by both increasing protein stability and DNA binding.

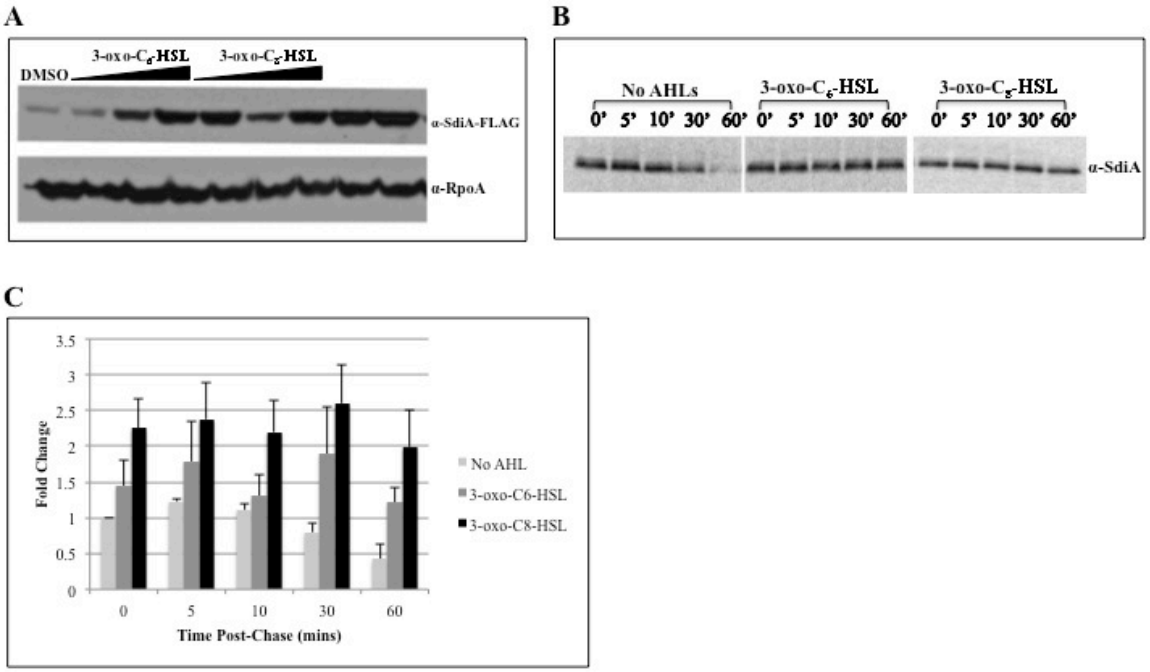


Figure 14. AHLs increase SdiA stability by preventing protein degradation *in vivo*. (A) Western blot of whole cell lysates of wild type EHEC with chromosomally Flag-tagged SdiA grown in DMEM supplemented with either increasing concentration of exogenous AHLs 3-oxo-C_6-HSL and 3-oxo-C_8-HSL or equivalent amounts of DMSO as no the AHLs control to late-log phase. The RpoA was used as a loading control. (B) Wild type EHEC cells expressing SdiA from a phage T7 promoter were treated with rifampicin to block host transcription, [^{35}S] methionine, and excess non-labeled methionine 10 min later to inhibit labeling. AHLs or DMSO were added 30 min before the addition of the radiolabel. Samples were collected at 0, 5, 10, 30, and 60 min after the addition of the non-labeled methionine. Intensity of the bands was quantified using the Storm Phosphorimager (C).

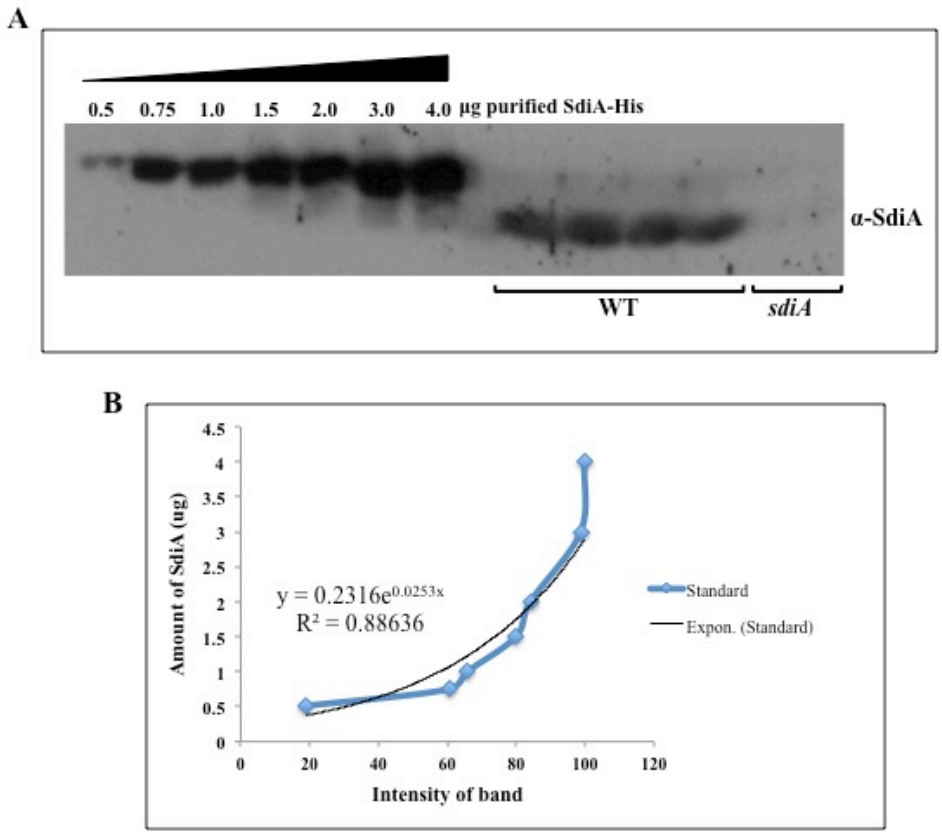


Figure 15. Intracellular concentration of SdiA. **(A)** Western blot of increasing amount of purified SdiA and of whole cell lysates of wild type EHEC grown in LB to OD_{600} of 0.5. Samples shown are duplicates of two biological replicates. Blots were probed with a custom polyclonal antibody against SdiA. (B) The graphs of the standard and of the best-fit exponential curve. Based on the derived equation from the standard curve $y = 0.2316e^{0.0253x}$ the intracellular concentration of SdiA is calculated to be approximately 11,400 molecules per cell or 29 aM.

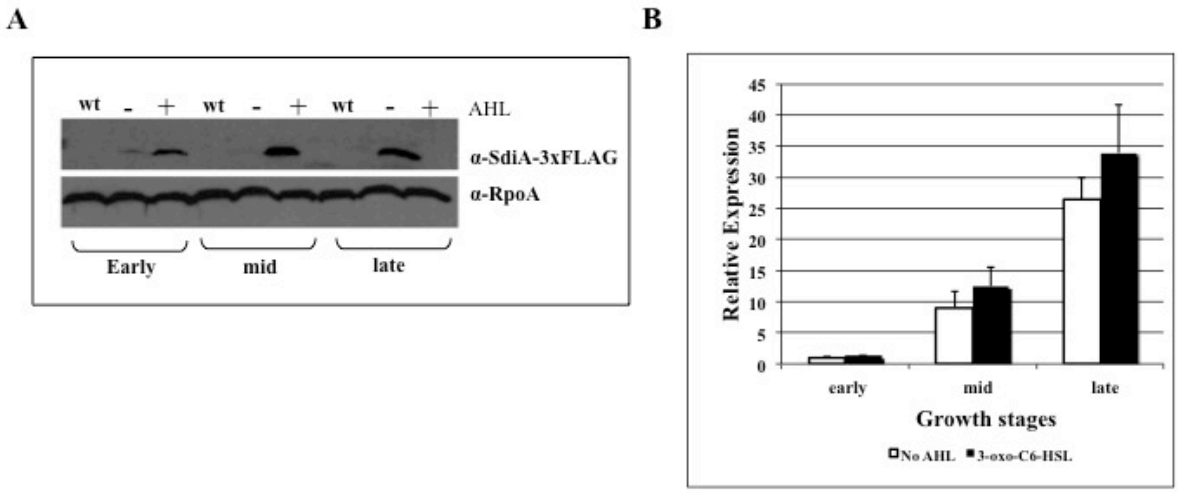


Figure 16. (A) Western blot of whole cell lysates of EHEC with untagged SdiA (WT) and chromosomally flag-tagged SdiA in the absence (DMSO) or presence of AHL grown in DMEM at early-, mid-, or late-log phases. RpoA was used as loading control. (B) The same cells were also collected for RNA extraction and qRT-PCR was performed to measure *sdiA* mRNA expressions. The mRNA levels are graphed as fold changes compared to WT grown in the absence of AHLs.

In addition to the subtle conformational change at the DBD between the structures with and without AHL, careful structural analysis also reveals two novel findings at the LBD. First, there are noticeable structural differences at the ligand binding sites between AHL-bound and the unbound states (Figure 17). AHL binding in SdiA is similar to other AHL-bound LuxR proteins [117,118] with the lactone ring forming specific AHL H-bond interactions with several highly conserved residues and the acyl chain is stabilized by hydrophobic residues. Specifically H-bonds are formed between W67 and the lactone ring, Y63 and the carbonyl on C1, D80 and amine, and S43 and carbonyl on C3. The mono-acyl chain of AHL packs within the cavity through hydrophobic interactions with F59, T61, Y63, Y71, and L77 (Figure 17B). The SdiA proteins complex with 3-oxo-C_6-HSL or 3-oxo-C_8-HSL align perfectly with each other, suggesting that both signals yield proteins in similar conformation (Figure 18). The bound AHL

is occluded from solvent access making the protein-AHL a tightly packed entity. In the AHL-unbound state, on the other hand, the ligand-binding pocket becomes an open chamber that traverses the LBD with solvent exposure on both ends (Figure 17C). Compared to the AHL-bound state, this open chamber formation in the absence of AHL results mainly from side-chain movement of three residues, F59, L77 and W107 (Figure 17D). In going from the AHL-unbound to the AHL bound state, L77 descends by 3.6 Å and F59 tilts by 1.6 Å, respectively, toward the acyl chain of AHL to occlude one end of the ligand-binding pocket, while W107 undergoes nearly a 147^{o} flip into the ligand-binding pocket to cap off the other end of the cavity (Figure 17D). Binding of 3-oxo-C_6-HSL and 3-oxo-C_8-HSL to the LBD is generally conserved, and the H-bond of Y63 with the C3 keto group is clearly important for specific and high affinity binding. The length of the acyl chain that can be accommodated in the hydrophobic pocket seems to be limited by the hydrophobic residues F59 and L77 (Figure 17B). A longer acyl chain would clash with these residues based on the structure of the LBD.

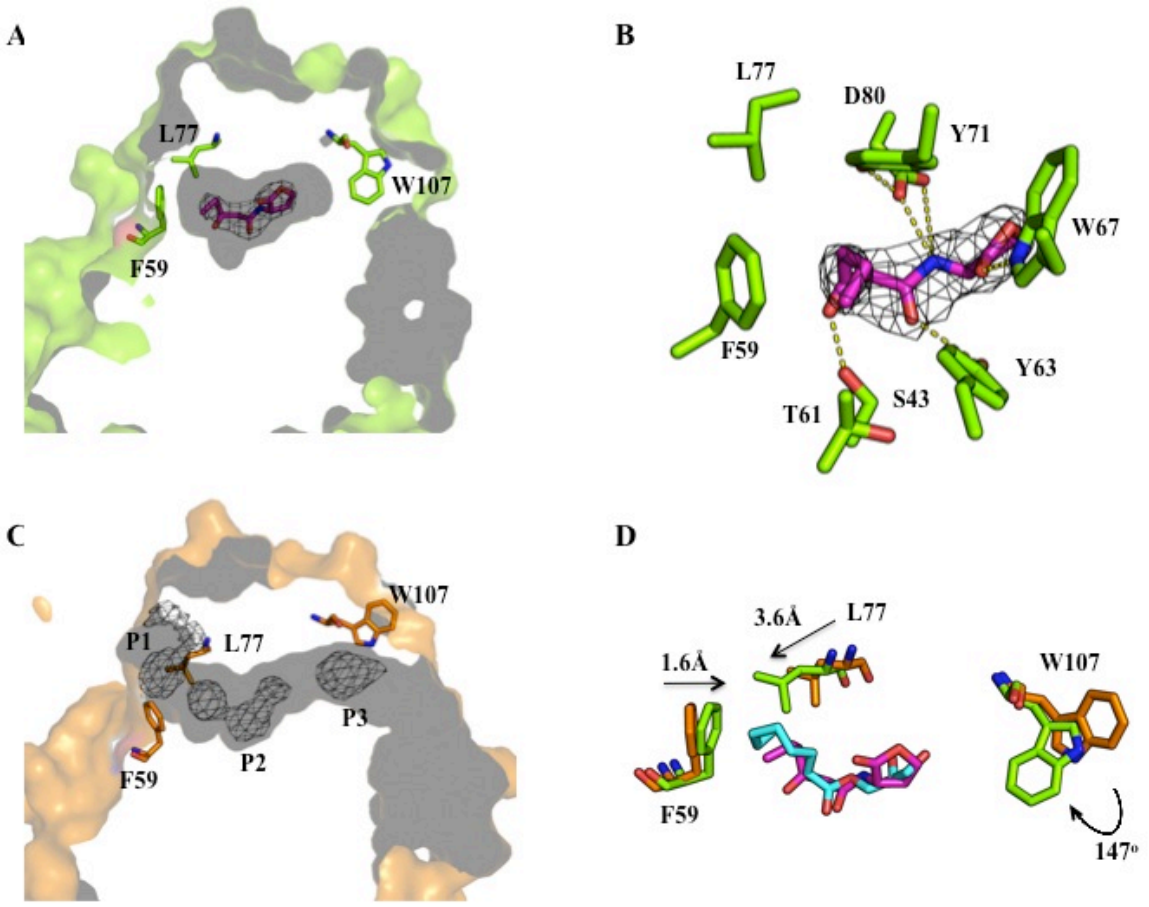


Figure 17. Binding pocket of SdiA-AHL bound and unbound. (A) The bound 3-oxo-C_6-HSL ($2F_o$-F_c electron density map, black mesh, contoured at 1σ) makes the protein-AHL a tightly packed entity. (B) 3-oxo-C_6-HSL forms four hydrogen bonds directly with surrounding residues as indicated by the dash lines. (C) In the absence of AHL the pocket adopts an open conformation that is solvent accessible and traverses the entire length of the cavity. The F_o-F_c map (black mesh), contoured at 3.5σ, shows three distinct electron densities (labeled as P1-P3) occupying the ligand-binding cavity. (D) Three residues contribute to the transition from an open (orange) to occluded (chartreuse) cavity. L77 descends by 3.6 Å and F59 tilts by 1.6 Å, respectively, toward the acyl chain of AHL to occlude one end of the ligand-binding pocket, while W107 undergoes nearly a 147^o flip into the ligand-binding pocket to cap off the other end of the cavity.

The second intriguing finding is that the ligand-binding pocket in the AHL-unbound state is actually not empty or filled with solvent. The structure of SdiA without AHL clearly shows multiple discrete electron density peaks, termed site P1 to P3 from one end to the other, indicating the binding of several non-AHL ligands, which have to come from the *E. coli* cell used for protein expression (Figure 17C). To identify this unknown mass, the ligand was extracted from purified SdiA with ethyl acetate, purified using HPLC, and subjected to NMR analysis. The extracted ligand was identified (Figure 19A) and confirmed (Figure 19B) to be 1-octanoyl-*rac*-glycerol. Upon the identification of the endogenous ligand, we could fit one OCL molecule into the P2 density at the center of the open chamber (Figure 19C). The P1 and P3 density peaks at the two ends of the chamber were modeled as two glycerol molecules. We believe these glycerol molecules could come from the glycerol moieties of the OCL ligands whose acyl chains are disordered. P2 OCL overlaps with the AHL binding site (Fig 19C) and forms H-bonds with D80, S43, and S134 (Figure 19C); P2 OCL appears to have higher occupancy than OCLs at P1 and P3, which could attribute to the partial loss of OCL ligands during the purification and crystallization process.

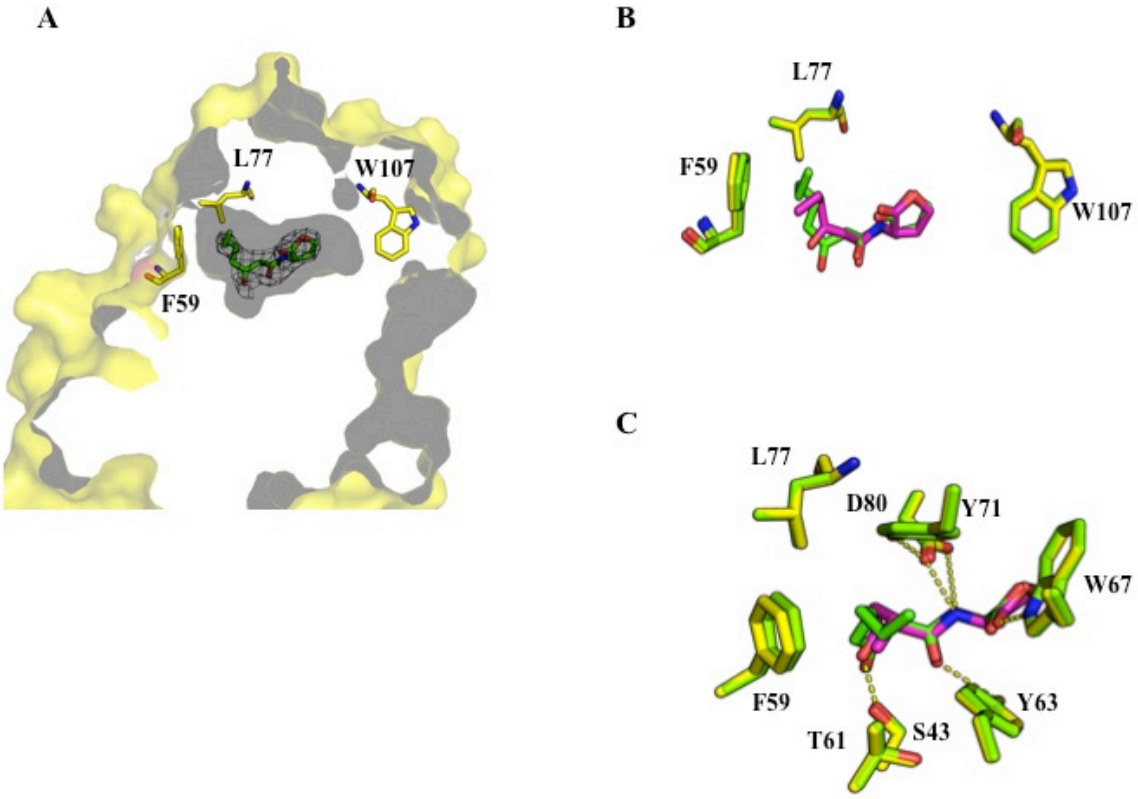
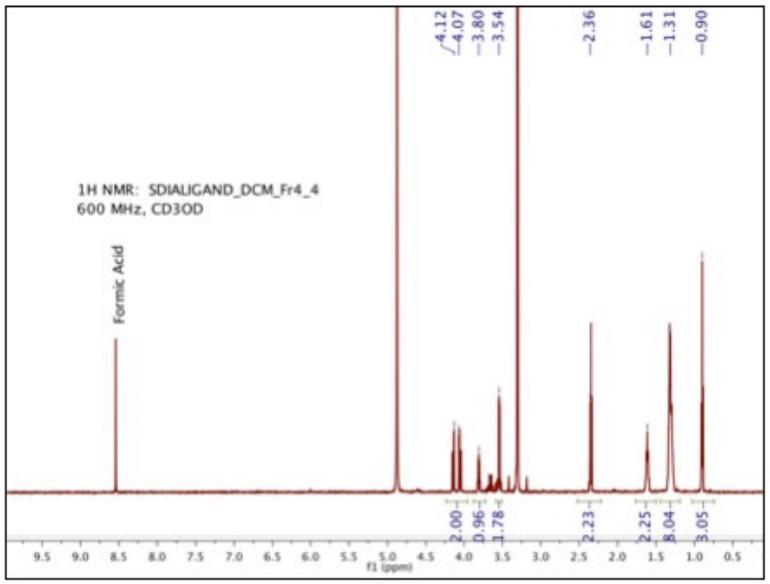


Figure 18. Binding pocket of SdiA-AHL bound. (A) The ligand-binding cavity of SdiA bound to 3-oxo-C_8-HSL (green). In the presence of 3-oxo-C_8-HSL the cavity collapses around the ligand forming an occluded, solvent inaccessible cavity. (B) Similar to SdiA bound to 3-oxo-C_6-HSL (magenta), three residues undergo the greatest conformational change in the transition from an open to a closed cavity when SdiA binds to AHL. (C) The same residues involved in atomic interactions between SdiA and 3-oxo-C_6-HSL are observed in the 3-oxo-C_8-HSL bound SdiA structure.

A



B

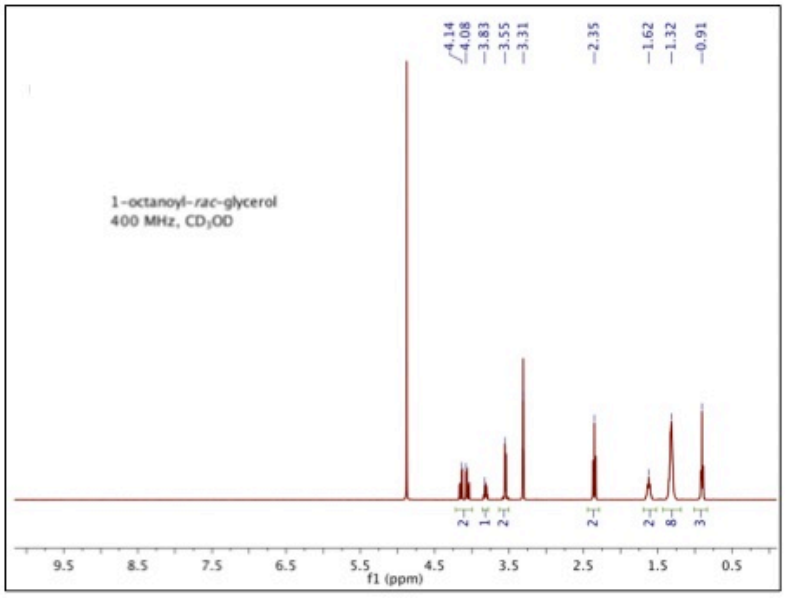


Figure 19. Identification of the endogenous ligand by NMR analysis. Endogenous ligand from *E. coli* is extracted from purified SdiA with ethyl acetate and purified by HPLC. The ligand is identified to be 1-octanoyl-*rac*-glycerol (OCL) (A) The NMR spectra of the purified ligand . (B) The NMR spectra of Sigma compound OCL.

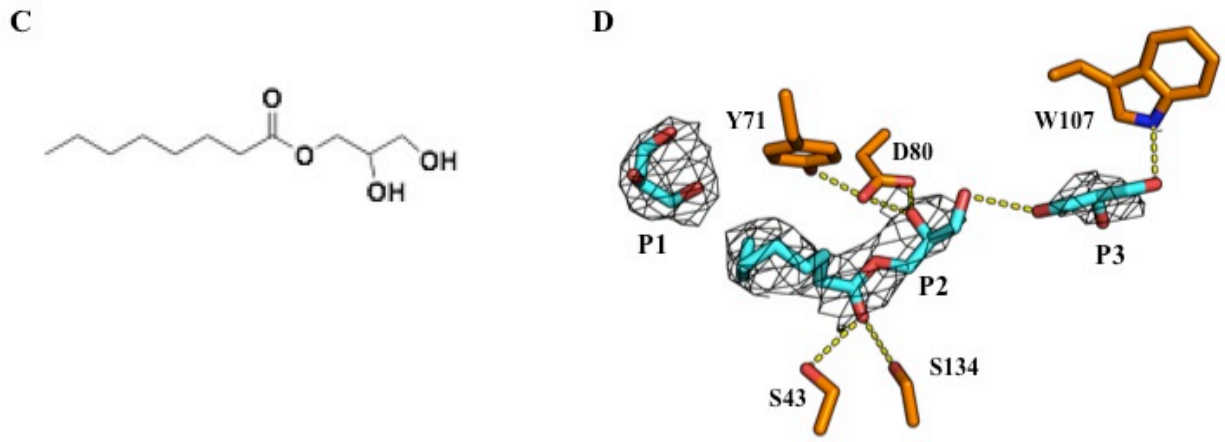


Figure 19. Identification of the endogenous ligand by NMR analysis. (C) The molecular structure of 1-octanoyl-*rac*-glycerol. (D) OCL modeled in the electron density at P2 site with a glycerol at P1 and P2 sites.

DISCUSSION

In summary, the structural studies of SdiA provide insights into the AHL regulation of LuxR proteins. The endogenous-ligand and the open transverse ligand-binding cavity adopted by SdiA in the absence of AHL suggest that SdiA, and potentially other LuxRs, detect non-AHL signals allowing bacterial adaptation to different environments. In a broader sense, the discovery of a monoacylglycerol as an SdiA-ligand breaks new ground in the understanding of LuxR-type proteins. Several LuxR-type proteins have their DNA binding properties inhibited by AHLs [116,125], and it has been largely assumed that these LuxRs are apo-proteins, when in fact they may be complexed with monoacylglycerols, and this could be a "molecular chaperone placeholder" for many LuxRs in the absence of AHLs. 1-octanoyl-*rac*-glycerol is a monoacylglycerol (MG), which are the building blocks of triacylglycerols, and are present in

both eukaryotes and prokaryotes [126]. Monoacylglycerols are highly abundant in the mammalian GI tract, and serve as energy sources, signaling molecules, and substrates for membrane biogenesis [127]. Since *E. coli* and *Salmonella* colonize the gut, these bacteria utilize SdiA to detect self, microbiota or host-derived monoacylglycerols to promote colonization in various eukaryotic hosts [2,4]. Thus far, monoacylglycerols have only been implicated in membrane biogenesis in prokaryotes, and here we show that these molecules can also be used as a chemical chaperone for a protein. Moreover, monoacylglycerols are highly prevalent in the mammalian intestine, suggesting that SdiA-OCL may have AHL-independent functions that are relevant to virulence of intestinal pathogens.

CHAPTER FOUR

THE ACYL-HOMOSERINE LACTONE SYNTHASE YENI FROM YERSINIA ENTEROCOLITICA MODULATES VIRULENCE GENE EXPRESSION IN ENTEROHEMORRHAGIC ESCHERICHIA COLI O157:H7 [δ]

INTRODUCTION

Enterohemorrhagic *E. coli* serotype O157:H7 (EHEC) is a human pathogen which causes complications that range from abdominal cramps and bloody diarrhea to the life-threatening sequelae known as hemolytic uremic syndrome [8-10]. Although EHEC colonizes the large intestine and causes disease in humans, EHEC is a member of the transient normal bovine microbial flora and naturally colonizes the RAJ mucosa of cattle and is then subsequently shed into the environment with the animal's feces [128]. To colonize the host, EHEC forms A/E lesions on epithelial cells [54]. These lesions are characterized by the effacement of the epithelium's microvilli, the intimate attachment of bacteria to the epithelial cells, and the rearrangement of the host actin cytoskeleton to form a pedestal-like structure cupping individual bacterium [129,130]. The majority of the genes required to form A/E lesions are encoded within a chromosomal pathogenicity island known as the LEE [55,56]. The LEE is comprised of 41 genes most of them organized in five major operons [56]. These genes encode for transcriptional regulators [131,132], a type III secretion system [58], the adhesin intimin [133] and its receptor Tir [133], as well as several effector proteins [134-138].

A complex network of proteins and genes has been shown to regulate the LEE, including H-NS [139], GadX [140], Per [132], EtrA, EivF [141], QseA [124], SdiA [142], CpxR [143], LexA [144], Pch [145], Hha [146], and Ler [132,147,148]. Ler, encoded by the first gene in the *LEE1* operon, is the transcriptional master regulator of the other LEE genes [132,147,148]. The nucleoid-associated protein H-NS silences the LEE; however, Ler antagonizes H-NS to overcome silencing and to activate the LEE [139]. Recently, a member of the LuxR protein family, the transcription factor SdiA, was shown to modulate transcription of the LEE by directly repressing the expression of *ler* [2,70].

The first LuxR-I quorum sensing (QS) system was described in *V. fischeri* [77]. Lux I is a synthase while LuxR is a cognate transcription factor. The LuxI synthase produces small chemical signaling molecules known as AHLs that diffuse freely out of the bacteria into the environment. Once an external threshold concentration is reached, AHLs diffuse back into the cells and bind to their cognate cytoplasmic LuxR transcription factor. Ligand binding initiates an increase in LuxR protein stability and also promotes LuxR protein oligomerization [76]. The AHL-LuxR complex then binds to target promoters and regulates their expression [76]. For example, the LuxR-I system of *V. fischeri* activates the production of light by inducing the expression of genes important for bioluminescence [77]. Since this initial discovery, homologs of the LuxR-I system have been found in over 50 bacterial species, including the human pathogens *Y. enterocolitica* and *P. aeruginosa* and the plant pathogens *E. carotovora* and *A. tumefaciens* [76]. Majority of these species encode both a synthase and a transcriptional regulator, but interestingly, a subset of species only encodes for the LuxR homologs but not their cognate LuxI synthases. The LuxR homolog SdiA present in *E. coli* and *Salmonella* spp. is an example of such "orphan" LuxR proteins.

SdiA has been shown to be involved in inter-species communication, as evidenced by the fact that SdiA is able to detect and respond to AHLs produced by other bacteria [2-4,74]. Dyszel and colleagues demonstrated that SdiA from *S.* Typhimurium detects AHLs produced by the pathogen *Y. enterocolitica* in mice. To mimic a constant interaction with *Y. enterocolitica*, Dyszel *et al.* constructed a *S.* Typhimurium strain that constitutively express the *Y. enterocolitica* gene *yenI* that encodes an AHL synthase. SdiA-dependent genes activated by AHLs conferred a fitness advantage in *S.* Typhimurium $yenI^{+}$ $sdiA^{+}$ compared to $yenI^{+}sdiA^{-}$, indicating the importance of SdiA and AHLs in competition within a niche.

Additionally, recent evidence from studies in cattle indicates that EHEC may sense AHLs in an SdiA-dependent manner in order to discern the appropriate niche for bacterial colonization. An investigation of the bovine digestive tract determined that AHLs are present only in the rumen [2,84]. EHEC activates the *gad* acid resistance genes in response to AHLs, likely as a mechanism to safely passage through the subsequent stomach's acidic environment [2]. Conversely, rumen-derived AHLs repress the LEE [2,142]. The rationale for this inhibition is that rumen colonization is unfavorable, and the absence of AHLs in the RAJ, the prominent site of colonization, alleviates SdiA-AHL mediated-repression of the LEE, thus promoting colonization at the RAJ. Ruminants known as "super shedders" shed high numbers of EHEC in their feces over a prolonged period, and EHEC strains isolated from "super shedders" more intimately colonize the RAJ [149]. Epidemiologic studies have shown that "super shedders" account for approximately 95 % of all EHEC shed into the environment [30,150,151]. Since most humans become infected with EHEC by either ingestion of food products contaminated by infected animals [36-39] or through direct contact with infected animals [40-43], understanding how EHEC promotes intimate colonization at the RAJ in its natural host is crucial for

development of preventive strategies to decrease EHEC shedding into the environment and consequent transmission to humans.

In this study, we explore the role of AHLs in colonization of EHEC at the RAJ by constructing an AHL-producing EHEC strain to mimic constant exposure of AHLs in the environment. Our data provides evidence that continuous exposure of EHEC to AHLs decreases A/E lesion formation on epithelial cells *in vitro* but does not prohibit colonization of EHEC at the RAJ in cattle. These data suggest that EHEC colonization at the RAJ is complex with multiple factors contributing to efficient colonization.

RESULTS

The canonical LuxR/LuxI-type quorum-sensing system encodes a transcription factor LuxR and an associated AHL synthase LuxI. Several examples exist, however, where a LuxR regulator lacks an associated AHL synthase. In *E. coli*, for example, the LuxR regulator SdiA functions to regulate several important virulence and colonization genes, yet lacks a cognate synthase. The EHEC homolog of SdiA senses AHLs synthesized by other bacteria rather than self-produced AHLs to modulate virulence gene expression [2]. Previously, we demonstrated that an *sdiA*$^-$ EHEC colonizes the RAJ of cattle with reduced efficiency compared to *sdiA*$^+$ [2], suggesting that SdiA plays an important factor to promote colonization at the RAJ. Additionally, we demonstrated that AHLs repress the LEE pathogenicity island *in vitro*. An absence of AHLs promotes alleviation of SdiA-AHL mediated-repression of the LEE and likely contributes to successful colonization of EHEC at the RAJ. To explore the effect of AHLs on EHEC virulence

and colonization, we engineered an EHEC strain to constitutively express the *Y. enterocolitica* AHL synthase, *yenI* to mimic a constant exposure of AHLs. The United States Department of Agriculture (USDA) prohibits the use of Shiga toxin-producing *E. coli* in cattle

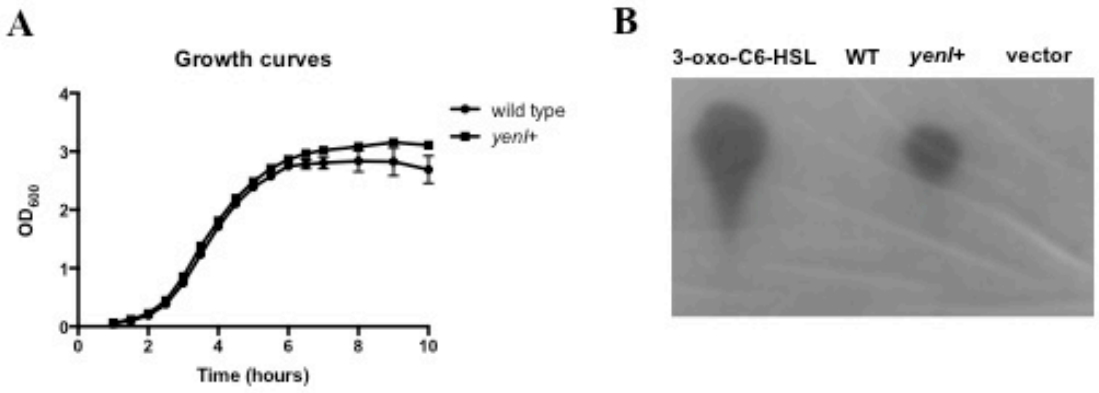


Figure 20. (A) Growth curves between wild type and *yenI*$^+$ grown in triplicates in high-glucose DMEM at 37 °C. Optical density at 600 nm (OD_{600}) were measured at the indicated times. (B) *yenI*$^+$ EHEC produces endogenous AHLs. TLC from AHLs extracted from wild type, *yenI*$^+$, and vector$^+$ strain, and 3-oxo-C_6-HSL was used as a positive control.

experiments; therefore, the *stx2a* gene was deleted from both wild type and *yenI*$^+$ EHEC strains. Initial studies confirmed that the chromosomal integration of *yenI* in EHEC did not affect growth (Figure 20A). Additionally, we confirmed that the *yenI*$^+$ EHEC strain also produced endogenous AHLs as detected by the thin layer chromatography compared to wild type and vector control (Figure 20B).

Endogenous AHLs repress the transcription of the LEE. In response to exogenous AHLs, SdiA represses transcription of the LEE genes by directly binding to the promoter and repressing the master transcriptional regulator *ler*, [2] (Figure 21A). Consistent with our previous results, addition of exogenous AHLs reduced the expression of the LEE. The endogenous production of AHLs in the *yenI*$^+$ EHEC strain decreased expression of *ler, espA,* and *tir* (all of them are LEE genes) to a similar level as the addition of exogenous signal. Transcriptional repression of the LEE in the *yenI*$^+$ strain was dependent on endogenous AHLs since chromosomal integration of the empty vector control had no effect on transcription of the LEE in the absence of exogenous AHL signal (Figure 21B).

***yenI*$^+$ EHEC form less A/E lesions.** Since endogenous AHLs reduced expression of the LEE, we next used fluorescent actin staining (FAS) to investigate whether *yenI*$^+$ EHEC have a reduced ability to form A/E lesions on epithelial cells compared to wild type. HeLa epithelial cells were infected for six hours with wild type, *yenI*$^+$ or vector only EHEC strains. Wild type and vector control EHEC formed A/E lesions on 88 % and 87 % of the HeLa cells, respectively (Figure 22 A and B). In contrast, *yenI*$^+$ produced significantly fewer A/E lesions, only infecting 3 % of the HeLa cells. These results suggest that endogenous AHLs decrease the ability for EHEC to form A/E lesions. Congruently with the decrease in LEE expression (Figure 21) and A/E lesion formation, the *yenI*$^+$ strain was unable to secrete the EspA and EspB LEE-Type three secreted proteins (Figure 22C).

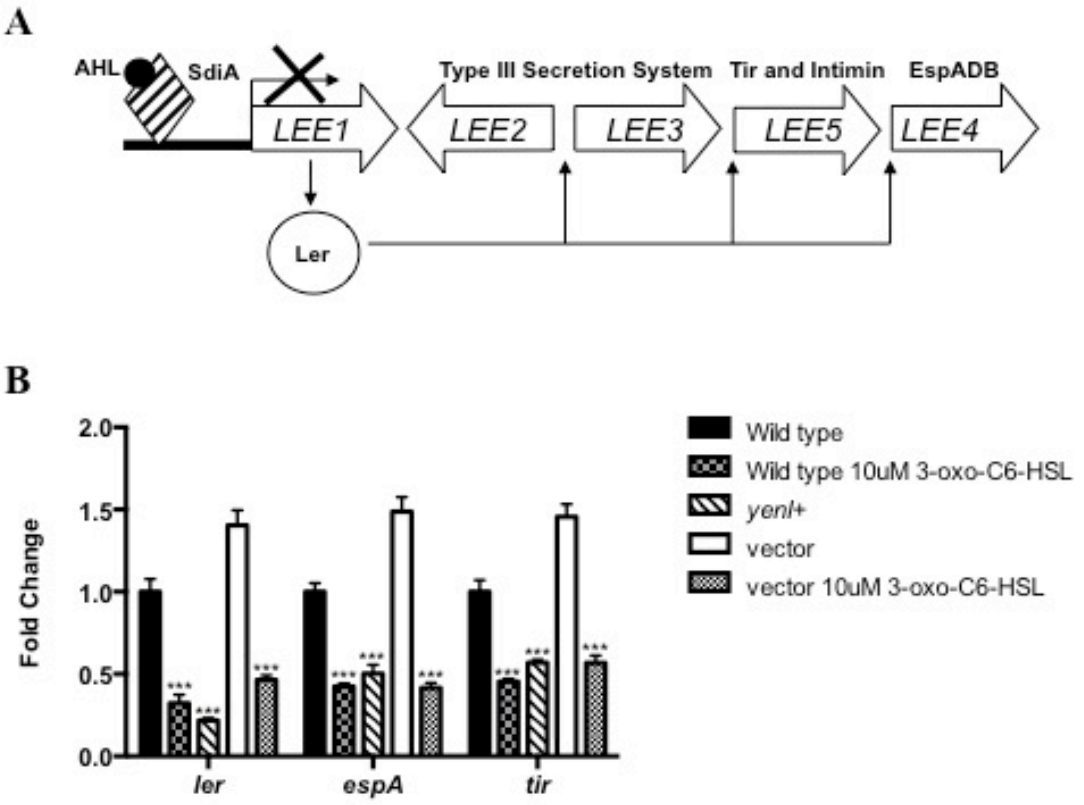


Figure 21. (A) Schematic of SdiA-AHL complex regulation of the LEE genes. (B) qRT-PCR analyses of *ler*, *espA*, and *tir* transcription. The mRNA levels are graphed as fold change compared to WT levels. Statistical significance was determined by Student's *t*-test, and *** indicated *P-value* of ≤ 0.001.

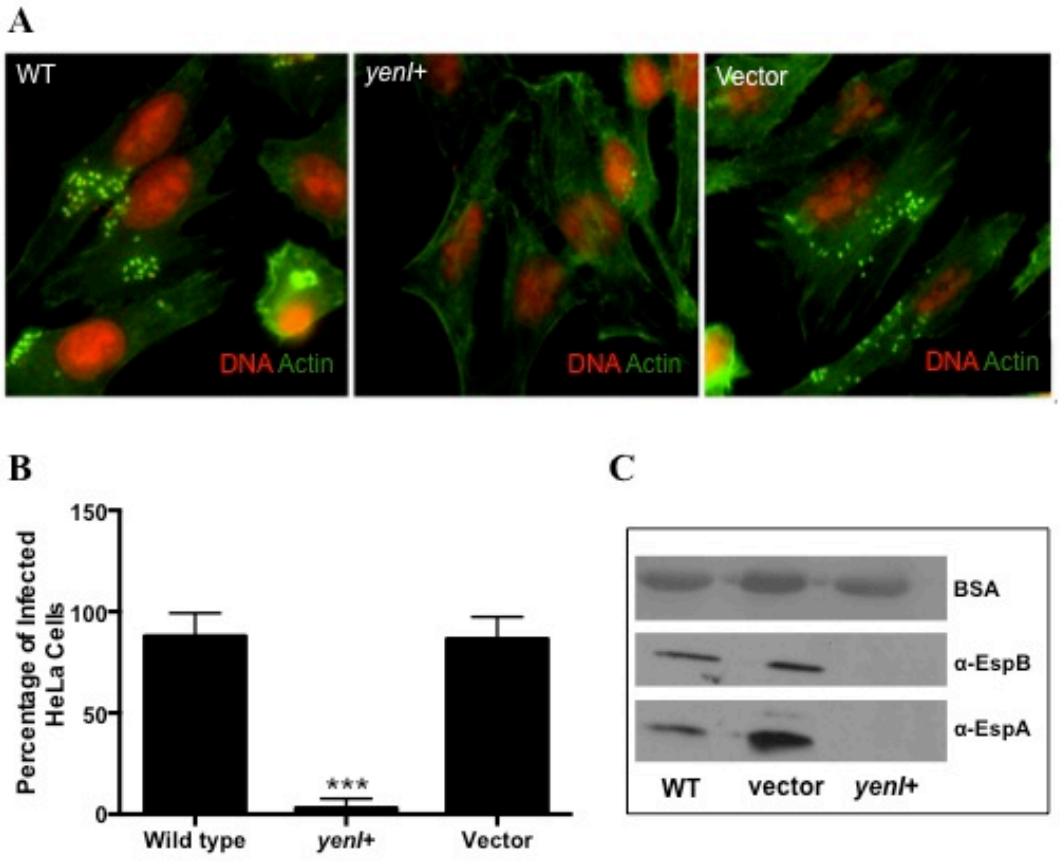


Figure 22. (A) Fluorescent actin staining assays. HeLa cells were infected with wild type, *yenI*$^{+}$, and vector control strains for 6 hours. Actin was stained in green with FITC-phalloidin and HeLa cell nuclei and bacteria stained in red with propidium iodide. A/E lesion formations were visualized as bright green cupping the red bacteria. (B) Quantification of the number of infected cells or number of cells with A/E lesions. (C) Western blots of the secreted proteins of wild type, vector, and *yenI*$^{+}$ strains probed with antiserum against EspB and EspA. Statistical significance was determined by Student's *t*-test, and *** indicated *P-value* of ≤ 0.001.

Endogenously produced AHLs activate gad gene expression. Although AHLs repress LEE gene expression, they activate expression of the gad acid resistance genes [2]. Here again we show that transcription of the *gadA, gadC* and *gadE* genes is significantly increased by both exogenous and endogenous (*yenI*$^+$) AHLs (Figure 23). These data combined suggest that the *yenI*$^+$ EHEC behaves as if it is constantly in the presence of this signal.

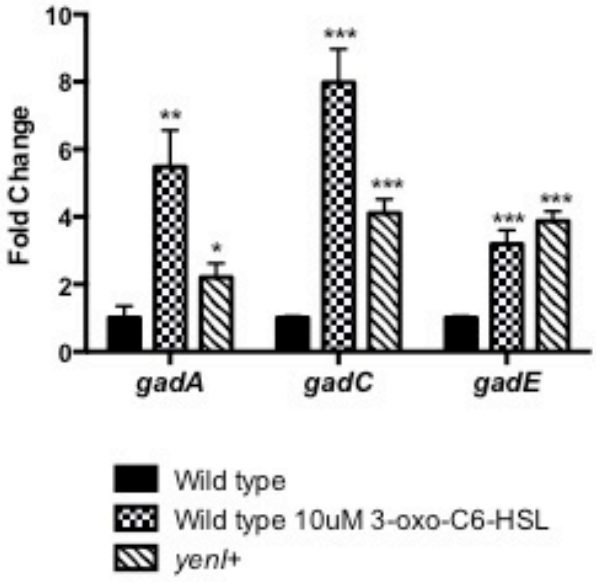


Figure 23. AHLs increase gad gene expression in EHEC. qRT-PCR analyses of *gadA*, *gadC*, and *gadE* transcription in the wild type, in the wild-type strain with exogenous AHLs, and in the *yenI*$^+$, strain (produces endogenous AHLs). The mRNA levels are graphed as fold changes compared to WT levels. Statistical significance was determined by Student's t test; **, *P-value* of ≤ 0.01; ***, *P-value* of ≤ 0.001.

Wild type and *yenI*+ EHEC colonize the RAJ mucosa of cattle similarly. Our *in vitro* studies suggest that endogenous AHL production decreases LEE expression and consequently, reduces A/E lesion formation on HeLa cells. Next, we investigated if *yenI*$^+$ EHEC colonizes the host less efficiently *in vivo* compared to wild type. Five steers were challenged with a single rectal application of wild type or *yenI*$^+$ EHEC. Wild type or *yenI*$^+$ EHEC cultured on day 0 prior to challenge and then on days 1, 3, 7, 10, 14, 17, 21, 24, 28, and 42 post-challenge. All animals were O157 culture negative prior to challenge and all animals were culture positive for at least 14 days post-challenge. The wild type and *yenI*$^+$ EHEC colonized the bovine RAJ similarly, although the wild type tended to persist slightly longer than the *yenI*$^+$ EHEC (Figure 24).

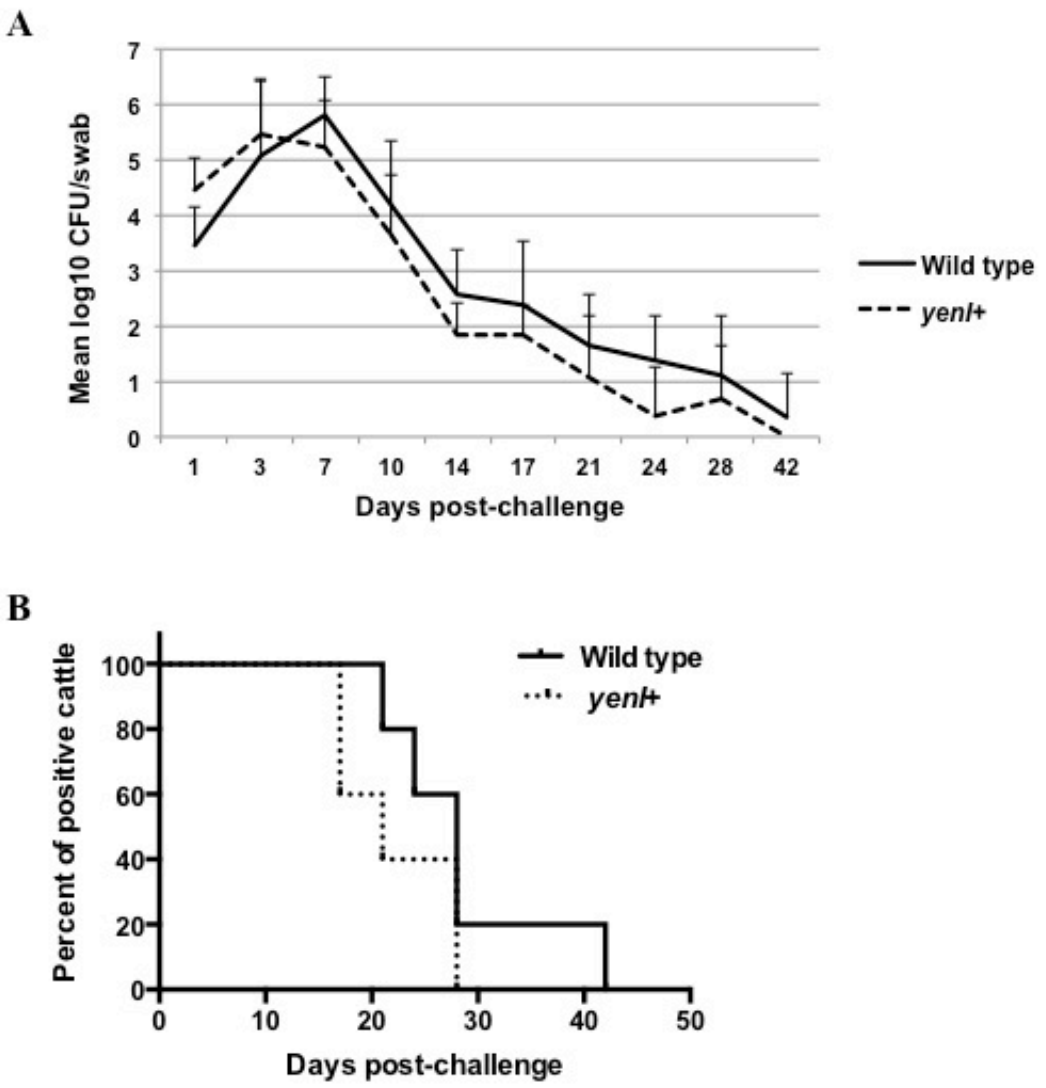


Figure 24. The recto-anal junctions of cattle were inoculated with 10^{10} CFU of either wild type or *yenI*$^{+}$ EHEC. Inoculated bacterial strains were isolated from RAJ mucosal swabs after the indicated post-challenge days. (A) The mean log CFUs per swab of either wild type or *yenI*$^{+}$ (B) The percentage of animals positive with EHEC at 14, 28, 42, or 56 days post-challenge.

DISCUSSION

EHEC can colonize multiple hosts. In order to survive host defenses and successfully colonize specific niches, EHEC must rely on environmental cues to modulate appropriate gene expression. Gram-negative bacteria use the chemical signal acyl-homoserine lactones to monitor their own population density and mount appropriate responses [76]. EHEC can hijack this bacterial cell-to-cell communication by sensing AHLs produced by other bacteria in the environment to regulate their genes for survival and colonization in its natural reservoir, cattle [2]. To establish colonization in cattle at the RAJ [128], EHEC must survive the acidic environment of the stomachs and activate A/E lesion formation at the RAJ. Construction of a type III secretion system for injection of bacterial effector proteins into the host cells to form A/E lesions requires a lot of energy resources; therefore, expression of the LEE genes that are required for A/E lesions has to be tightly regulated to prevent waste of energy in the wrong gastrointestinal compartment. Previous findings suggest that activation of acid resistance genes by AHLs synthesized by other bacteria present in the rumen of cattle prepares EHEC to survive the acidic environment of cattle stomachs while decreasing the LEE since the rumen is not a preferable niche for colonization [2]. Interestingly, the RAJ contains no AHLs [2,84]. The absence of AHLs in the RAJ alleviates AHL-mediated repression of the LEE to allow for A/E lesion formation on the mucosal epithelial cells at the RAJ, favoring colonization at the RAJ. These data suggest that AHLs produced by the bacterial population in cattle guide EHEC to survive and establish a niche in an appropriate environment once inside the cattle.

If AHLs provide cues to EHEC that the rumen is an unfavorable environment for colonization, then an interesting question to explore is whether AHLs present in the RAJ would

decrease bacterial colonization of EHEC at the RAJ and decrease subsequent shedding of EHEC into the environment. We explored this idea by creating an EHEC strain that contains the LuxI-like synthase gene *yenI* gene from *Y. enterocolitica* to imitate constant exposure of AHLs that are found in the environment. *YenI*$^{+}$ EHEC produced endogenous AHLs that significantly decreased expression of the LEE genes, while increasing expression of the *gad* acid resistance genes (Figure 20-23). Lowered transcriptional expression of the LEE also resulted in a significant decreased production of secreted proteins and consequently, A/E lesion formation on HeLa cells (Figure 22). Although the *in vitro* data is consistent with our previous data demonstrating the importance of AHLs in the downregulation of the LEE, *yenI*$^{+}$ EHEC colonizes the RAJ similarly to wild type, albeit there is a trend that *yenI*$^{+}$ EHEC sheds EHEC for a shorter period of time than WT (Figure 24). Diffusion of AHLs into the large environment of the RAJ, as compared to an enclosed system *in vitro*, may account for the lack of AHL-mediated repression of EHEC colonization of this site.

The data also suggest that unidentified signals sensed by SdiA are contributing to EHEC colonization at the RAJ, as supported by our previous findings that the wild type EHEC outcompeted the *sdiA*$^{-}$ mutant in colonization at the RAJ [2]. Since AHLs are not naturally present in the RAJ, it is also possible that there are enzymes or factors expressed by the epithelial cells in the RAJ that can readily degrade AHLs. For example, a class of AHL-degrading enzymes found in mammals called paraoxonases (PONs) has been shown to degrade AHLs and inhibit the quorum sensing regulation of bacteria [152]. PON2 has the highest enzymatic capacity to degrade AHLs compared to other PONs [153,154]. Interestingly, various tissues including epithelial cells of the gastrointestinal tract, where EHEC colonizes in humans and AHLs are absent [155], express PON2 [156]. This suggests that EHEC utilizes other available

signals such as such as epinephrine or norepinephrine [157-159] to modulate the LEE genes to promote colonization in the human gut. Paraoxonases are also found in cattle [160] and bovine serum has been shown to degrade AHLs [154]. This infers that either paraoxonases or other similar AHL-degrading enzymes could degrade the AHLs produced by *yenI*$^{+}$ in the RAJ and as a result, EHEC is utilizing other more abundant signals to promote colonization.

Other non-LEE factors may also important for colonization of EHEC at the RAJ. For example curli and other fimbriae have been implicated to be important for colonization of EHEC in cattle [161]. This demonstrates how EHEC can utilize an array of complex mechanisms and signals to regulate genes required for colonization in cattle. Further identification of new potential signals and elucidation of the mechanisms used by EHEC to colonize its natural host will help develop better preventive strategies to reduce EHEC colonization at the RAJ and consequent, shedding of EHEC into the environment and transmitting to human.

CHAPTER FIVE

GENERAL DISCUSSION, BROAD IMPLICATIONS, AND FUTURE

Microorganisms and their eukaryotic hosts have co-existed for millions of years. In the mammalian GI tract, bacteria establish a niche that can be beneficial or detrimental to the host. Enterohemorrhagic *E. coli* serotype O157:H7 can thrive as a commensal or as a pathogen depending on its host. For example, while EHEC colonization in its natural host, cattle, is asymptomatic, accidental ingestion of EHEC leads to clinical complications ranging from bloody diarrhea to HUS in humans [162]. Since EHEC is carried by approximately 70-80 % of the cattle herds in the United States and the majority of the reported outbreaks involve contaminated bovine products [163], preventive strategies to reduce colonization in cattle and diminish EHEC shedding in the environment would not only reduce human disease but would relieve economical costs the for beef industry. Various studies were implemented to find alternative strategies to reduce EHEC shedding into the environment. Some of these ongoing studies include supplementation with probiotics, administration of antibiotics, and vaccination against the T3SS machinery [164,165]. Conflicting results from these studies has thwarted efforts to control EHEC populations within cattle [166-169], and emphasizes the necessity for additional research to be performed. The dearth of knowledge on the mechanisms regulating intestinal colonization of ruminants by EHEC has hindered these strategies. A better understanding of the biology of EHEC colonization in cattle is vital to the development of new preventative strategies.

Intervention in QS can provide an alternative strategy to reduce carriage in cattle [2]. AHL molecules, produced by other bacteria in cattle, serve as signaling molecules that guide

EHEC to the appropriate colonization site. Inference in this signaling cascade can result in inhibiting EHEC from successfully colonizing its host. To exploit the SdiA-AHL signaling, we engineered an EHEC strain expressing endogenous AHLs and analyzed its ability to downregulate the LEE genes and consequently, colonization at the RAJ in cattle [107]. Although wild type EHEC persisted slightly longer than AHL-producing EHEC, both strains colonized the RAJ similarly [107]. It is possible that the concentration of AHLs were insufficient in the RAJ, due to either enzymatic breakdown or chemical degradation of AHLs from the alkaline environment [152,156,170], to repress the LEE genes.

In addition to the LEE, SdiA induces the GAD resistance system, which is required for EHEC survival in the acidic stomachs of cattle [47], by sensing AHLs produced by the gastrointestinal microflora of cattle. Surviving the acidic stomachs is critical for colonization of EHEC at the RAJ. Therefore, an alternative strategy to prevent EHEC from colonizing cattle is to reduce EHEC tolerance for surviving in acidic conditions. Since AHL signals are important for inducing the SdiA-mediated GAD system, interference with this QS signal by AHL degrading enzymes in the rumen can potentially reduce EHEC survival and correspondingly, colonization in cattle. One example is the AHL lactonase encoded by the *aiiA* gene from Gram-positive *Bacillus* species [171]. The lactonase inactivates AHLs by hydrolyzing the lactone ring [172].

Several groups have demonstrated the effectiveness of lactonase enzyme to interfere with the QS of pathogens. For example, the AHL lactonase producing bacterium *Bacillus thuringiensis* (*B. thuringiensis*) was able to suppress QS-dependent virulence of the plant bacterial pathogen *E. carotovora* when they were co-inoculated in plants. *B. thuringiensis* had no effect on *E. carotovora* growth but was able to effectively stop the QS-dependent spreading of *E.*

carotovora in plants. Lactonase deficient strains of *B. thuringiensis* failed to inhibit pathogenesis of *E. carotovora* [173]. Additionally, they showed that virulence was attenuated in *E. carotovora* strains that expressed the *B. thuringiensis* lactonase gene *aiiA* [171]. This demonstrates that AHL-degradation enzyme can eliminate the QS signals and suppress QS-dependent virulence gene expression by pathogens. The efficiency of QS quenching enzymes, such as *aiiA* encoded AHL lactonase to attenuate survival and colonization of EHEC in cattle have not been explored. To investigate this idea, we can construct an AHL-degrading EHEC strain by cloning the *aiiA* gene from *B. thuringiensis* into the chromosome of EHEC, as performed previously with the *yenI*$^+$ EHEC strain. For assessing the hydrolysis of AHLs by endogenous lactonase, we can culture the *aiiA*$^+$ EHEC with exogenous AHLs, then extract and detect the degraded AHLs from the supernatant and whole cell lysate using ethyl acetate and TLC, respectively, as performed previously. Quantitative real-time PCR can be used to measure the expressions of the *gad* genes in the *aiiA*$^+$ EHEC strain. The GAD assay can be used to investigate the tolerance of lactonase-expressing EHEC at low pH *in vitro*. Finally, we can investigate the efficiency of the *aiiA* encoded AHL lactonase to attenuate survival and colonization of EHEC in its natural host by challenging cattle orally with equal ratio of WT to *aiiA*$^+$ EHEC to mimic the natural route of infection. After various post-challenge time points, samples from the rumen and RAJ can be isolated and the competitive index can be calculated to access the effects of lactonase on bacterial colonization in cattle. Although disrupting SdiA-AHL signaling in cattle by quenching AHLs can serve as a potential alternative strategy, degradation of these signals can have a negative effect on the intestinal microflora of cattle. The role of AHLs (if any) on the function of the gut microbiota and on the physiology of cattle has not been studied; therefore, depleting AHL from the rumen must be investigated with caution.

Lactonase inhibits QS signaling by hydrolyzing the AHL signals. The use of AHL antagonists, such as halogenated furanones, can serve as an alternative strategy to interfere with QS signaling without destroying the AHL signals from the environment. Halogenated furanone compounds are naturally produced by marine macro algae *Delisea pulchra*. The furanones are synthesized in the cell then are released into the environment [174]. Both the natural halogenated furanones isolated from algae and their synthetic derivatives can interferes QS by inhibiting AHL-mediated gene expression [174-178]. For example, furanones reduced gene expression of the QS regulon, resulting in the subsequent decrease in expression of the QS mediated virulence genes in *P. aeruginosa* [175]. *P. aeruginosa* is opportunistic human pathogen that causes high morbidity and mortality in cystic fibrosis patients [179]. Using a pulmonary mouse model to assess the role of *P. aeruginosa* in lung disease, mice that are treated with furanones after challenge with *P. aeruginosa* had accelerated bacterial clearance and reduced lung pathology compared to the untreated control group [175,177]. These studies suggest that halogenated furanones can be used to successfully inhibit QS, thereby, reducing virulence of pathogens in their host.

The use of halogenated furanones to disrupt the SdiA-AHL signaling in the rumen as an alternative strategy to reduce EHEC fitness in cattle has not been investigated. To explore this idea, commercially available furanones can first be tested for efficacy in the repression of SdiA-AHL mediated genes, such as the *gad* and the LEE genes, using qRT-PCR. Furanones function as an antagonist by competing AHLs for binding on the LuxR-like proteins [176] and reducing protein stability *in vivo* [180]. Therefore, in addition to gene expression, we can assess the effect of furanones on SdiA stability by performing pulse-chase experiments of EHEC grown in the absence and presence of furanone and AHL. The effects of algae rich in furanones on EHEC

colonization has not been explored. Because algae are rich in nutrients and cheap to produce, scientists are becoming more interested in testing the effects of feeding cattle with different types of marine algae on the quality of dairy products [174,181-184]. To investigate the effect of furanones on EHEC survival and colonization in cattle, we can feed cattle with furanone-producing algae or food supplemented with synthetic furanones. After post-furanone treatment, we can orally inoculate cattle with wild type EHEC and collect samples from the rumen and RAJ as performed previously. Furanone treatment could potentially have a deleterious effect on gut microbiota, however, since furanone can also interfere with the QS used by these resident bacteria. Testing will be needed to determine if this is a viable strategy.

The regulation of EHEC colonization is complex. EHEC must rely on multiple cues from the environment to guide itself to its appropriate colonization. For instance, sensing AHLs in the rumen warns the bacterium that it has reached a hostile environment. But after safe passage through the acidic stomachs, it is still unclear how EHEC navigates its way through the intestinal tract of cattle to reach the RAJ, its prominent site of colonization [29] (Figure 1). The observation that in addition to AHLs, SdiA also uses OCL as the endogenous ligand has several important implications in EHEC pathogenesis, as well as other enteric bacteria species that also harbor SdiA (e.g. *Salmonella* and *Klebsiella*). Monoacylglycerols are the building blocks of triacylglycerols, and present in both eukaryotes and prokaryotes. Triacylglycerols constitute an important energy reservoir, and a source of signaling molecules and substrate for membrane biogenesis [127]. Monoacylglycerols are abundant in the gut of cattle and humans; opening the possibility that SdiA has a function in the intestinal tract of cattle to aid EHEC colonization at the RAJ as well as promoting disease in humans.

To assess the role of SdiA regulation of virulence gene expression within the mammalian intestine, we can use the *Citrobacter rodentium* (*C. rodentium*) murine infection model since EHEC is exclusively a human pathogen and cannot colonize the intestinal mucosa of mice [185]. *C. rodentium* shares 67 % of its genome with EHEC including the LEE [186], which allows *C. rodentium* to form A/E lesions on the intestine of mice and cause colonic hyperplasia [131,187]. Our preliminary data shows that 3-oxo-C_6-HSL also repress LEE gene expression in *C. rodentium* (Figure 25). In addition, all of the known virulence genes of EHEC, such as non-LEE encoded T3SS translocated factor NleA and NleH, have been validated *in vivo* using *C. rodentium* murine infections [131,185,188-191].

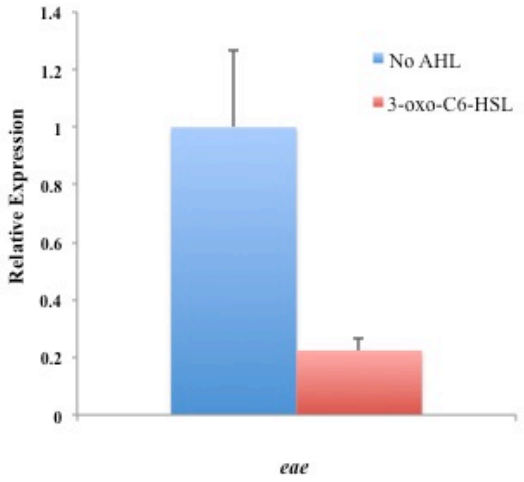


Figure 25. The expression of *eae* in the absence or presence of 3-oxo-C_6-HSL in *C. rodentium* by qRT-PCR.

To investigate the role of SdiA in the pathogenesis of *C. rodentium* during murine infections, we first need to confirm that SdiA in *C. rodentium* have a similar function as SdiA in EHEC. The λ red recombination system can be used to generate an *sdiA* mutant in *C. rodentium,* and the effect of SdiA on expression of the *gad* and LEE genes can be measured using qRT-PCR. For the *in vivo* experiments, mice can be challenged with either WT or the *sdiA* mutant. Feces and organs can be collected at various time points to assess bacterial burden and RNA from the isolated bacteria can be extracted to measure gene expressions using qRT-PCR. Haematoxylin and eosin (H/E) staining of the intestine can be used to survey the pathology such as vasculitis, bacterial attachment, apoptosis, neutrophil, and crypt intestinal edema of the inoculated mice. Survival and weight loss of the mice can also be monitored to assess the morbidity and the progression of the disease caused by either WT or *sdiA* mutant. The results from these experiments will provide valuable insights into the role of SdiA in the intestine.

The function of monoacylglycerol in prokaryotes, apart from membrane biogenesis, is unknown. Preliminary data suggests that the SdiA endogenous ligand OCL acts as a chemical chaperone placeholder to stabilize SdiA since dialyzing the ligand resulted in the precipitation of SdiA. Currently, the OCL synthase has not been identified. Identification of this synthase is crucial for assessing the biological relevance of OCL. Monoacylglycerol synthesis is achieved through glycerol-phosphate acyltransferases, which can utilize endogenous sources of lipids such as acyl-ACPs, or external sources of lipids imported by the bacteria through acyl-CoA [127]. Lipases can also generate monoacylglycerol by catalyzing the hydrolysis of the triacylglycerol substrate [192]. Using the *E. coli* Keio Knockout Collection, which contains a set of single-gene, knockout mutants for all nonessential genes in *E. coli* K-12, we generated a list of either putative acyltransferases or lipases (Table 5). Based on our hypothesis that OCL acts to stabilize SdiA,

cells that lack the OCL synthase will result in reduced cellular level of SdiA. Since we have already generated an antibody against SdiA, we can screen the selected Keio strains by western blot for mutants with leading to reduced levels of SdiA compare to wild type. Positive mutants from the screen will be selected for further analysis including molecular and biochemical characterization of the synthase including reconstituting the synthesis of OCL *in vitro.*

SdiA binds not only to OCL and AHLs, but it can also potentially respond to other types of monoacylglycerols that are found in the gut. Ligand specificity analysis of SdiA has not been well studied. Up until now, it has been a challenge to purify SdiA in the absence of AHLs to perform any biochemical studies. Although we overcame this challenge, the observation that SdiA binds OCL without AHLs makes the assessment of the role of ligand binding challenging. Identification of the OCL synthase will allow us for the first time to study SdiA in its true potential apo-state. Using either circular dichroism (CD) or isothermal titration calorimetry (ITC), we can calculate the binding affinities of SdiA to different ligands. This will provide further understanding of the specificity of ligand binding of SdiA. We can use this information to develop drug targets against SdiA by to inhibit SdiA signaling in EHEC.

The crystal structures of SdiA provide useful information on the residues that may play a key role in ligand recognition. For example, both OCL and AHLs form four hydrogen bonds directly with W67, D80, Y71, Y63, S43, or 143 (Figure 26A). In addition, F59, L77, and W107 undergo large shifts to accommodate binding to an AHL molecule (Figure 26B). The biological relevance of these residues has not been investigated. We have generated single point mutations of these key residues to an alanine by site-directed mutagenesis. To investigate the role of these residues in binding to AHL molecules, we overexpressed the SdiA mutants in the absence (just with the endogenous OCL) or presence of either 3-oxo-C_6-HSL or 3-oxo-C_8-HSL and measure

SdiA stability by western blot (Figure 26C). Site-directed mutagenesis studies suggest that there are differences in the role of different conserved residues within the LBD when bound to OCL or different AHLs in the stabilization of SdiA (Figure 26C). Mutation of D80 abolishes SdiA stability, suggesting that this residue is critical for the function of OCL and both AHLs. Mutations in W67 and Y71 decrease the stability of SdiA but the addition of 3-oxo-C_8-HSL to Y71 mutant increases stability, although not to wild type levels. The longer acyl chain in 3-oxo-C_8-HSL can still be stabilized by hydrophobic interactions when Y is mutated to an A. Mutations in Y63 slightly decrease the protein stability, and mutation in the least conserved residue Y43 did not alter SdiA stability. The Y63 mutant has a mild phenotype. Interestingly F59, L77, and W107 mutants have no phenotype (Figure 26C). A mild to no phenotype may be due to the fact that the loss of interaction caused by a single point mutation can be compensated by other interactions in the binding pocket. For example, changing a S43 to A abolish a hydrogen bond, but the ligand can still be stabilized by the hydrophobic interactions of the acyl chain and by the other remaining hydrogen bonds (Figure 26A). Therefore, to better investigate the relevance of these residues on ligand recognition, we can generate either double or triple mutants to abolish multiple interactions in the binding pocket. Additionally, overexpression of SdiA may not be the best strategy to study the stability of SdiA. Alternatively, we can generate chromosomal mutations and measure endogenous protein level in the presence or absence of AHLs. Additionally, we can perform pulse-chase experiments to further investigate its stability *in vivo* over time. ITC or CD can also be used to measure changes in ligand binding of these mutants. Functional studies such as qRT-PCR and FAS can also be executed to evaluate the role of these residues in SdiA function. SdiA is important for guiding EHEC to its site of colonization in

cattle. Understanding how SdiA detect cues, such as AHLs and OCL, from the environment is valuable especially for developing strategies to inhibit SdiA-signaling in cattle.

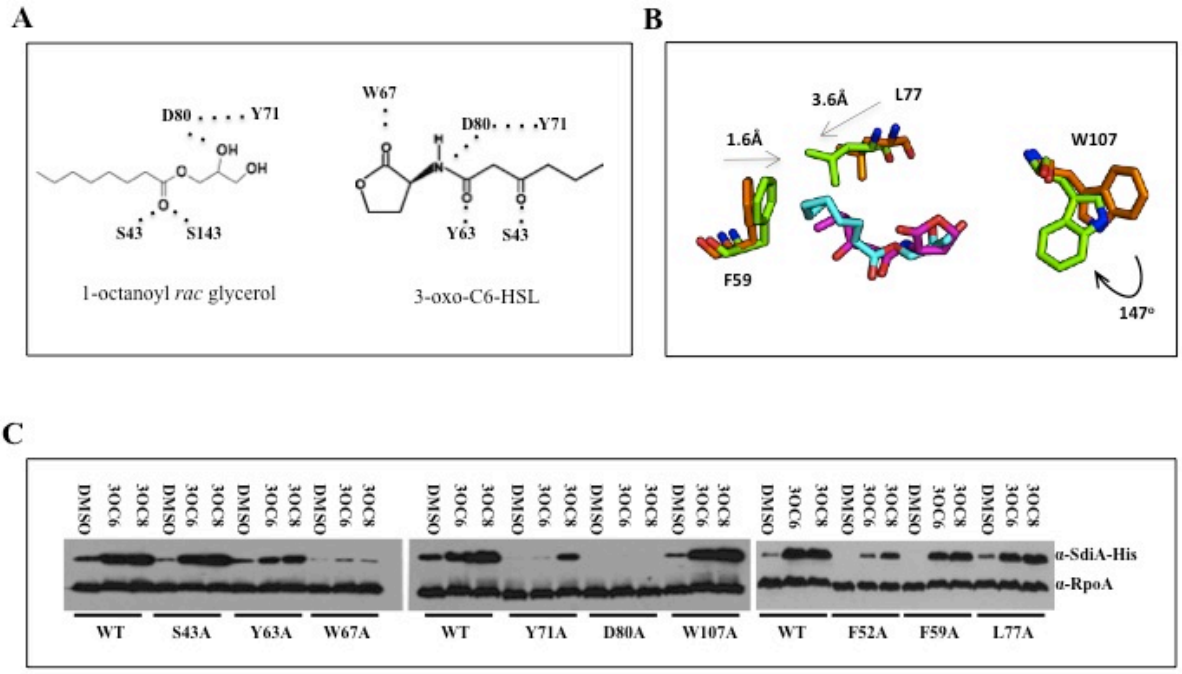


Figure 26. (A) Cartoon showing the H-bonds (dotted lines) between key residues of SdiA to the 1-octanoyl-*rac*-glyecerol and 3-oxo-C_6-HSL. (B) Side-chain movements of F59, L77, and W107 in the LBD of SdiA with and without AHLs. (C) Western blot of single point mutant SdiA expressed from pBAD/Myc-His vector.

This work highlights how different chemical signaling systems can be employed by bacteria to adapt to either pathogenic or commensal lifestyles in different hosts, and that this signaling system aids this human pathogen to adapt to a commensal life-style in cattle, its main reservoir. The mammalian gastrointestinal tract is colonized by a complex community of bacteria. However, how these microbial communities structure themselves, and prefer certain

niches within the GI tract of its animal host is largely unknown. Bacterial communities utilize chemical signaling to coordinate population behavior. One of the long-standing questions in this field is whether bacterial cells utilize chemical signals to select their niche within the host, and establish a commensal relationship. Our work constitutes the very first description of chemical sensing in the GI tract of mammals facilitating a host bacterial commensal association. It is also the very first time chemical signaling is shown to occur in the complex system of the mammalian GI tract *in vivo* utilizing a natural animal reservoir of the commensal bacteria, and that this signaling determines the niche specificity for colonization of a specific area of the GI tract [2]. This work also has implications on basic science and public health giving that this bovine commensal is also a deadly human pathogen. These studies open exciting and novel avenues to control shedding of this human pathogen in the environment

Additionally this research will solve a 40-year withstanding question in the QS field, which is what is the mechanism of action of the orphan SdiA LuxR-type sensor? We crystallized three forms of SdiA, bound to two AHLs, and identified 1-octanoyl-*rac*-glycerol, a monoacylglycerol, as an endogenous ligand for this protein. Our studies also show that the SdiA-AHL bound form of this protein has higher DNA binding affinity. This suggests that the endogenous-ligand-bound-SdiA can only recognize promoter regions that have high affinity binding sites for SdiA, while the AHL-bound form can also regulate promoters with a less conserved SdiA-binding consensus sequence. These different DNA binding affinities of SdiA can finally explain the withstanding conundrum of why certain genes can be regulated by SdiA only in the presence of AHLs, and without AHLs they can only be regulated by SdiA upon overexpression of this transcription factor. Furthermore, this also explains why certain genes depict SdiA-dependent regulation in the absence of AHL. In a broader sense the discovery of a

monoacylglycerol as an SdiA ligand breaks new ground in EHEC pathogenesis, and also in our understanding of LuxR-type proteins at large. Several LuxR-type proteins have their DNA binding properties inhibited by AHLs, which seems to be the exact opposite of the role of AHLs in SdiA DNA binding. Furthermore, it has been largely assumed that these AHL-inhibited LuxR-type proteins are apo-proteins, when in fact they may very well be complexed with monoacylglycerols, and this could be a "molecular chaperone placeholder" for many LuxR-type proteins in the absence of AHLs. Finally, the description that the endogenous ligand of SdiA is a monoacylglycerol, which is a building block for triacylglycerol, conserved throughout the tree of life, has important physiological and evolutionary implications. Although the synthetic pathways for these molecules is very well defined in mammalian cells, almost nothing is known about it in prokaryotes [3]. In addition, thus far the only function of monoacylglycerols in prokaryotes has to do with membrane biogenesis, and here we show that these molecules can also be used in cell signaling.

Figure 5. List of potential targets of OCL synthase in *E. coli* K-12

Gene	B#	Description
lpxL	b1054	lauryl-acyl carrier protein (ACP)-dependent acyltransferase
lpxM	b1855	myristoyl-acyl carrier protein (ACP)-dependent acyltransferase
aas	b2836	fused 2-acylglycerophospho-ethanolamine acyltransferase/acyl-acyl carrier protein synthetase
lpxP	b2378	palmitoleoyl-acyl carrier protein (ACP)-dependent acyltransferase
yihG	b3862	inner membrane protein, inner membrane acyltransferase
mnaT	b1448	methionine N-acyltransferase; L-amino acid N-acyltransferase
yihG	b3862	inner membrane protein, inner membrane acyltransferase
rssA	b1234	putative patatin-like family phospholipase
pldB	b3825	lysophospholipase L2
mhpC	b0349	2-hydroxy-6-ketonona-2,4-dienedioic acid hydrolase
ysgA	b3830	putative hydrolase
ybfF	b0686	acyl-CoA esterase

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