HIGH-THROUGHPUT IDENTIFICATION OF REGULATORS OF THE LOCUS OF ENTEROCYTE EFFACEMENT FROM ENTEROHEMORRHAGIC *E. COLI*

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Dedication

To Kurt, Matthew, Jasmine, and Janet.

HIGH-THROUGHPUT IDENTIFICATION OF REGULATORS OF THE LOCUS OF ENTEROCYTE EFFACEMENT FROM ENTEROHEMORRHAGIC *E. COLI*

BY

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Identification of pathways involved in the regulation of the type III secretion system (T3SS) encoded by the locus of enterocyte effacement (LEE) of Enterohemorrhagic *E. coli* (EHEC) has been underway for more than twenty years, but significant knowledge gaps remain. We have created a generalizable, high-throughput method of identifying *E. coli* core genome components that facilitate T3S. Using this tool, we have generated a dataset of regulatory pathways that control the LEE under anaerobic conditions, a largely neglected but essential consideration for this enteric pathogen. As proof of principle as to the efficacy of this method, we have characterized two transcription factors, *cutR* and *fadR*, as previously unknown LEE regulators in EHEC and *Citrobacter rodentium*. We have determined that CutR functions as a transcriptional activator of the LEE in the presence of L-cysteine, in EHEC and *C. rodentium*. We have observed that CutR is capable of directly binding to the *LEE1* regulatory region, suggesting a direct mechanism of action, while simultaneously controlling a network of LEE-governing genes. We have also determined that FadR functions as a transcriptional repressor of the LEE in EHEC and *C. rodentium*, directly binding to *LEE1* of both organisms. Finally, we explore the broader dataset to begin assessing the potential of yet uncharacterized LEE regulators.

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Prior Publications

Pifer R., Sperandio V. (2014) The Interplay between the Microbiota and Enterohemorrhagic Escherichia coli. Microbiol Spectr. 2014 Oct;2(5).

Pifer R., Curtis M.M., Sperandio V. Redox, amino acid and fatty acid metabolism intersect with bacterial virulence in the gut. Submitted.

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86-24	Enterohemorrhagic *E. coli* strain 86-24
ACP	Acyl carrier protein
A/E	Attaching and effacing
aEPEC	Atypical Enteropathogenic E. coli
AHL	Acyl homoserine lactone
ANOVA	Analysis of variance
Bp	Base pairs
BSA	Bovine serum albumin
BW25113	*E. coli* strain BW25113
ChIP	Chromatin immunoprecipitation
CoA	Coenzyme A
DBS770	*Citrobacter rodentium* strain DBS770
DMEM	Dulbecco's Modified Eagle Medium
ECL	Enhanced chemiluminescence
EHEC	Enterohemorrhagic *E. coli*
EMSA	Electromobility shift assay
EPEC	Enteropathogenic *E. coli*
FAD	Flavin adenine dinucleotide
FFRP	Feast/famine regulatory protein
GCS	Glycine cleavage system
GI	Gastrointestinal
HRP	Horseradish peroxidase
HUS	Hemolytic uremic syndrome

HTH	Helix turn helix domain
K-12	Laboratory adapted *E. coli* strain
Kd	Dissociation constant
LB	Luria-Bertani
LCFA	Long chain fatty acid
LEE	Locus of enterocyte effacement
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NANA	N-acetylneuraminic acid
NAP	Nucleoid associated protein
Ni-NTA	Nickel-nitrilotriacetic acid
NO	Nitric oxide
OD	Optical density
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PHB	Poly-3-hydroxybutyrate
PNK	T4 polynucleotide kinase
qPCR	Quantitative polymerase chain reaction
ROS	Radical oxygen species
SPI-1	*Salmonella* pathogenicity island-1
Stx	Shiga toxin
T3S	Type III secretion
T3SS	Type III secretion system

TA	Toxin-antitoxin
TBE	Tris borate EDTA
TCA	Tricarboxylic acid cycle
TCS	Two component system
TMB	3,3’,5,5’-Tetramethylbenzidine
UFA	Unsaturated fatty acid
WT	Wild type

CHAPTER ONE

Literature Review

Attaching and Effacing Pathogens

Attaching and Effacing (A/E) pathogens are a subset of *Enterobacteriaceae* that can cause enteric infections in humans or other animals. These pathogens have horizontally acquired a type III secretion system (T3SS) encoding island called the locus of enterocyte effacement (LEE) (Jarvis et al., 1995; Jerse et al., 1990; McDaniel et al., 1995). This T3SS is used to colonize the surface of the intestinal epithelium, without invasion as seen with other T3SS containing enteric pathogens, such as *Salmonella enterica*, *Shigella flexneri*, or *Vibrio parahaemolyticus.*

Four A/E pathogens with clinical relevance to humans are Enterohemorrhagic *E. coli* (EHEC) (Sherman et al., 1987), Enteropathogenic *E. coli* (EPEC) (Moon et al., 1983), Atypical EPEC (aEPEC) (Pelayo et al., 1999), and *Escherichia albertii* (Ooka et al., 2015).

Type III secretion systems are molecular machines that form a syringe and needle apparatus, an injectosome, that enable the direct translocation of proteins from the bacterial cytosol into a mammalian target cell cytoplasm (Jarvis et al., 1995; Salmond and Reeves, 1993; Wolff et al., 1998). These translocated proteins, called effector proteins, can alter many aspects of the physiology of the target cell, including cytoskeletal rearrangement (Rosenshine et al., 1996), cell signaling (Sham et al., 2011), immune activation (Hemrajani et al., 2008; Nadler et al., 2010), apoptosis (Crane et al., 2001), and vesicular trafficking (Clements et al., 2011). The T3SS is composed of multiple segments, each performing a necessary function for activity: a C-

ring segment that interacts with effector proteins within the bacterial cytoplasm, catalyzing the unfolding of proteins to be translocated (Akeda and Galan, 2005); a basal body, composed of a series of rings which cross from the bacterial plasma membrane, the periplasmic space, and the bacterial outer membrane (Sukhan et al., 2001); an extracellular needle that bridges the bacterial cell and the mammalian cell (Knutton et al., 1998; Sekiya et al., 2001); and a translocon complex that embeds in the host cell plasma membrane forms a pore through which effector proteins can enter the host cell (Ide et al., 2001).

The LEE is composed of forty one genes arranged into five major operons, *LEE1-5*. *LEE1*, *LEE2*, and *LEE3* encode for the basal body proteins (Pallen et al., 2005). *LEE1* also encodes the Ler activator protein which positively regulates transcription of the remaining operons (Mellies et al., 1999). *LEE4* encodes for the proteins of the extracellular apparatus and the translocon pore. *LEE5* encodes intimin, an outer membrane adhesin, and the translocated intimin receptor, Tir, which embeds in the host plasma membrane to mediate tight binding to between the bacterium and the host cell (DeVinney et al., 1999). This tight binding interaction begins the process of actin rearrangement that is characteristic of EHEC infections. EHEC creates a unique niche in the form of an actin enriched pedestal protruding from the enterocyte membrane upon which the the bacterium sits (Knutton et al., 1989). Pedestal formation is dependent upon Tir and the cryptic prophage encoded effector protein, EspFu. Tir recruits the host proteins IRTKS and IRSp53, which in turn recruit EspFu (Crepin et al., 2010; Vingadassalom et al., 2009). EspFu activates the host actin nucleation complex N-WASP-Arp2/3, which then facilitates actin filament formation beneath the bacterium (Campellone et al., 2004; Garmendia et al., 2006; Garmendia et al., 2004; Goosney et al., 2001).

EHEC is a commensal for ruminant species, residing at the rectal anal junction of cattle (Sheng et al., 2006). While not part of the natural life cycle for EHEC, the organism can cause life threatening, short term infections in humans. During the course of infection, EHEC colonizes the colonic epithelial layer, promoting bloody diarrhea and severe abdominal cramping. In some cases, including those patients mistakenly treated with antibiotics or morphine, a second virulence factor, Shiga toxin (vero toxin) is induced (Yoh and Honda, 1997). Shiga toxin is an AB toxin that enters mammalian cells and catalyzes the inactivation of ribosomes (Obrig et al., 1985). As the damage to host tissues accumulates, a condition known as hemolytic uremic syndrome (HUS) sets in, which is characterized by renal failure, thrombocytopenia, and microangiopathic hemolytic anemia (Karmali, 1989; Karmali et al., 1983).

EHEC species fall into several known serotypes: O157:H7, O26:H11, O45:H2, O103:H2, O111:H8, O121:H19, O145:H28 (Delannoy et al., 2013a, b). These lineages differ importantly from EPEC lineages in that they encode the bacteriophage-borne Shiga toxin. A subset of *E. albertii* strains encode a Shiga toxin isoform, but the clinical importance of this is not clear (Ooka et al., 2015). The O157:H7 lineage is believed to have evolved from the EPEC lineage O55:H7 (Whittam et al., 1993) and is now the most common EHEC outbreak lineage in the US, causing 13 of 15 multistate outbreaks in 2009-2010 (Centers for Disease and Prevention, 2013). The vast majority of infections with EHEC are from the consumption of contaminated beef, leafy vegetables, or dairy products (Gould et al., 2013).

In addition to the human pathogens, *C. rodentium* is a LEE-containing A/E pathogen of mice (Frankel et al., 1996). EHEC is able to colonize but not cause disease in mice and is

incapable of forming A/E lesions in the murine intestinal tract; therefore *C. rodentium* is often used as an *in vivo* experimental model. *C. rodentium* does not natively encode Shiga toxin, however recently a strain has been engineered to carry this virulence factor (Mallick et al., 2012).

A critical difference between EHEC and *C. rodentium* is that the LEE has undergone rearrangements that affect the regulation of this pathogenicity island (Deng et al., 2004; Russell et al., 2007). In EHEC, the putative *LEE1* regulatory region extends upstream of the *ler* open reading frame by 1262 bp whereupon the ORF of the effector *espG* begins. In *C. rodentium*, the operon consisting of *espG* and *rorf1* has been moved downstream of *LEE4* (Deng et al., 2004). Additionally, a mobile insertional element has substantially altered the upstream regulatory region of *LEE1* of *C. rodentium*, rendering the vast majority of this region non-homologous to EHEC. It is unclear what regulatory functions have been altered by this genetic change. *C. rodentium* has accumulated mutations to the remaining *LEE1* upstream regulatory region that draw a distinction with EHEC. In EHEC, there are two distinct *LEE1* promoters: a proximal promoter with a transcription start site 35 bp upstream of the *ler* translation start site, and a distal promoter with a transcription start site 167 bp upstream of the *ler* translation start site. In *C. rodentium* and EPEC, the region corresponding to the proximal promoter has a 6 bp insertion that has moved the -10 site away from a potential transcription start site, rendering these organisms with only one active promoter, corresponding to the distal promoter of EHEC (Russell et al., 2007).

Regulation of the Locus of Enterocyte Effacement

The LEE encodes three transcription factors that control its own expression: *ler*, *grlR*, and *grlA*. Ler is the *LEE1* encoded master regulator of the LEE, activating the expression of LEE2-5 operons. Ler exerts its positive effect on LEE transcription by displacing the negative regulator, H-NS (Bustamante et al., 2001; Winardhi et al., 2014). H-NS is a histone-like protein that represses transcription of approximately 5% of genes, particularly those acquired by horizontal gene transfer, by inducing supercoiling (Hommais et al., 2001; Kahramanoglou et al., 2011; Navarre et al., 2006). H-NS directly binds to and silences all operons within the LEE and the relief of this inhibition is a common motif in LEE transcriptional activation (Bustamante et al., 2001; Haack et al., 2003; Sanchez-SanMartin et al., 2001; Umanski et al., 2002). In addition to activation of the LEE transcripts, Ler also promotes transcription of non-LEE encoded virulence factors, including *nleA*, *nleB*, *nleH*, and *nleF* (Bingle et al., 2014). However, not all T3SS effectors are under Ler control, such as *espFu* (Garmendia and Frankel, 2005).

GrlR and *grlA* are cotranscribed from a small operon located between *LEE1* and *LEE2*. GrlR functions as a repressor of the LEE, while GrlA is an activator (Deng et al., 2004). GrlA functions in part by displacing H-NS from the LEE1 promoter, however it retains some activator function in the absence of *hns* (Barba et al., 2005; Jimenez et al., 2010). Interestingly, Ler activates grlRA transcription via H-NS displacement, allowing the formation of a Ler-GrlA positive feedback loop (Barba et al., 2005). GrlA functions as a repressor of flagella gene expression via inhibition of *flhDC* transcription (Iyoda et al., 2006). This inhibition of the flagellar

type III secretion system is likely a prerequisite for epithelial cell colonization, as overexpression of flhDC is capable of inhibiting pedestal formation by EHEC (Iyoda et al., 2006). GrlR is an accessory protein of GrlA that is capable of directly binding to the HTH domain of GrlA, preventing GrlA from binding to DNA (Padavannil et al., 2013). GrlR functions as an apparent repressor of the LEE (Deng et al., 2004), while acting to disinhibit flagella gene expression (Kitagawa et al., 2011).

In addition to H-NS, LEE expression is controlled by several other nucleoid associated proteins (NAPs), including IHF, Hha, and Fis. Integration host factor (IHF) is a nucleoid associated protein that was originally described as being essential for lambda phage integration, but has since been implicated in the control of ~13% of the E. coli genome (Prieto et al., 2012). IHF binds to DNA in a sequence specific manner and dramatically bends the chromosome (Rice et al., 1996). IHF serves to activate LEE expression by directly binding to the LEE1 regulatory region (Friedberg et al., 1999). Hha is thought to interact with and augment the function of H-NS in gene silencing (Ueda et al., 2013). Hha acts as a repressor of the LEE via direct binding to the *LEE1* promoter, downregulating *ler* expression (Sharma and Zuerner, 2004). Fis, which preferentially binds to AT rich tracts of DNA, has approximately 900 binding sites in the MG1655 (K-12) E. coli genome (Cho et al., 2008b). Fis acts as an activator of *LEE1* and *LEE4* expression (Goldberg et al., 2001).

The ferric uptake regulator (Fur) protein consists of an N-terminal DNA binding domain and a C-terminal Fe(II) binding domain (Bagg and Neilands, 1987; Stojiljkovic and Hantke, 1995). As iron can catalyze the Fenton reaction, causing oxidative stress and DNA damage, iron levels

within a cell must be strictly controlled. Fur functions as a repressor of iron utilization genes when bound by Fe(II), but allows for the transcription of these genes when iron is limiting. Increasing intracellular iron concentrations reduces LEE gene expression (Tobe et al., 2014).

BirA is a bifunctional transcriptional repressor and biotin-protein ligase (Wilson et al., 1992). The biotin biosynthetic genes, *bioA* and *bioBFCD*, are expressed when cellular biotin concentrations are low; as biotin is produced, BirA catalyzes the formation of biotinyl 5'-adenylate, which can be transferred to the AccB protein involved in fatty acid synthesis (Chakravartty and Cronan, 2012). When biotin levels exceed the capacity of AccB, biotinyl 5' adenylate functions as a corepressor with BirA to reduce *bioA* and *bioBFCD* expression. Biotin supplementation diminishes LEE expression in a *birA* dependent manner (Yang et al., 2015). This LEE repressor ability of BirA requires *fur*, which is downregulated by BirA binding to the *fur* promoter. Fur behaves as a positive regulator of LEE expression, however it is believed to function through indirect activation (Tobe et al., 2014; Yang et al., 2015). It is not presently clear what mechanism is utilized by Fur to regulate the LEE.

The nitric oxide (NO) responsive transcription factor NsrR is a direct activator of LEE1, LEE4, and LEE5 promoters (Branchu et al., 2014). NO can bind iron-sulfur cluster containing enzymes, such as aconitase and fumarase, and inhibit their activity (Justino et al., 2007). NsrR is an iron-sulfur cluster containing protein that represses genes responsible for coping with nitrositive stress, including the nitric oxide dioxygenase, *hmpA,* and the iron-sulfur cluster repair protein, *ytfE* (Bodenmiller and Spiro, 2006). NsrR can bind NO via its iron-sulfur cluster, relieving the inhibition of transcription of these genes (Crack et al., 2015). Treatment of EHEC

with the NO donor, NOR-4, downregulates *LEE1*, *LEE4*, and *LEE5* transcripts in a *nsrR* dependent manner. NsrR is also a repressor of the gad acid resistance system regulators *gadE* and *gadX* (Branchu et al., 2014). The gad acid resistance system consists of two glutamate decarboxylase enzymes, GadA and GadB, that consume a proton to produce γ-aminobutyrate (GABA) and CO_2 from glutamate, enabling survival at low pH (Castanie-Cornet et al., 1999). This reaction is facilitated by the gadC encoded glutamate:GABA antiporter that refreshes the supply of cytoplasmic glutamate. The LuxR family protein, GadE, is the direct activator of the *gadA* and *gadBC* genes (Ma et al., 2003). GadX, an AraC type transcription factor, activates transcription of *gadE* and, thus, indirectly activates the gad acid resistance system (Sayed et al., 2007). GadE and GadX participate in a feedback loop in which GadE activates the expression of *gadX* (Branchu et al., 2014). In EPEC, GadX functions as repressors of the *perA*, and thus represses the LEE, however *perA* is absent in EHEC (Shin et al., 2001). In EHEC, like in EPEC, GadX functions as repressor of *LEE1* via an unknown mechanism, however it functions as an activator of *LEE4* and *LEE5* (Branchu et al., 2014). GadE functions as a repressor of the LEE (Branchu et al., 2014) and is capable of binding in vitro to the EHEC *LEE1* promoter (Tree et al., 2011), suggesting that GadE may be a direct repressor of LEE expression.

The gad acid resistance system can be activated by a second LuxR-type transcription factor, SdiA (Hughes et al., 2010). SdiA, when overexpressed, diminishes production of LEE4 and LEE5 (Kanamaru et al., 2000). SdiA is a regulator that senses acyl homoserine lactone (AHL) signaling molecules and aids EHEC survival and colonization of the bovine GI tract. AHLs are prominent within cattle rumen but absent in the other sections of the GI tract. Through SdiA, transcription of the LEE genes is decreased by rumen AHLs, while transcription of the gad acid-

resistant system is increased (Hughes et al., 2010; Sheng et al., 2013). SdiA is able to control LEE expression through a direct interaction with the *LEE1* promoter, binding to which can be enhanced by AHLs (Nguyen et al., 2015).

In addition to control through NO responsive NsrR, the LEE is regulated by a nitrogen responsive AraC-type transcription factor, EutR. EutR is the activator of the ethanolamine utilization pathway in *Salmonella* and E. coli, which requires the presence of ethanolamine and vitamin B-12 for induction (Luzader et al., 2013; Sheppard and Roth, 1994). Ethanolamine is capable of inducing LEE expression in EHEC, through direct binding of EutR to the *LEE1* promoter (Kendall et al., 2012; Luzader et al., 2013).

QseE is a sensor histidine kinase that is capable of sensing the host derived catecholamine hormones epinephrine and norepinephrine (Clarke et al., 2006; Reading et al., 2009). QseE form a two component system with its cognate response regulator QseF (Yamamoto et al., 2005). QseF indirectly activates expression of the non-LEE encoded effector *espFu* (Reading et al., 2007) via a pair of small RNAs, GlmY and GlmZ (Gruber and Sperandio, 2014). GlmY and GlmZ are capable of destabilizing *LEE4* and *LEE5* transcripts (Gruber and Sperandio, 2014). A second catecholamine responsive histidine kinase is capable of phosphorylating QseF, QseC (Hughes et al., 2009). QseC activates expression of *qseE* (Reading et al., 2007). QseC forms a two component system with its cognate response regulator QseB, but can phosphorylate QseF and another response regulator, KdpE (Hughes et al., 2009).

Njoroge and colleagues uncovered the importance of glucose availability in regulating T3SS by EHEC (Njoroge et al., 2012). From the observation that high-glucose growth media

suppressed type III secretion (T3S) while low-glucose conditions induced LEE expression, the authors uncovered a role for the catabolite repressor/activator protein (Cra) in ler regulation. Indeed, a *cra*-deficient mutant of EHEC exhibited diminished attaching and effacing (A/E) lesion formation, LEE1 promoter activity, transcript levels, and EspA secretion under low-glucose conditions. However, under glucose-rich conditions no effect was seen. A Cra-binding site was identified upstream of the distal LEE1 promoter, and binding to this site in an electrophoretic mobility shift assay could be prevented by the inclusion of the glycolytic intermediates fructose-1-phosphate or fructose-1,6-bisphosphate. Cra was found to directly interact with the response regulator KdpE, previously found to positively regulate *LEE1* (Hughes et al., 2009). Interestingly, KdpE dependent LEE regulation was also found to be in effect only in low-glucose conditions, and KdpE binding in vitro to the *LEE1* promoter was diminished by phosphorylation (Njoroge et al., 2012). Presumably, under high-glucose conditions, KdpE is phosphorylated by its cognate sensor kinase, KdpD, which is activated by the glucose-sensitive IIA^{Ntr} phosphotransfer system (Luttmann et al., 2009). These data suggest that Cra and KdpE act in concert to induce T3S by inducing *ler* expression and that this control is only active under glucose limiting conditions, such as those found within the colon.

Pacheco et al. describe a novel two-component system that enables regulation of virulence gene expression and carbon-source choice by EHEC upon sensing fucose (Pacheco et al., 2012). The *fusKR* operon is within the genomic O-island 20, found only in O55:H7 descendant strains and within *C. rodentium*. FusK is a transmembrane HK that autophosphorylates at a histidine residue and transfers the phospho-group to an aspartate of FusR to regulate DNA binding. The authors observe that the FusKR two component system

functions as a repressor of T3S. Given that FusK shares sequence homology with UhpB, a glucose-6-phosphate sensor kinase, the authors explored whether FusK was responsive to sugar monomers and found that fucose, but not other sugars, was sufficient to induce autophosphorylation of the kinase. Expression of *LEE1* is diminished in wild-type EHEC grown with fucose as the sole carbon source when compared with glucose; however, no difference is seen in *fusK*-deficient EHEC. Similarly, mucin was found to diminish LEE1 expression when EHEC was grown in the presence of *B. thetaiotaomicron*, which expresses fucosidases capable of liberating fucose from mucin. The effect was not seen with cocultures grown in the presence of fucose rather than mucin. Altogether, these data suggest that fucose serves as a signal to downregulate T3S via *fusKR* when EHEC is in the lumen of the colon (Pacheco et al., 2012).

The Feast/Famine Regulatory Protein family has three representatives in *E. coli*: *asnC*, *lrp*, and *ybaO* (since renamed as *cutR*) (Yokoyama et al., 2006). This family consists of an N-terminal DNA binding domain and a C-terminal oligomerization domain (Kolling and Lother, 1985). To bind DNA, these protein form multimers, with dimers and octomers being the predominant species (Koike et al., 2004; Willins et al., 1991; Yokoyama et al., 2006). AsnC is an asparagine-inhibited activator of the asparagine synthase gene *asnA* (Kolling and Lother, 1985). Lrp is said to be a global regulator of gene expression, activating expression of some genes while repressing others (Cho et al., 2008a). Lrp is thought to be a mediator of nitrogen utilization by amino acid uptake and synthesis (Ernsting et al., 1992; Newman et al., 1992). Lrp, the leucine responsive protein, binds to leucine to prevent of encourage DNA binding (Newman et al., 1992). For example, Lrp promotes expression of *ilvIH*, but *ilvIH* transcription is diminished by exogenous leucine. Lrp binding to the *ilvIH* promoter *in vitro* is disrupted by the presence of

leucine (Willins et al., 1991). However, there are promoters that are governed by Lrp and insensitive to leucine, such as the glycine cleavage system, *gcvTHP,* which is directly bound by Lrp and requires Lrp for regulation by the GcvA-GcvR system (Cho et al., 2008a; Lin et al., 1992; Stauffer and Stauffer, 1994). In addition to being responsive to leucine, Lrp is regulated by the short chain fatty acid butyrate (Nakanishi et al., 2009). Butyrate and leucine are capable of inducing LEE gene expression in a manner that is dependent upon the presence of *lrp,* which activates the LEE (Nakanishi et al., 2009). Interestingly, *lrp* behaves as a LEE repressor in *C. rodentium* (Cordone et al., 2011).

Recently, *ybaO*/*cutR* has been implicated in certain aspects of L-cysteine and D/L-serine metabolism. CutR, as it was originally named in *Salmonella*, is a positive regulator of the adjacent gene *cdsH*, a cysteine desulfhydrase that is not present in *E. coli*. CdsH protects *Salmonella* from cysteine toxicity in minimal media, presumably by mediating the decomposition of cysteine to sulfide, ammonia, and pyruvate (Oguri et al., 2012). CutR is capable, in the BW25113 strain of *E. coli*, of directly binding to the promoter of YhaO in a cysteine-dependent manner and positively regulating the expression of YhaO (Shimada et al., 2016). YhaO is a L/D-serine inner membrane transport protein (Connolly et al., 2016). YhaO serves as an indirect activator of the LEE, functioning through the YhaJ LysR type transcription factor, which is capable of directly binding to and activating LEE1 expression.

Cysteine Metabolism

Cysteine levels in the cell are carefully managed, as under certain conditions, namely high oxidative stress, cysteine is thought to be deleterious to the cell. Cysteine can catalyze the

reduction of Fe(III) to Fe(II), with the concomitant production of the oxidized form of the amino acid, cystine. Fe(II) catalyzes the Fenton reaction, in which Fe(II) reduces hydrogen peroxide to produce a hydroxyl radical, a hydroxide ion, and Fe(III) (Park and Imlay, 2003). The hydroxyl radical is capable of creating strand breaks in nearby DNA (Tullius and Dombroski, 1986). As such, *E. coli* encodes a number of cysteine transport systems.

The oxidized amino acid cystine is thought to be the imported by two transport systems, encoded by *ydjN* and *fliY-yecSC*. YdjN is a low affinity, high activity cystine: proton symporter that is thought to be the primary import system under normal conditions (Ohtsu et al., 2015). FliY is a periplasmic L-cystine binding protein that associates with the YecS ATPase and YecC membrane spanning protein to form an ATP-binding cassette transport system (Ohtsu et al., 2010). The *fliY-yecSC* system is thought to function under conditions of oxidative stress or when cysteine is scarce (Chonoles Imlay et al., 2015; Ohtsu et al., 2015). It is interesting to note that the *fliY* and *yecSC* genes are located adjacent to the LEE regulator *sdiA*.

Cysteine export from the cytoplasm is thought to combat oxidative stress in two ways: to limit the cytoplasmic pool of L-cysteine that can contribute to DNA damage through the iron mediated Fenton reaction and to attenuate oxidative damage in the periplasm (Ohtsu et al., 2010). Radical oxygen species (ROS), specifically superoxide, can be produced in the periplasm by respiratory enzymes such as fumarate reductase and NADH dehydrogenase (Imlay, 1995; Korshunov and Imlay, 2010; Seaver and Imlay, 2004). If not reduced, superoxide can damage iron-sulfur cluster containing enzymes (Jang and Imlay, 2007; Varghese et al., 2003). Superoxide dismutase in the periplasm catalyzes the reduction of superoxide to hydrogen peroxide

(Gregory et al., 1973). The AhpCF alkyl hydroperoxide reductase is an enzyme that resides in the periplasmic space and catalyzes the reduction of hydrogen peroxide to water using NADH (Cha et al., 1995). Interestingly, unlike K-12 strains, EHEC strains express a periplasmic enzyme encoded on the pO157 plasmid that exhibits catalase activity (Brunder et al., 1996). While the enzymes damaged by ROS are cytosolic, lipid peroxidation damage can occur to the plasma membrane. Cysteine can be used directly to reduce hydrogen peroxide to water (Ohtsu et al., 2010). The cysteine exporter, YdeD, is required for tolerance to externally derived hydrogen peroxide (Ohtsu et al., 2010). YdeD is a transporter of the drug/metabolite exporter (DME) family of proteins (Jack et al., 2001). It is believed that *ydeD* and the *fliY-yecSC* transport systems function as a cysteine/cystine shuttle that enables the continuous reduction of the periplasm during periods of oxidative stress (Ohtsu et al., 2010). Indeed, strains lacking either *ydeD* or *fliY* exhibit increased lipid peroxidation (Ohtsu et al., 2015). However, overwhelming oxidative stress can create a detrimental effect via the YdjN import system (Chonoles Imlay et al., 2015). Under such circumstances where exported cysteine is continually reoxidized to cystine and reimported via *ydjN*, a futile cycle can result which consumes a significant quantity of the cellular reducing capacity (Reitzer, 2015). In addition to cysteine export by YdeD, *E. coli* express the YfiK O-acetylserine/cysteine export protein belonging to the RhtB family (Franke et al., 2003). The *bcr* encoded major facilitator family transporter involved in tetracycline resistance, is also capable of exporting L-cysteine (Yamada et al., 2006).

Serine is used for the feed stock to produce cysteine. The serine transacetylase, CysE, catalyzes the acetylation of L-serine by acetyl-CoA, producing O-acetyl-L-serine (Denk and Bock, 1987). Assimilation of inorganic sulfate or thiosulfate provides the sulfur component to

produce cysteine. Import of either sulfate or thiosulfate requires the ABC transport system encoded by *cysPUWAM*. CysP is the periplasmic binding protein for sulfate; the binding protein for thiosulfate is encoded by *sbp*. CysA is the ATP binding component of the transport system. CysU and CysW form the transmembrane channel (Sirko et al., 1995).

Thiosulfate and O-acetylserine are substrates for CysM, an O-acetylserine thiolyase, which produces S-sulfocysteine (Sirko et al., 1987). S-sulfocysteine is then cleaved to produce L-cysteine and sulfite by an unknown mechanism thought to involve the glutaredoxin enzymes (Nakatani et al., 2012).

Sulfate must be reduced to sulfide before being assimilated. Sulfate is coupled to ATP by the *cysD* and *cysN* encoded sulfate adenyltransferase to create Adenosine 5' phosphosulfate (APS) and PPi (Leyh et al., 1988). APS is phosphorylated on the 3' hydroxyl by CysC, adenylylsulfate kinase, to create 3'-Phosphoadenosine 5'-phosphosulfate (PAPS) (Leyh et al., 1988). PAPS undergoes a reaction mediated by CysH, the phosphoadenosine phosphosulfate reductase , and thioredoxin 1 (Trx1) as a cofactor; in this reaction, sulfite is liberated alongside Adenosine 3', 5' bisphosphate, at the expense of oxidizing Trx1, which will be reduced by NADPH (Miranda-Vizuete et al., 1997; Russel et al., 1990). Sulfite will then be reduced by the *cysJI* encoded, NADPH dependent sulfate reductase to sulfide (Tei et al., 1990). L-cysteine can be produced from sulfide and O-acetylserine by one of two cysteine synthases, CysK or CysM (Claus et al., 2005).

In circumstances where L-cysteine levels are low, the LysR type transcription factor CysB facilitates biosynthesis. CysB responds to two small molecules, O-acetyl-L-serine and thiosulfate

(Hryniewicz and Kredich, 1991). O-acetyl-L-serine binds to CysB and facilitates DNA interactions in circumstances where serine accumulation outweighs the cytoplasmic pool of sulfur; thiosulfate accumulation antagonizes CysB activity in circumstances where the O-acetyl-L-serine pool is inadequate to assimilate the excess sulfur. CysB drives expression from the promoters of *cysDNC* (Malo and Loughlin, 1990), *cysPUWAM* (Hulanicka et al., 1979), *cysJIH* (Ostrowski and Kredich, 1989), *cysK* (Hulanicka et al., 1979), *ydjN*, *fliY*, and *yecSC* (Chonoles Imlay et al., 2015) in response to sulfur scarcity. Interestingly, in microarray studies to determine the regulon of Ler in EPEC, *cysPUWA*, *cysDNC*, and *cysJI* were all found to be positively regulated by Ler. CysB was not found to be regulated and this phenotype was not witnessed in EHEC (Bingle et al., 2014).

Intracellular pools of cysteine are maintained at low levels, with the vast majority of sulfur stored as glutathione. Glutathione production requires three amino acid components: L-cysteine, L-glutamate, and L-glycine. The first step of glutathione synthesis is catalyzed by GshA, which, in an ATP dependent process, ligates the amino group of L-cysteine to the R-group carboxylate of L-glutamate to form glutamyl-L-cysteine (Apontoweil and Berends, 1975a, b). In a second ATP dependent step, GshB catalyzes the ligation of L-glycine to glutamyl-L-cysteine to produce glutathione (Fuchs and Warner, 1975).

Methionine is produced from L-cysteine, along with L-aspartate and TCA intermediates. L-aspartate is converted to L-4-aspartyl phosphate by the action of one of three aspartate kinases, MetL, ThrA, or LysC (Cassan et al., 1986; Zakin et al., 1983). L-4-aspartyl phosphate is reduced by the *asd* encoded aspartate-semialdehyde dehydrogenase, with the concomitant

oxidation of NADPH, to produce L-Aspartate-4-semialdehyde (Haziza et al., 1982). MetL or ThrA, which have the additional homoserine dehydrogenase activity, then catalyze the reduction to L-homoserine, at the expense of NADPH (Zakin et al., 1983). MetA, the homoserine O-transsuccinylase, ligates succinate derived from the TCA intermediate succinyl-CoA to L-homoserine to produce O-succinylhomoserine (Born and Blanchard, 1999). O-succinylhomoserine is then ligated by MetB to L-cysteine to form L-cystathionine releasing succinate (Krueger et al., 1978). MetC, the cystathionine beta-lyase, then cleaves L-cystathionine into L-homocysteine, ammonia, and pyruvate (Belfaiza et al., 1986). Two homocysteine methyltransferases can complete the synthesis of L-methionine by methylation of the sulfhydryl group of L-homocysteine, MetH and MetE (Old et al., 1988). These enzymes differ in that MetH utilizes a cobalamin cofactor as an intermediate carrier for the methyl group derived from methyltetrahydrofolate (CH_3-H_4folate), while MetE transfers the group from CH_3-H_4folate (Pejchal and Ludwig, 2005).

In addition to the use of L-cysteine as a feed stock for protein, glutathione, and methionine production, the amino acid can be cleaved to liberate component molecules. There are five cysteine desulhydrases that can mediate the decomposition of L-cysteine to pyruvate, hydrogen sulfide, and ammonia in E. coli: *tnaA*, *metC*, *cysK*, *cysM*, and *malY* (Awano et al., 2005). There are three cysteine desulfurase enzymes in *E. coli: iscS*, *sufS*, and *nifS* (Lauhon and Kambampati, 2000; Loiseau et al., 2003; Mihara et al., 1997). These catalyze the transfer of the sulhydryl group from cysteine to an acceptor molecule, generating alanine. These liberated sulfur groups participate in a huge number of metabolic processes, including the formation of thiamin, biotin, lipoic acid, molybdopterin, and iron-sulfur cluster proteins (Mueller, 2006).

Fatty Acid Metabolism

The β-oxidation pathway of long chain fatty acid catabolism (Fig. 1) begins with import of exogenous LCFAs into the periplasm by the outer membrane transporter, FadL (Nunn et al., 1986; Nunn and Simons, 1978). The fatty acyl-CoA synthase, FadD, catalyzes the formation of a thioester between Coenzyme A (CoA) and the carboxylate group of the LCFA (Greenway and Silbert, 1983). Transport into the cytoplasm is mediated by FadD and is thought to occur simultaneously with CoA derivatization (Weimar et al., 2002). The fatty acyl-CoA thioester then undergoes a FadE catalyzed dehydrogenation reaction to an unsaturated 2-trans-enoyl-CoA, with a concomitant reduction of flavin adenine dinucleotide (FAD) to FADH2. FabB then catalyzes the hydration of the newly created C2-C3 double carbon bond to create a 3-hydroxyacyl-CoA (Campbell and Cronan, 2002; Lu et al., 2003). This hydroxyl group is then oxidized to a carbonyl in a second FabB catalyzed reaction, with the reduction of nicotinamide adenine dinucleotide (NAD+) to NADH (D'Agnolo et al., 1975). The FabB product, a 3-ketoacyl-CoA, is cleaved by FadA to release acetyl-CoA and, consuming CoA from solution, an acyl-CoA chain that has been shorted by two carbons (Yang et al., 1990). This acyl-CoA molecule is then available to FadE to repeat the process.

Under anaerobic growth conditions, a second enzyme, FadJ (YfcX), can substitute FadB for both enoyl-CoA hydratase and 3-hydroxy acyl-CoA dehydrogenase functions, while FadI (YfcY) can substitute FadA in ketoacyl-CoA thiolase activity (Campbell et al., 2003).

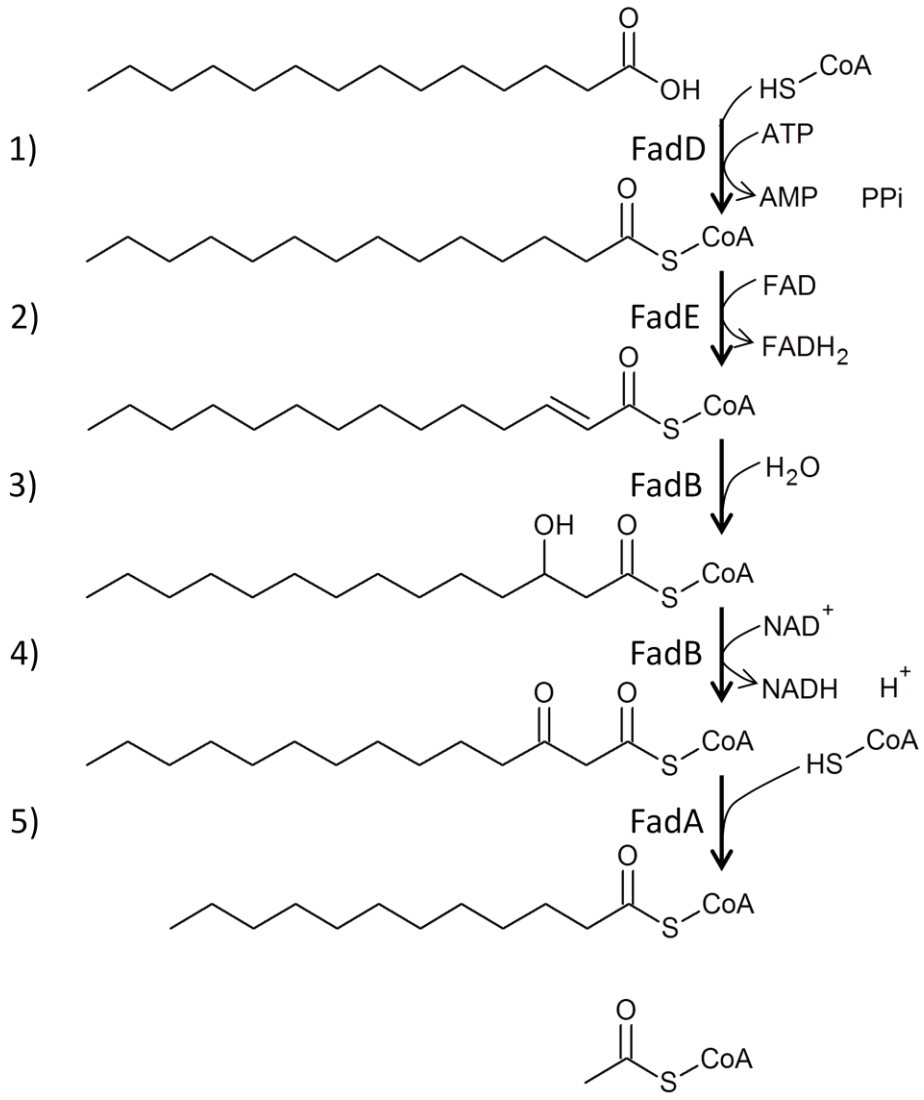

Figure 1. Diagram of β-oxidation of a fatty acid in *E. coli*

The fourteen carbon unsaturated myristic acid is processed to the twelve carbon lauric acid through a single round of the β-oxidation process. The uncoupled fatty acid is derivatized by conjugation to Coenzyme A by FadD at the expense of ATP (1). The acyl chain is oxidized by FadE to an enoyl group, reducing FAD (2). FabB catalyzes a hydration reaction to create a hydroxyacyl chain (3). FabB then catalyzes a second oxidation reaction to create a ketoacyl group, with the accompanying reduction of NAD^+ (4). FadA catalyzes the final step of the process which renders acetyl-CoA and, in this example, lauroyl-CoA, which can serve as a FadE substrate (5).

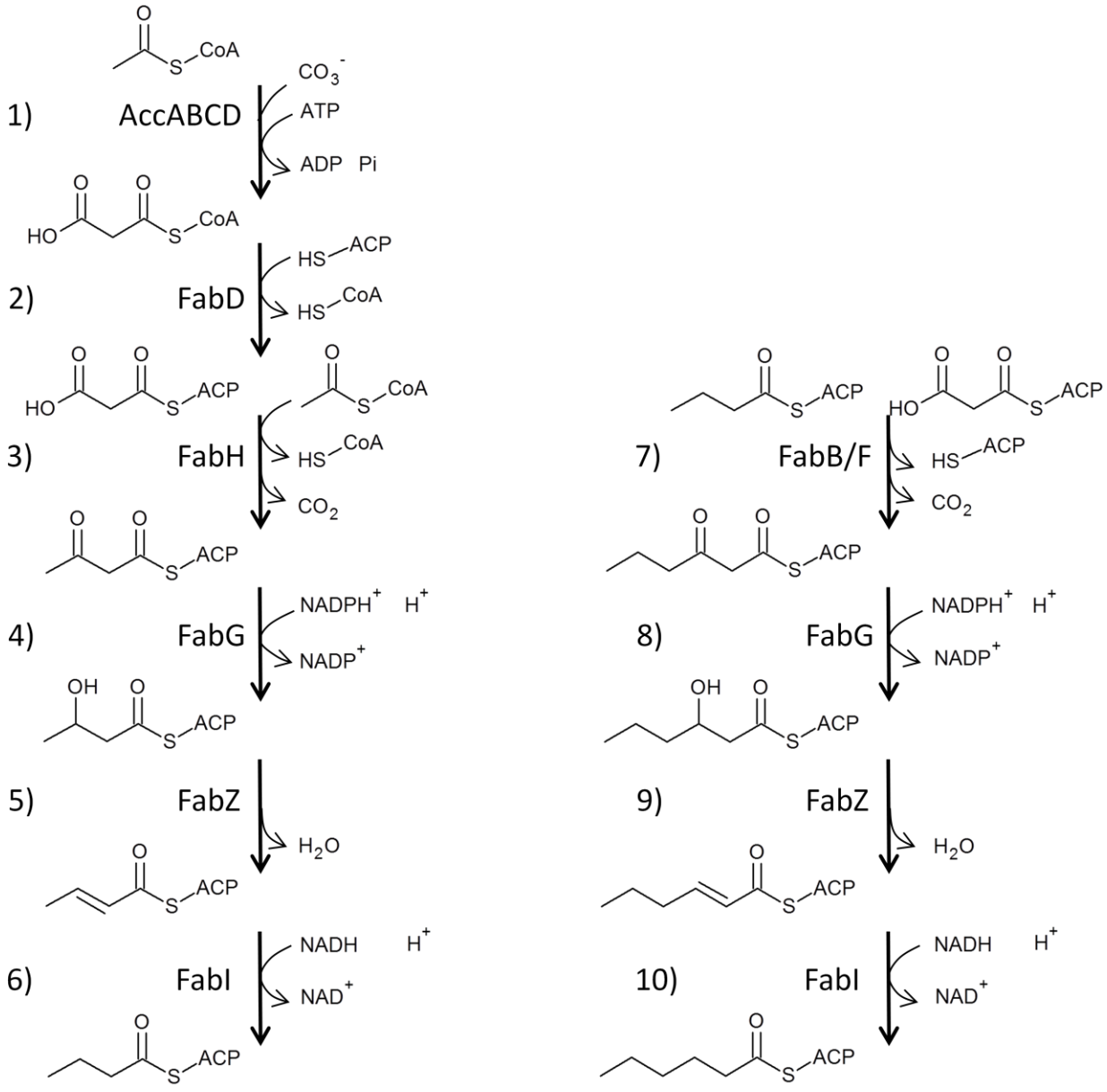

Figure 2. Fatty acid synthesis as it occurs in *E. coli*

Beginning with acetyl-CoA, a six carbon hexanoyl fatty acid chain is produced to exemplify the fatty acid synthesis process. Malonyl-CoA is produced by carboxylation of acetyl-CoA by the enzyme complex AccABCD (1). The acyl chain of malonyl-CoA is transferred to acyl carrier protein by FabD (2). FabH catalyzes a condensation of malonyl-ACP with acetyl-CoA (3). FabG catalyzes the reduction of the 3-ketoacyl chain (4). FabZ dehydrates the 3-hydroxyacyl chain (5). FabI reduces the enoyl-ACP (6). FabB or FabF catalyze a condensation of a preexisting acyl-ACP (such as that produced by Step 5) with malonyl-CoA (6). FabG, FabZ, and FabI catalyze analogous steps 8-10.

Unlike in *Salmonella*, where β-oxidation can process acyl-CoA molecules fully to acetyl-CoA, this process is unable to run until completion in *E. coli* as FadA is are unable to efficiently cleave the four carbon 3-keto-butyryl-CoA derived from butyrate (Iram and Cronan, 2006). In order to utilize this shorter product, *E. coli* must utilize the acetoaetate degradation pathway, encoded by the *atoSCDAEB* operon. In this pathway, AtoB functions functions in place of FadA in the capacity as acyl-CoA thiolase to release two molecules of acetyl-CoA from 3-keto-butyryl-CoA (Jenkins and Nunn, 1987). The gene products of *atoADE* encode a transport and CoA transferase system analogous to FadD for short acyl chains (Jenkins and Nunn, 1987)(Jenkins and Nunn 1987 Jbac). Interestingly the *ato* locus, which is located between *rcsC* and *yfaP* in K-12 *E. coli*, is absent in EHEC.

The synthesis of fatty acids (Fig. 2) is initiated by the ATP dependent incorporation of bicarbonate into acetyl-CoA by the AccABCD enzyme complex to produce the three carbon malonyl-CoA (Li and Cronan, 1993). This malonate group must be transferred to the *acpP* encoded acyl carrier protein (ACP) by FabD for further extension (Magnuson et al., 1992). The first round of extension from the three carbon malonyl-ACP to the four carbon acetoacetyl-ACP is catalyzed by FabH, consuming acetyl-CoA (Tsay et al., 1992). Acetoacetyl-ACP undergoes a reduction by nicotine adenine dinucleotide phosphate (NADPH), catalyzed by FabG to produce a 3-hydroxyacyl-ACP (Zhang et al., 2003). This molecule is then dehydrated by one of two 3-hydroxyacyl-ACP dehydratases, FabZ or FabA (Mohan et al., 1994). These enzymes are partially redundant, differing in their acyl chain length preferences (Heath and Rock, 1996). FabZ preferentially acts on substrates under six carbons or over fourteen carbons in length, while FabA targets intermediate acyl groups. In most circumstances, the product of FabZ or FabA is a

C2-C3 double bonded trans enoyl-ACP. This enoyl-ACP is reduced by NADH and FabI to produce an unsaturated acyl-ACP (Heath and Rock, 1995) that can be further extended by the action of FabB or FabF, which like FabH, are 3-oxoacyl-ACP synthases (D'Agnolo et al., 1975; Garwin et al., 1980a, b). Unlike FabH, FabB or FabF mediated reactions are primed by malonyl-ACP rather than acetyl-CoA. FabB/F catalyze the condensation of malonyl-ACP with an n-length acyl-ACP, producing a chain n+2 length 3-ketoacyl-ACP, which serves as a FabG substrate. FabB and FabF differ in that FabB participates in unsaturated fatty acid (UFA) synthesis. The production of UFA is initiated upon a ten carbon trans-2-decenoyl-ACP by the action of FabA, utilizing a second enzyme activity as an isomerase to produce cis-2-decenoyl-ACP. FabA catalyzes all analogous dehydration steps for extensions of unsaturated acyl chains, while FabZ participates in saturated fatty acid elongation (Heath and Rock, 1996). FabB is capable of condensing malonyl-ACP with cis-2-decenoyl-ACP to form cis-5-ene-3-ketododecenoyl-ACP, a FabG substate. The UFA synthesis continues with FabA and FabI activity, to produce elongated unsaturated acyl chains. Release of an acyl chain from ACP is mediated by thioesterases, including TesA, but the relative importance of various enzymes is not clearly understood (Zheng et al., 2004).

FadR is the master regulator of fatty acid synthesis in *E. coli*. FadR was originally identified as a result of a spontaneous mutation which enabled growth on medium chain length fatty acids as a sole carbon source (Nunn and Simons, 1978). Indeed, FadR is a GntR-type transcription factor that can be influenced by long chain, but not medium chain fatty acids. Transcription factors of this type contain an N-terminal HTH domain and a C-terminal small molecule binding and oligomerization domain (Haydon and Guest, 1991; Rigali et al., 2002) binds to the promoter regions of both genes involved in β-oxidation and fatty acid synthesis.

FadR acts as a repressor for the β-oxidation genes *fadD* (Black et al., 1992), *fadE* (Clark, 1981), *fadB* (DiRusso et al., 1992), *fabA*, and *fadL* (DiRusso et al., 1993). FadR acts as an activator of genes involved in fatty acid synthesis including *fabHDG* (My et al., 2013), *fabA*, and *fabB* (Campbell and Cronan, 2001). Upon binding to a derivative of the β-oxidation pathway, long chain fatty acyl-CoA thioesters, DNA binding is inhibited (DiRusso et al., 1992), inducing the expression of the β-oxidation pathway and inactivating fatty acid synthesis. FadR is capable of high affinity binding to long chain fatty acyl coenzyme A thioesters such as Oleoyl-CoA (C18:1 (cis-9), Kd~63 nM) or Myristoyl-CoA (C14:0, K_d~59 nM), however no binding occurs with medium chain length thioesters such as Octanoyl-CoA (DiRusso et al., 1992; DiRusso et al., 1998).

A second regulator of fatty acid metabolism exists in *E. coli*. FabR is a TetR-type transcription factor and a regulator of unsaturated fatty acid synthesis in *E. coli*. FabR is a repressor of *fabA* and *fabB* and directly binds to the promoters of both genes, an interaction that is facilitated by the presence of unsaturated fatty acid thioesters coupled to either ACP or coenzyme A, enabling a shut off of the synthesis of unsaturated fatty acids when concentrations reach sufficient levels (Feng and Cronan, 2011; Marrakchi et al., 2002). FabR does not regulate the fatty acid oxidation pathway (Zhang et al., 2002).

It is interesting to consider the possibility of uncharacterized fatty acid responsive transcription factors. In *Salmonella*, expression of the SPI-1 encoded activator, HilA can be downregulated by exogenous fatty acids (Kollanoor-Johny et al., 2012). This downregulation occurs through inhibition of the *hilA* activator HilD, which is capable of directly interacting with

fatty acids such as oleate (Golubeva et al., 2016). In EHEC, the LEE-encoded GrlA accessory protein, GrlR has been shown to directly bind to lipid molecules, specifically 1-hexanoyl-2-hydroxy-*sn*-glycero-3-phosphate, however it is unclear what influence this may have on GrlR activity (Jobichen et al., 2009).

CHAPTER TWO

Overall Objectives and Synopsis

Enterohemorrhagic *E. coli* (EHEC) O157:H7 is a food-borne pathogen of humans that colonizes colonic epithelial cells and causes severe diarrhea. The locus of enterocyte effacement (LEE) of EHEC encodes a type III secretion system that is essential for virulence. The LEE mediates the secretion of effector molecules that induce cytoskeletal rearrangements within colonized epithelial cells to form a unique environmental niche. Expression of the LEE is a significant metabolic burden for EHEC and thus it must be carefully regulated. Prior research from us and others have substantiated the importance of small molecule metabolites in controlling LEE expression and EHEC virulence; however, discerning the mechanisms of these signals is often challenging. We have established a high-throughput method using readily available tools to define these LEE regulatory mechanisms. We have developed an ELISA-based approach for evaluating the expression level of EspB, a component of the LEE-encoded translocon. Using the *E. coli* K-12 Keio knockout library (Baba et al., 2006), we created a library of LEE-expressing, transcription factor-deficient strains capable of type III secretion and a screen was undertaken to identify genes present in the *E. coli* core genome that can regulate the LEE. As a proof of principal of the utility of these tools, we have explored the potential role

of cysteine as a small molecule regulator of the LEE. We have subsequently identified a putative transcription factor, CutR, which is capable of governing type III secretion by *E. coli* K-12. We observed that a *cutR*-deficient strain of EHEC exhibits reduced LEE transcript levels and defective secretion. We also have determined that this is dependent upon the presence of cysteine within the growth medium. Using *Citrobacter rodentium* as a surrogate EHEC infection model, we have observed that a *cutR*-deficient strain exhibits reduced mortality compared to wild type, suggesting that *cutR* is required for full virulence *in vivo*. To further validate the findings of our screen, we have evaluated a second hit, FadR, for its relevance to LEE regulation. FadR is the master regulator of long chain fatty acid metabolism in *E. coli.* We have observed that FadR is capable of functioning as a repressor of LEE transcription in EHEC and *C. rodentium*. We have observed that *fadR*-deficient *C. rodentium* has delayed lethality as compared to wild type in an *in vivo* infection model. The LEE is differentially regulated by a variety of transcription factors, such as FadR and CutR, and small molecule metabolites, as exemplified by our findings with cysteine. As such, our work substantiates a platform method that can be used by researchers to quickly determine the roles of growth conditions and/or various metabolites in governing key signaling events by EHEC.

CHAPTER THREE

Materials and Methods

Bacterial Strains and Growth Conditions

Overnight cultures of 86-24 EHEC strains were grown for 18 hours in LB Miller broth supplemented with streptomycin or other antibiotic as determined by plasmid being

maintained. *C. rodentium* strain DBS770 (Mallick et al., 2012) was maintained similarly with chloramphenicol. Knockout strains were derived using lambda red gene replacement (Datsenko and Wanner, 2000). Strains used or prepared in this manuscript as described in Appendix A. K-12 *E. coli* knockouts were obtained from the Keio knockout library and grown in kanamycin or chloramphenicol when carrying pJAY1512. The plasmids pACYC184 (NEB) and pASKIBA32 (IBA-lifesciences) were used as backbones for complementation vectors created with the Gibson cloning kit (NEB). Appendix B describes the primers used to clone into ScaI and EcoRI linearized pACYC184 and to linearize and clone into pASKIBA32. For experimental cultures, overnight cultures were inoculated into low glucose DMEM (Gibco) supplemented with pyruvate, sodium thioglycolate, with or without 4 mM cysteine, as appropriate. Growth curves were performed in 96-well plates and analyzed for optical density at 600 nm on a BMG Labtech Fluostar Optima plate reader. Generation times were calculated as [Log2]/slope of semi-log plot of OD per time by Graphpad Prism 6. Cultures were grown at 37^{o}C under strict anaerobic conditions using a ShelLabs Bactron chamber containing 5 % H_2, 5 % CO_2, 90 % N_2.

EspB Production Screening in K-12

Keio library derived K-12 knockout strains were transformed with pAY1512, a cosmid harboring the locus of enterocyte effacement derived from O157:H7 EHEC strain 85-170, using the rubidium chloride method. These strains were grown as overnights in 96-well plates in LB media and subsequently inoculated into experimental plates in triplicate. Growth occurred under anaerobic conditions and monitored with by OD at 600nm with a plate reader. After reaching stationary phase at 6 hr, the cultures were halted by transfer to 4^{o}C and

supplementation with 15 mg/mL Sodium Azide and Sigma Protease Inhibitor Cocktail. Halted cultures were diluted in PBS into Immulon microtiter plates. EspB levels determined with ELISA using anti-EspB rabbit polyclonal antisera followed by goat anti-rabbit HRP-conjugated antibody developed with TMB colorimetric substrate and quantified at 450 nm. Absolute concentrations were interpolated from standard curves from titrations of recombinant EspB protein. The Δ*lacA* keio K-12 knockout strain with and without pJAY1512 were used as controls for normalization and background subtraction. All mutants were screen in triplicate; statistical significance were calculated in Graphpad Prism 6 by ANOVA followed by Dunnett's multiple comparisons test.

Chromatin Immunoprecipitation

ChIP was performed using N-terminally V5-tagged CutR protein was cloned into PCR linearized pASK IBA32 using Gibson cloning with primers described in Appendix B. WT and mutant strains of 86-24 were transformed by electroporation with pASKempty vector or pASKnV5YbaO, as appropriate. These strains were grown with supplementation of 12 ng/mL Anhydrotetracycline. The cells were harvested and split for use in evaluation of protein expression, mRNA expression, or for ChIP. ChIP samples were treated with 1 % formaldehyde for 20 minutes. Fixed cells were washed, resuspended in 10 mM Tris pH 8, 150 mM NaCl, 1 mM EDTA, 1 % Triton X-100, 0.1 % Sodium Deoxycholate, 0.1 % RNaseA, Sigma Protease Inhibitor Cocktail and sonicated to fragments of 100-600 bp by 7 cycles of 30 sec on/60 sec off at 95 % power on a Qsonica Q125 sonicator. Lysed sample were cleared and quantified by nanodrop. Equivalent loadings of nucleic acids were used for each ChIP replicate and coupled at 37^{o}C by adding 10 ug of anti-V5 antibody (Abcam) per reaction. These reaction were precipitated with

1.5 mg Protein A Dynabeads, and washed 10 mM Tris pH 8, 500 mM LiCl, 1 mM EDTA, 0.5 % Nonidet P40, 0.5 % Sodium Deoxycholate. ChIP samples were decrosslinked for 18 hr at 65^{o}C in 10 mM Tris pH 8, 50 mM NaCl, 10 mM EDTA, 1 % SDS. Protein was degraded by the addition of 80 μg Proteinase K per reaction and incubation at 55^{o}C for 4 hr, then 95^{o}C for 10 min. DNA was purified with Qiagen Minelute kits. qRTPCR was used to evaluate the percent input of each sample captured during ChIP. Standard curves for each input sample were performed for each probe set (Appendix B) and used to calculate the percent of sequence precipitated.

PAGE and Western Blotting

Samples were grown in triplicate in 50 mL falcon tubes as described above. Samples of equivalent optical densities were pelleted and the supernatants were filtered through Millipore Steriflip vacuum filter devices. Samples were combined with sample buffer containing BSA and boiled. Samples were run on precast Biorad Stain-Free TGX minigels and transferred onto low fluorescence PVDF membranes. Loading was evaluated using stain free settings on Biorad Chemidoc imaging system. Blots were blocked with PBS with 0.1 % Tween-20 containing 5 % fat free milk. Anti-EspB staining was performed with rabbit polyclonal antisera, followed by goat anti-rabbit HRP-conjugated antibody, and developed with Biorad Clarity ECL.

Protein purification and EMSA

FadR was cloned into the NdeI and BamHI sites of pET28 by gibson cloning (Appendix B) to create an N-terminal His-tagged construct. This was transformed into NiCO21 (NEB) chemically competent cells. Protein expression was performed as follows: cultures were grown in LB broth with kanamycin at 37^{o}C with vigorous shaking until OD ~ 0.6. Culturing temperature

was dropped to 20^{o}C, IPTG was added to 1 mM, and vigorous shaking was continued for 18 hr. Cells were pelleted, washed with PBS, and resuspended in 20 mM Na_2HPO_4-KH_2PO_4 buffer pH 7.7, 1.5 mM DTT, 20 mM imidazole, with Sigma Protease Inhibitors cocktail added. The cell suspension was lysed on ice by sonication on a Qsonica Q125 sonicator at 75 % power for 6 minutes total with 30sec on and 55 sec off pulses. Lysates were cleared by centrifugation and filter sterilized. Samples were incubated with Ni-NTA beads for 1 hr at 4^{o}C with shaking. Samples were applied to a gravity column and washed extensively with lysis buffer. Samples were eluted from the column with lysis buffer containing 250 mM imidazole. Samples were dialyzed and concentrated using 10,000 MWCO Amicon spin concentrators into 20 mM Na_2HPO_4-KH_2PO_4 buffer pH 7.7, 1.5 mM DTT. For EMSA, DNA probes were prepared by PCR (Appendix B for primers) from genomic templates. Probes were purified by gene electrophoresis and labeled with ^{32}P γ-ATP by T4 PNK (NEB). Labeled probes were further purified by Qiagen PCR Purification kit. EMSA reactions were prepared as protein diluted into a 2X EMSA buffer composted of 50 mM Na_2HPO_4-KH_2PO_4 buffer pH 7.5, 100 mM NaCl, 1.5 mM DTT, 100 μg/mL BSA, 250 μg/mL sonicated salmon sperm DNA. Binding was resolved on 5% polyacrylamide gels in TBE. Gels were dried onto filter paper and exposed to phosphoimager screens and assessed on GE Typhoon Scanner.

QRT-PCR and Microarray

Samples were grown in triplicate in 50mL falcon tubes as described above. Samples of equivalent optical densities were pelleted and cells resuspended in Trizol reagent. RNA was purified using Ambion Bacterial RNA Extraction kit. QPCR was performed with Invitrogen SYBR

Green Real-Time PCR Master Mix according to manufacturers specification on a ABI QuantStudio 6 Flex instrument. Data was analyzed by the $\Delta\Delta$Ct method. Statistics were calculated as *t*-test with Graphpad Prism. For microarrays, RNA samples were converted to cDNA and labeled as described in the Affymetrix Gene Expression manual. Samples were hybridize to Affymetrix *E. coli* 2.0 chips according to manufacturers' recommendations. Data analysis used GeneSpring software, using MAS5 normalization. We report only genes found to be differentially expressed by 2-fold.

Animal Experiments

Female C3H/HeJ mice were purchased from Jackson Labs and housed under SPF conditions at UTSW. All experiments were performed under IACUC approved protocols. We used 8-10 animals per experimental group that were between 4-6 weeks of age at the time of experiment. Animals were sorted for homogenous weights between groups and housed 3-4 animals per cage, with weekly cage changes. Animals were infected by oral gavage of 10^9 CFU of DBS770 or mutants in PBS or PBS alone. Animals were checked daily for survival, weight change, and turds were collected for analysis of CFU from feces. In some experiments, animals were precleared of their microbiota by including in their water 1 g/L Ampicllin sodium salt, 1 g/L Neomycin trisulfate salt hydrate, 1 g/L Metronidazole, 0.5 g/L Vancomycin. This treatment was continued for 10 d, followed by a single day without antibiotics before gavage. Statistical significance was determined by Prism 6. Survival was evaluated by Gehan-Breslow-Wilcoxon test.

CHAPTER FOUR

Redox, amino acid and fatty acid metabolism intersect with bacterial virulence in the gut

SUMMARY

The gut metabolic landscape is complex and is influenced by the microbiota, host physiology and enteric pathogens. To dissect the important metabolic pathways that influence virulence of enterohemorrhagic *E. coli* (EHEC), we conducted a high throughput screen. We generated a dataset of regulatory pathways that control EHEC virulence expression under anaerobic conditions. This unraveled that the cysteine-responsive regulator, CutR, converges with the YhaO serine import pump and the fatty acid metabolism regulator FadR to optimally control virulence expression in EHEC. Moreover, CutR and FadR are also necessary for murine infection by *Citrobacter rodentium*, which is a murine pathogen extensively employed as a surrogate animal model for EHEC. This high throughput approach proved to be a powerful tool to map the web of cellular circuits that allows an enteric pathogen to monitor the gut environment, and adjust the levels of expression of its virulence repertoire towards successful infection of the host.

INTRODUCTION

The gastrointestinal (GI) tract is a complex and diverse environment. The availability of metabolites and signaling molecules changes in different microenvironments, and is affected by microbiota composition, host physiology and immunology, and by pathogenic insults (Baumler and Sperandio, 2016; Cameron and Sperandio, 2015). Enteric pathogens employ various metabolic and virulence strategies to outcompete and/or exploit the resident microbiota to

successfully colonize a GI niche. These strategies include utilization of certain carbon and nitrogen sources as preferred nutrients and/or signals, exploitation of the host inflammation, and acquisition of metals amongst others (Anderson et al., 2015; Behnsen et al., 2014; Carlson-Banning and Sperandio, 2016; Curtis et al., 2014; Ferreyra et al., 2014; Fox et al., 2009; Kendall et al., 2012; Liu et al., 2012; Mellin et al., 2014; Ng et al., 2013; Pacheco et al., 2012; Thiennimitr et al., 2011; Winter et al., 2010; Winter et al., 2013).

Enterohemorrhagic *E. coli* (EHEC) O157:H7 is a food borne pathogen of humans that colonizes colonic epithelial cells and causes severe diarrhea. EHEC virulence and intestinal colonization, as well of its surrogate murine infection model, *C. rodentium*, is regulated by sugar, nitrogen, organic acid, short chain fatty acid and oxygen availability (Autieri et al., 2007; Carlson-Banning and Sperandio, 2016; Curtis et al., 2014; Fabich et al., 2008; Kamada et al., 2012; Kendall et al., 2012; Lopez et al., 2016; Nakanishi et al., 2009; Njoroge et al., 2012; Pacheco et al., 2012; Tobe et al., 2011; Tobe et al., 2014). The locus of enterocyte effacement (LEE) of EHEC encodes a type III secretion system (T3SS) that is essential for virulence. The LEE mediates secretion of effector molecules that induce cytoskeletal rearrangements within colonized epithelial cells to form a unique environmental niche. This niche takes the form of characteristic attaching and effacing (A/E) lesions formed by the organism on the intestinal epithelium (Kaper et al., 2004). Expression of the LEE is a significant metabolic burden for EHEC and must be carefully regulated (Pacheco et al., 2012). We have established a high-throughput method using readily available tools to define these LEE regulatory mechanisms. We have identified two transcription factors, CutR and FadR, that we show are capable of governing LEE expression in K-12, EHEC, and *C. rodentium*.

CutR (aka YbaO/DecR) is a member of the feast/famine regulatory protein (FFRP) family of transcription factors. These transcription factors have a N-terminal helix-turn-helix (HTH) domain and a C-terminal ligand binding/oligomerization domain (Yokoyama et al., 2006). Lrp, the canonical example of FFRP transcription factors, has been demonstrated to regulate the LEE in response to butyrate levels (Nakanishi et al., 2009). The short chain fatty acid butyrate is the principal, microbiota derived carbon source for colonic enterocytes (Roediger, 1982). CutR is a cysteine responsive transcription factor (Shimada et al., 2016). Free L-cysteine cannot be detected in the cecal contents of adult SPF mice (Sasabe et al., 2016), but upon infection with *C. rodentium*, a bloom of L-cysteine is observed (Curtis et al., 2014). Of note cysteine is important in the maintenance of the mucosal integrity through its luminal redox status (Circu and Aw, 2012). In *Salmonella*, CutR is essential for the transcription of an adjacent cysteine desulfhydrase, *cdsH*, and contributes to the detoxification of cysteine (Oguri et al., 2012). CutR has been identified as an activator of the *yhaOM* locus in the *E. coli* K-12 strain BW25113 (Shimada et al., 2016). CutR directly binds the *yhaO* (*dslT*) promoter in a cysteine-dependent manner. YhaO is a HAAAP family amino acid transporter of D- and L- serine (Connolly et al., 2016), which has been shown to activate expression of the LEE through YhaJ, a LysR-type transcription factor. The authors found that YhaJ directly binds the LEE regulatory region to drive its expression.

FadR is a member of the GntR family of transcriptional regulators, composed of an N-terminal HTH domain and a C-terminal ligand binding and dimerization domain. FadR is responsible for maintaining a balance of expression of long chain fatty acid (LCFA) synthesis and catabolism, activating expression of genes required for the former while repressing those of the

latter. FadR activity is regulated by the products of FadD, the enzyme facilitating the first step of β-oxidation, conversion of LCFAs to acyl-CoA derivatives. FadR-DNA interactions are disrupted by binding to long acyl-CoA molecules. LCFAs have been shown to influence the virulence of the enteric pathogens *V. cholera* and *S. enterica* serovar Typhimurium. In the El Tor biotype of *V. cholera*, FadR indirectly activates the expression of the master virulence regulator toxT, which activates the expression of cholera toxin and toxin-co-regulated pilus (Kovacikova et al., 2017). FadR also participates in ToxT regulation by activating the expression of fabA, encoding an enzyme required for unsaturated fatty acid (UFA) synthesis. The UFA linoleic acid directly binds to ToxT to disrupt binding to DNA (Plecha and Withey, 2015; Thomson et al., 2015). In *Salmonella* Typhimurium, a transposon disrupting *fadD* was found to decrease expression of the *Salmonella* pathogenicity island (SPI)-1 T3SS transcriptional activator, *hilA* (Lucas et al., 2000). Exogenous LCFAs are capable of downregulating *hilA* levels through a mechanism that is dependent upon the outer membrane LCFA transport protein, FadL, while being independent of FadR. DNA binding of the *hilA* activator, HilD, can be abolished by the presence of the LCFA oleate (Golubeva et al., 2016).

Here we designed a high throughput screen that allowed us to establish a data set of transcription regulators and metabolic pathways that affect LEE gene regulation. Notably CutR, YhaO and FadR intersect and converge to regulate LEE gene expression in EHEC and. *C. rodentium*. CutR and FadR virulence gene regulation also occurs during mammalian infection, with *cutR* and *fadR C. rodentium* mutants being attenuated for murine pathogenesis. This links redox, amino-acid and fatty acid metabolism with virulence gene expression in an enteric

pathogen. In summary our findings indicate that a complex web of metabolic interactions intersects with virulence regulation to promote enteric disease.

RESULTS

Survey of E. coli core genome transcription pathways that influence LEE gene expression

The LEE contains 41 genes, the majority of which are organized in five operons (LEE1-5). The first gene within *LEE1* encodes the Ler transcriptional activator of all LEE genes (Mellies and Lorenzen, 2014) (Figure 3A). The *LEE4* operon encodes the EspB protein that is part of the T3SS translocon (Garmendia et al., 2005). We have developed an ELISA-based approach for evaluating the expression of EspB (Figure 1B). The pJAY1512 LEE encoding cosmid was transformed (Elliott et al., 1999) into a subset of the *E. coli* strains from the BW25113 Keio knockout library (Baba et al., 2006), generating a library of LEE expressing K-12 strains (Appendix C). This library included 372 strains deficient in transcription factors, transcription antiterminators, two component systems, and the DNA-binding type II antitoxin proteins (Figure 3C, Appendix C). This library was screened in cysteine-supplemented DMEM under anaerobic conditions, as oxygen availability is an important factor governing LEE expression (Carlson-Banning and Sperandio, 2016), and the pathways controlling LEE expression under anaerobic conditions have been understudied.

This screen yielded 58 strains that met the cutoff criterion of a 2-fold change in EspB expression (Figure 3C, Appendix C). As a testament to the validity of the screen we also identified several factors previously described to control LEE gene expression such as Fis (Goldberg et al., 2001), HNS (Bustamante et al., 2001), Hha (Sharma and Zuerner, 2004), EutR

(Kendall et al., 2012), Fur (Tobe et al., 2014; Yang et al., 2015), GadX (Branchu et al., 2014; Shin et al., 2001), and Lrp (Nakanishi et al., 2009) (Figure 3E). The majority of the genes involved in LEE regulation were in the other category. The second most common was anti-terminators, followed by two component systems. Transcription factors of the AraC and GntR family were found at similar numbers, and the least common class of transcription factors to regulate the LEE was LysRs (Figure 3D, Appendix C). From these previously uncharacterized LEE regulators, three promising targets were selected for validation and further investigation: CutR, a FFRP family transcriptional regulator; FadR, a GntR family transcription factor; and YehU, a two component system histidine sensor kinase. CutR is a cysteine-sensitive activator of the D-serine transporter YhaO, a previously described LEE-controlling protein (Connolly et al., 2016; Shimada et al., 2016). FadR is the master regulator of fatty acid synthesis and degradation, which has been well described with regards to metabolism, but has not been characterized as having a virulence related function in pathogenic E. coli. YehU is a peptide and amino acid sensitive histidine sensor kinase known to activate the expression of *yjiY* (Behr et al., 2014), that encodes a transporter that is important during APEC infections (Tuntufye et al., 2012). We generated deletion mutants of these genes in the 86-24 strain of EHEC. Upon rescreening these mutants by ELISA, we find that all three genes contribute to EspB regulation as suggested by our screen in K-12 (Figure 3F).

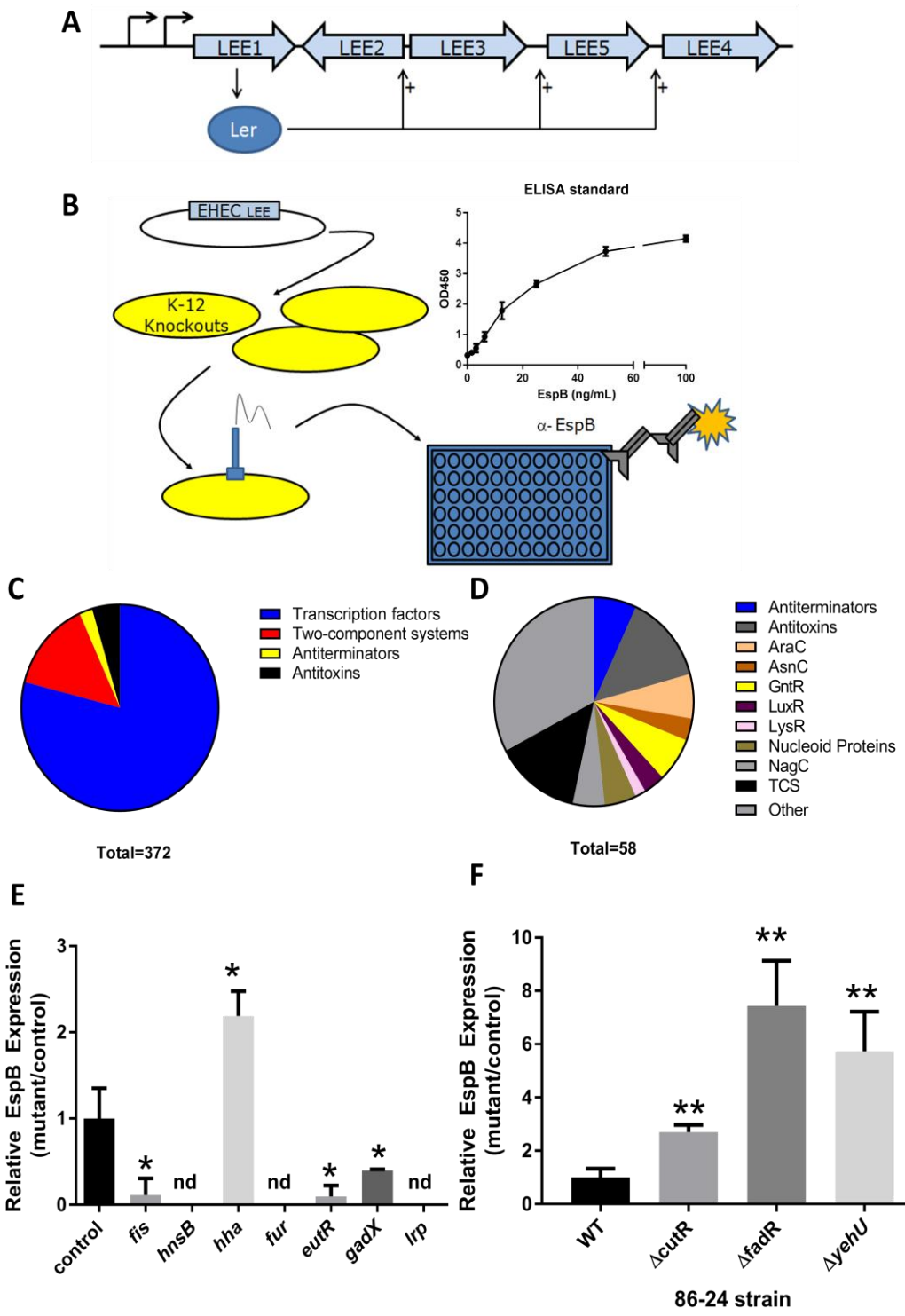

Figure 3. Screening for regulators of the Locus of Enterocyte Effacement.
A) Schematic of the T3SS-encoding Locus of Enterocyte Effacement (LEE) operon arrangement. The *LEE1* encoded Ler transcription factor positively regulates expression of the downstream LEE operons. B) Schematic of the screening process used to identify putative LEE regulators. BW25113 K-12 deletion strains were transformed with the LEE-encoding cosmid, pJAY1512. All strains were grown in DMEM under anaerobic conditions in 96-well plate format and supernatants were evaluated by ELISA for EspB

protein production. Inset, validation of EspB-directed ELISA procedure by addition of recombinant EspB to culture supernatants of BW25113. C) The class makeup of the BW25113 deletion stains included for screening by EspB production. D) The makeup of gene families found in which knockout in BW25113 resulted in at least a two-fold change in EspB production. Statistical significance was calculated as ANOVA with Dunnett's post-hoc test. See Appendix C. E) K-12 strains identified during the screen which harbor knockouts of genes previously known to regulate the LEE. F) EspB ELISA of EHEC (86-24) deletion strains used for validation of screen results. Significance representations of * ($p<0.05$), ** ($p<0.01$), nd indicates results below limit of detection.

Assessing the phenotypes of CutR, FadR, and YehU in *C.rodentium*

To investigate whether the role of these transcription factors in virulence regulation translated into in vivo phenotypes, we employed the *C.rodentium* murine infection model. *C. rodentium* is an A/E lesion forming pathogen that is extensively used as a surrogate organism for a mouse model of EHEC infection. *C. rodentium* contains the LEE and forms A/E lesion on murine colonocytes (Deng et al., 2004). We generated Δ*cutR*, Δ*fadR*, and Δ*yehU* strains of DBS770, a *C. rodentium* strain harboring the Shiga toxin encoding phage (Mallick et al., 2012). Shiga toxin (Stx) is responsible for the hemolytic uremic syndrome in EHEC infections (Karmali et al., 1983). The *C. rodentium* Stx model more closely resembles all of the facets of EHEC infection (Mallick et al., 2012).

To evaluate whether these pathways have *in vivo* relevance, we infected C3H/HeJ mice by oral gavage and monitored their survival and weight through the course of infection. We observe that both the Δ*cutR* and the Δ*fadR* mutants are attenuated for murine infection compared to WT (Figures 4A-D). The Δ*yehU* mutant is not attenuated for murine infection (Figure 4 E and F), suggesting that this gene is not critical in this infection model. The attenuation phenotypes of Δ*cutR* and Δ*fadR* cannot be explained by a difference in bacterial

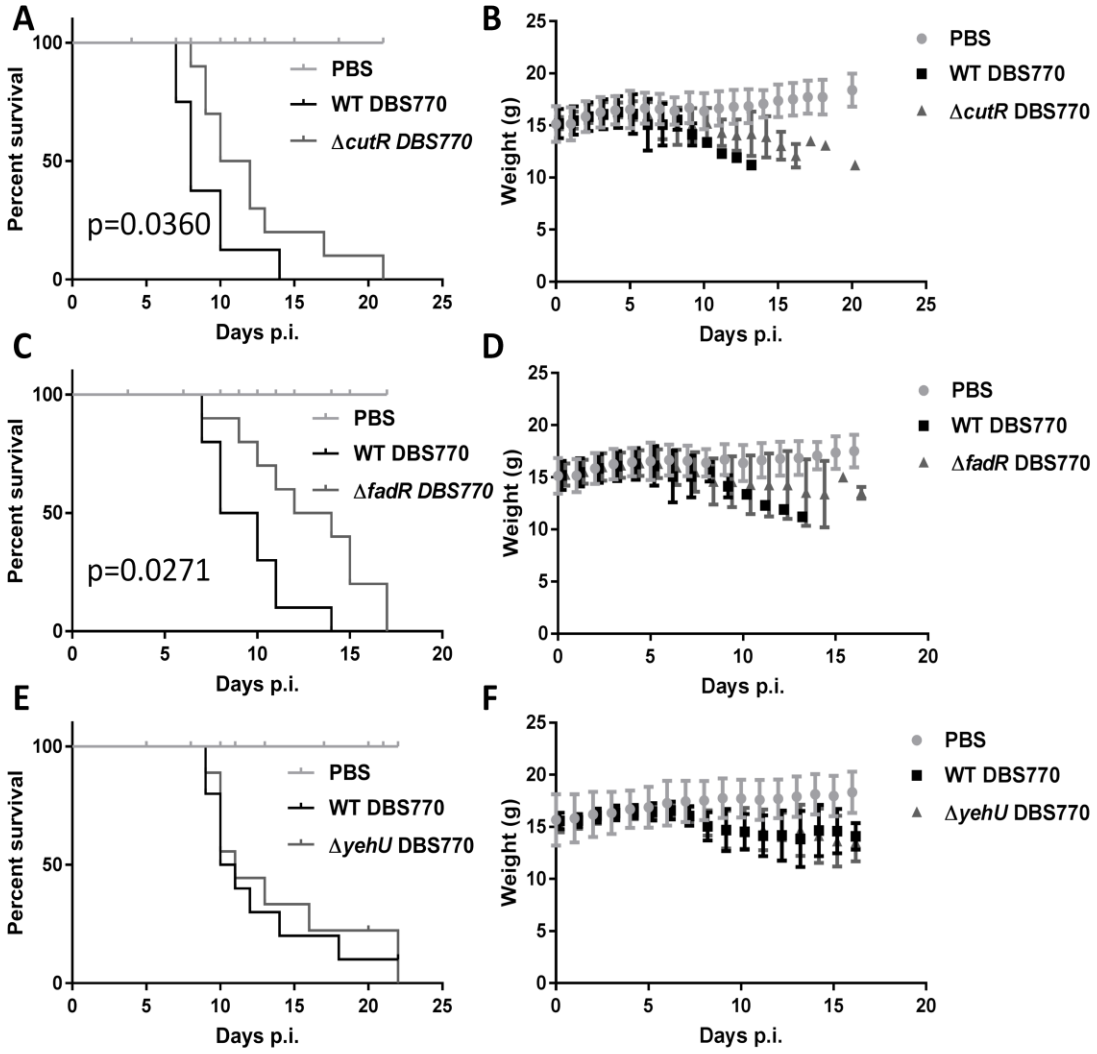

Figure 4. Evaluation of representative screen hits in *C. rodentium* infection model
Survival curves of C3H/HeJ animals infected by oral gavage with 10^9 CFU of WT, (A) Δ*cutR*, (C) Δ*fadR*, or (E) Δ*yehU* DBS770 *C. rodentium* or with PBS control. The data represents 10 animals per infection group and 8 animals for PBS controls. Statistical significance calculated by Gehan-Breslow-Wilcoxon test and p-values reported directly when significant. Weight of living experimental animals throughout the course of infection with (B) Δ*cutR*, (D) Δ*fadR*, or (F) Δ*yehU* DBS770 infected or WT infected or mock (PBS) infected. See Figure 5.

burden, as these strains colonized to levels equivalent to WT throughout the infection (Figure 5). Similarly, there is no significant difference in generation time *in vitro* for Δ*cutR* DBS770 (82±8 min) or Δ*fadR* DBS770 (71±10 min) as compared to WT (81±9 min) (Figure 7E-G).

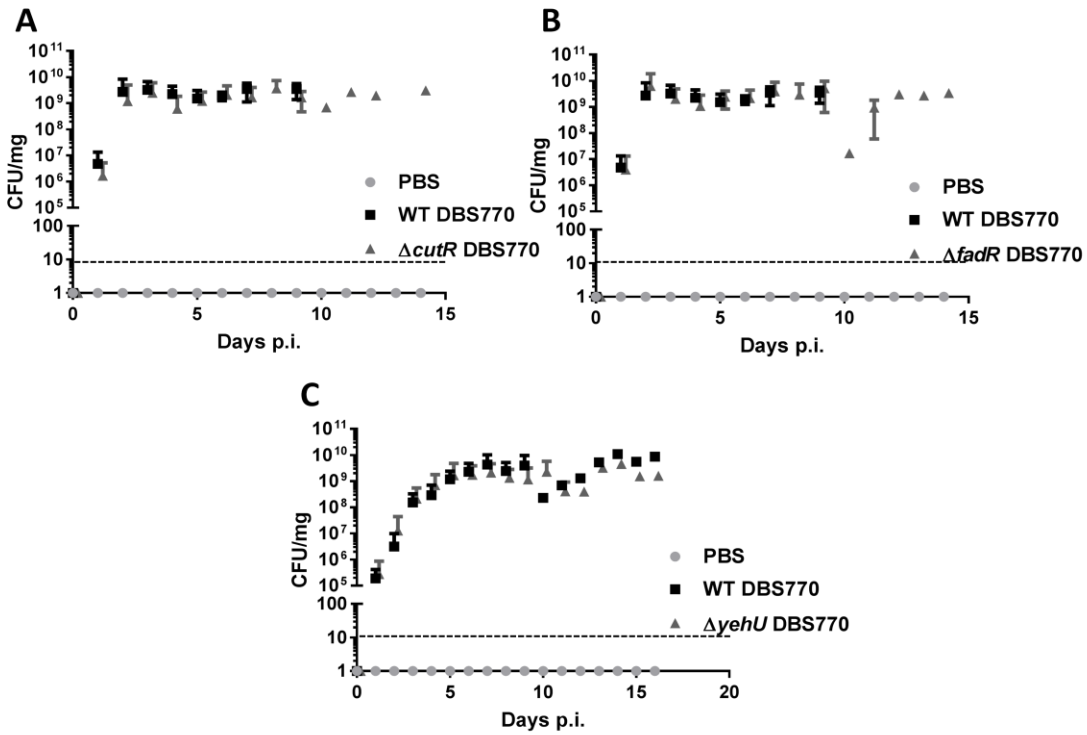

Figure 5. Extent of colonization *in vivo* for *C. rodentium* mutant strains
Bacterial load time series from feces from animals infected by oral gavage with WT or (A) Δ*cutR*, (B) Δ*fadR*, (C) Δ*yehU* DBS770 *C. rodentium*. Only animals healthy enough to pass a turd are included. Limit of detection of colony forming units is represented as a dotted line. See Figure 4.

We have previously investigated the intestinal metabolic profile of DBS770 infected or uninfected animals (Curtis et al., 2014). Cysteine was the second most increased (155-fold) metabolite in *C. rodentium*-infected animals when compared to uninfected (PBS control) animals, following antibiotic pretreatment to deplete the resident microbiota. As CutR has been demonstrated to require cysteine to function (Shimada et al., 2016), we investigated the course of infection for Δ*cutR* under depletion of the microbiota to assess whether the bloom of cysteine was microbiota dependent. Following antibiotic treatment, Δ*cutR* was still attenuated

compared to WT, suggesting that changes in the cysteine levels within the intestine are not being dictated by the microbiota (Figure 6), and may reflect the host immune responses in attempting to restore mucosal integrity (Circu and Aw, 2012).

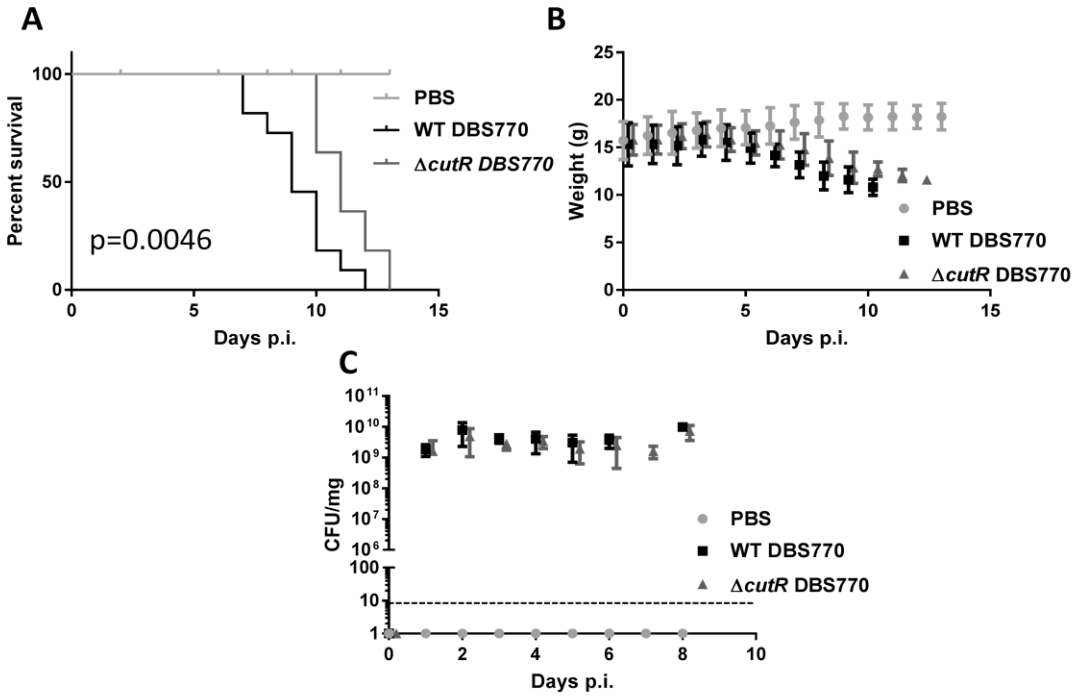

Figure 6. Effect of microbiota depletion on CutR *in vivo* phenotype
Oral gavage of WT, Δ*cutR* DBS770, or mock infection of C3H/HeJ animals treated with antibiotic cocktail prior to infection. Evaluation of survival (A), animal weight (B), and bacterial burden from feces (C). Limit of detection of colony forming units is represented as a dotted line. Statistical significance for survival curve calculated by Gehan-Breslow-Wilcoxon test.

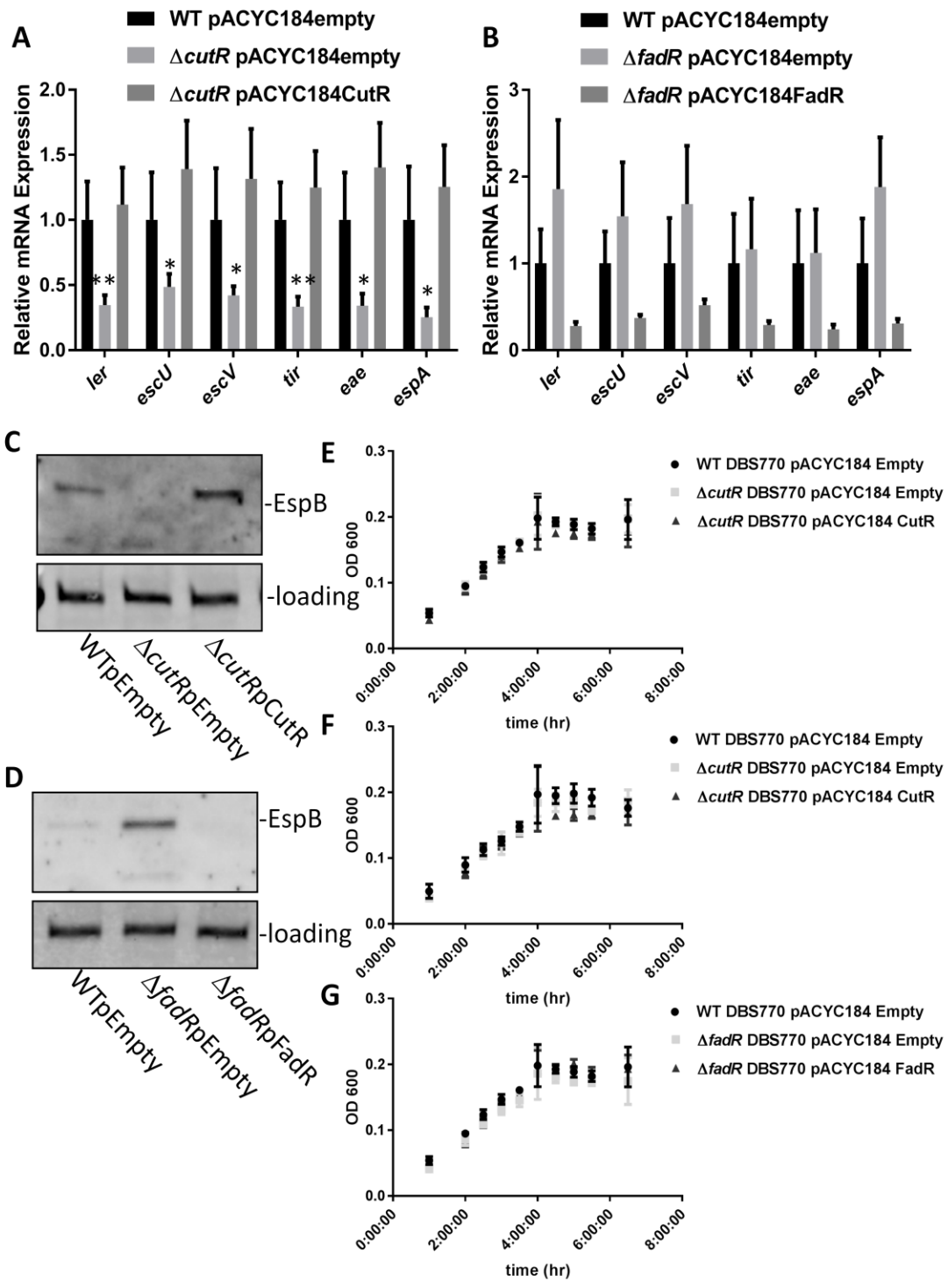

Figure 7. Evaluation of *cutR*- and *fadR*-deficiency in *C. rodentium in vitro*
A) qRT-PCR quantification of *C. rodentium* LEE mRNAs from *in vitro* anaerobically grown WT or Δ*cutR* DBS770 strains either complemented with pACYC184 carrying an empty cassette or a *cutR* expression cassette. B) qRT-PCR quantification of *C. rodentium* LEE mRNAs from *in vitro* anaerobically grown WT or

Δ*fadR* strains either complemented with pACYC184 carrying an empty cassette or a *fadR* expression cassette. C) Western blotting for EspB from *in vitro* culture supernatants from WT or Δ*cutR* DBS770 either carrying an empty pACYC184 vector or a *cutR* complementation vector. D) Western blotting for EspB from *in vitro* culture supernatants from WT or Δ*fadR* DBS770 either carrying an empty pACYC184 vector or a *fadR* complementation vector. E-F) *In vitro* growth curve of anaerobically grown WT or ΔcutR DBS770 in (E) DMEM reduced with sodium thioglycolate or (F) DMEM reduced with sodium thioglycolate and cysteine. G) *In vitro* growth curve of anaerobically grown WT or fadR DBS770 in DMEM reduced with sodium thioglycolate. Statistics are calculated as t-tests unless otherwise stated; significance representations of * ($p<0.05$), ** ($p<0.01$), # ($p<0.001$), ##($p<0.0001$).

Both CutR and FadR influence LEE expression in *C. rodentium*. The Δ*cutR* has reduced secretion of EspB and LEE mRNA levels compared to WT (Figures 7A and C). The Δ*fadR* secretes higher levels of EspB protein than WT DBS770 (Figure 7D) and is a repressor of the LEE at the mRNA level (Figure 7B). These results suggest that de-regulation of LEE expression, whether by decreased or increased expression, affects *C. rodentium* pathogenesis. A strain that poorly expresses the LEE, such as in a Δ*cutR*, is intuitively compromised in the capacity of the organism to colonize the epithelial layer. However, inappropriately high expression of the T3SS, such as with Δ*fadR*, likely represents a metabolic cost on the pathogen. Indeed, *S*. Typhimurium "defectors" deficient in SPI-1 expression via *hilD* mutation are emergent during animal infections and exhibit fast *in vitro* growth (Diard et al., 2013). In fact, over-expression of the LEE T3SS can be detrimental to the pathogen's virulence (Pacheco et al., 2012). Therefore, EHEC has to be very efficient in coordinating the right levels of expression of its virulence traits in order to successfully compete with the dense and highly adapted colonic microbiota for a colonization niche.

Intersection of CutR/YhaO and FadR LEE regulation in EHEC

Congruent with the *C. rodentium* results, CutR is also a transcription activator of LEE gene expression in EHEC (Figure 8A). EspB secretion is decreased in Δ*cutR* (Figure 8B), reflecting the overall decreased transcription of the LEE-encoded T3SS (Figure 8A). However, the levels of EspB in whole cell lysates are increased in Δ*cutR* (Figure 8C). The EspB transcript is highly post-transcriptionally regulated in EHEC (Gruber and Sperandio, 2014; Hansen and Kaper, 2009; Lodato and Kaper, 2009; Shakhnovich et al., 2009), and the discrepancy in the overall levels of EspB protein versus secreted EspB are probably a reflection of this regulation. This also explains the elevated levels of overall EspB protein in the ELISA performed with the EHEC Δ*cutR* (Figure 3F). The *cutR* gene is located adjacent to the *cdsH* (*cysK*) gene In *C. rodentium* and *Salmonella* Typimurium, which encodes the cysteine desulfhydrase (Figure 8D). EHEC also harbors *cysK*, but it is encoded in a different chromosome location. CutR is a cysteine dependent transcription factor (Shimada et al., 2016), and regulates the expression of the *cdsH* gene that encodes cysteine desulfhydrase that contributes to the detoxification of cysteine (Oguri et al., 2012). CutR is as a cysteine-dependent transcription factor in the regulation of its previously described target *yhaO*. Therefore, we attempted to determine whether the LEE regulatory phenotype was dependent upon the presence of cysteine by excluding this amino acid from the growth media. We observe that the ability of CutR to govern LEE transcript level requires the presence of cysteine (Figure 8E-G), consistent with its dependency on cysteine for transcription activity.

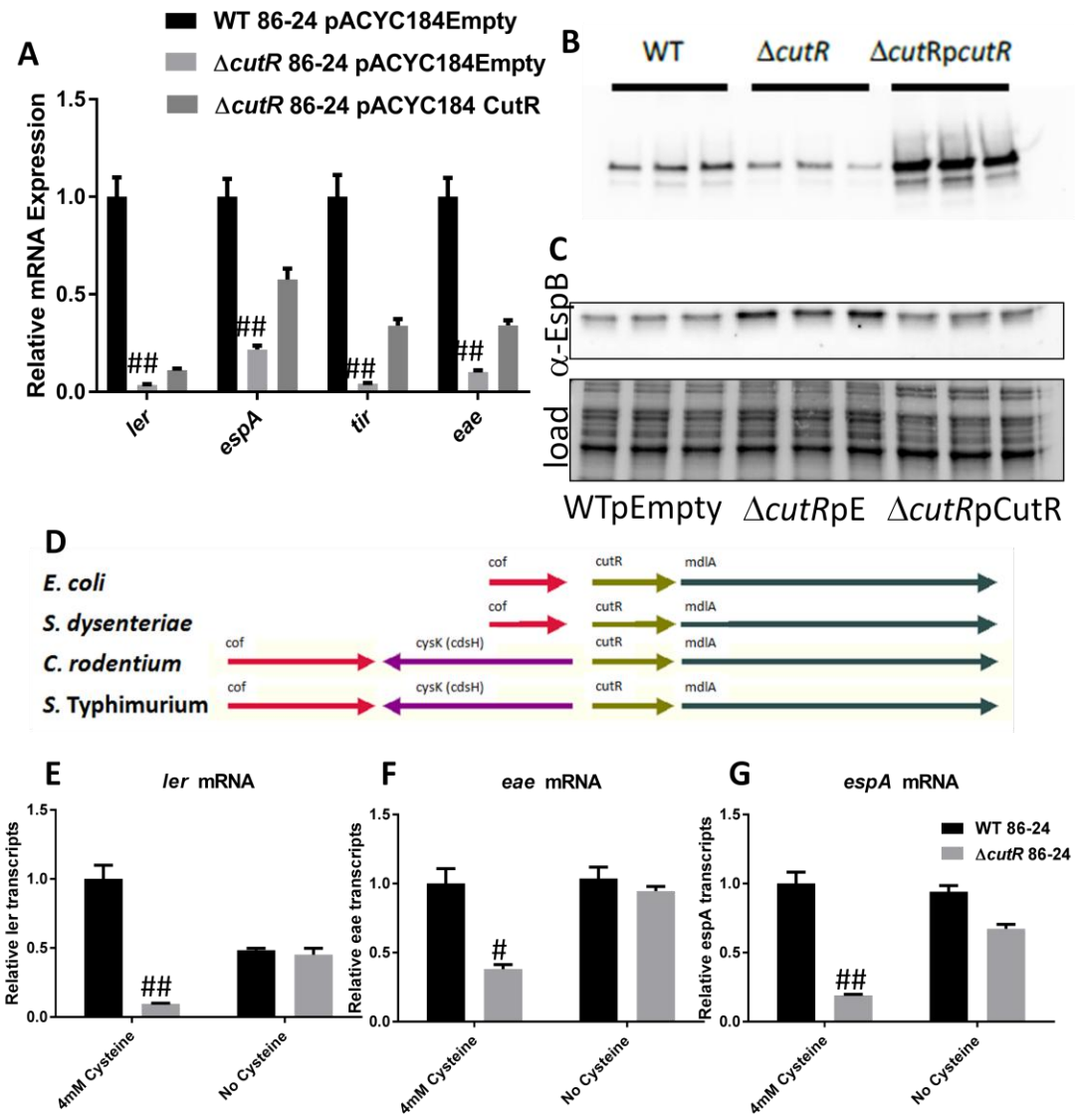

Figure 8. Evaluation of LEE expression from Δ*cutR* EHEC
A) qRT-PCR of LEE genes from WT or Δ*cutR* 86-24 EHEC grown anaerobically in the presence of cysteine. Strains are complemented either with pACYC184 carrying an empty cassette or a *cutR* expression cassette. B) Western blotting for EspB secreted from WT or Δ*cutR* 86-24 EHEC grown anaerobically in the presence of cysteine. C) Western blotting for EspB from whole cell lysates of WT or Δ*cutR* EHEC. D) Schematic comparing the *cutR* locus from *E. coli*, *S. dysenteria*, *C. rodentium*, and *S.* Typhimurium. *E. coli* has lost the adjacent *cysK*-like gene with cysteine desulfhydrase activity found in *Salmonella*. E-G) qRT-PCR of LEE transcripts for (E) *ler*, (F) *eae*, and (G) *espA* from WT or Δ*cutR* 86-24 EHEC grown anaerobically in DMEM either with or without cysteine supplementation. Statistics are calculated as t-tests unless otherwise stated; significance representations of * ($p<0.05$), ** ($p<0.01$), # ($p<0.001$), ##($p<0.0001$).

CutR is an activator of *yhaO*, which enhances serine import to increase activity of the YhaJ transcription factor that directly activates LEE transcription (Connolly et al., 2016) (Figures 9A and B). Therefore, it is conceivable that CutR-dependent LEE transcriptional activation acts through YhaO. We evaluated whether CutR-dependent LEE transcriptional activation occurs through *yhaO* by generating a double knockout of Δ*cutR*Δ*yhaO*. This double knockout had an additive effect beyond the single *yhaO* deletion mutant, suggesting that CutR LEE transcriptional regulation also occurs independently from YhaO (Figure 9C). YhaO exerts its control over the LEE through the *yhaJ* encoded transcription factor 10 that directly activates LEE transcription (Connolly et al., 2016). Therefore, we hypothesized that *cutR*-deficiency might diminish *yhaJ* expression as it does *yhaO*, and in turn diminish LEE transcript levels. However, we do not observe a decrease in *yhaJ* in Δ*cutR*, suggesting that CutR is not involved in transcriptional regulation of this gene, as probably YhaO modulates YhaJ activity through the levels of imported serine (Figure 11G). Altogether, these data indicate that there is a CutR-YhaO arm, and a CutR-YhaO independent arm to control LEE transcription. These data suggest that CutR may directly regulate transcription of the LEE genes. Because we were unable to purify soluble and folded CutR protein, to evaluate if CutR directly regulates transcription of the LEE, we constructed a tet-inducible V5 N-terminal tagged CutR plasmid for use as a ChIP construct. This construct is capable of complementing the *cutR* mutant (Figure 9E). We then performed ChIP-qPCR to evaluate if segments of the LEE1 promoter (that encodes the Ler master regulator of the LEE genes) are capable of interacting with CutR protein *in vivo* (Figure 9F). We observed that CutR is capable of interacting with the *yhaO* promoter as expected, while not interacting with the negative control *rpoZ*. We observe that CutR interacts with the LEE1 regulatory region,

suggesting that direct regulation of *ler* may be a mechanism by which *cutR* regulates T3S in EHEC (Figure 9F).

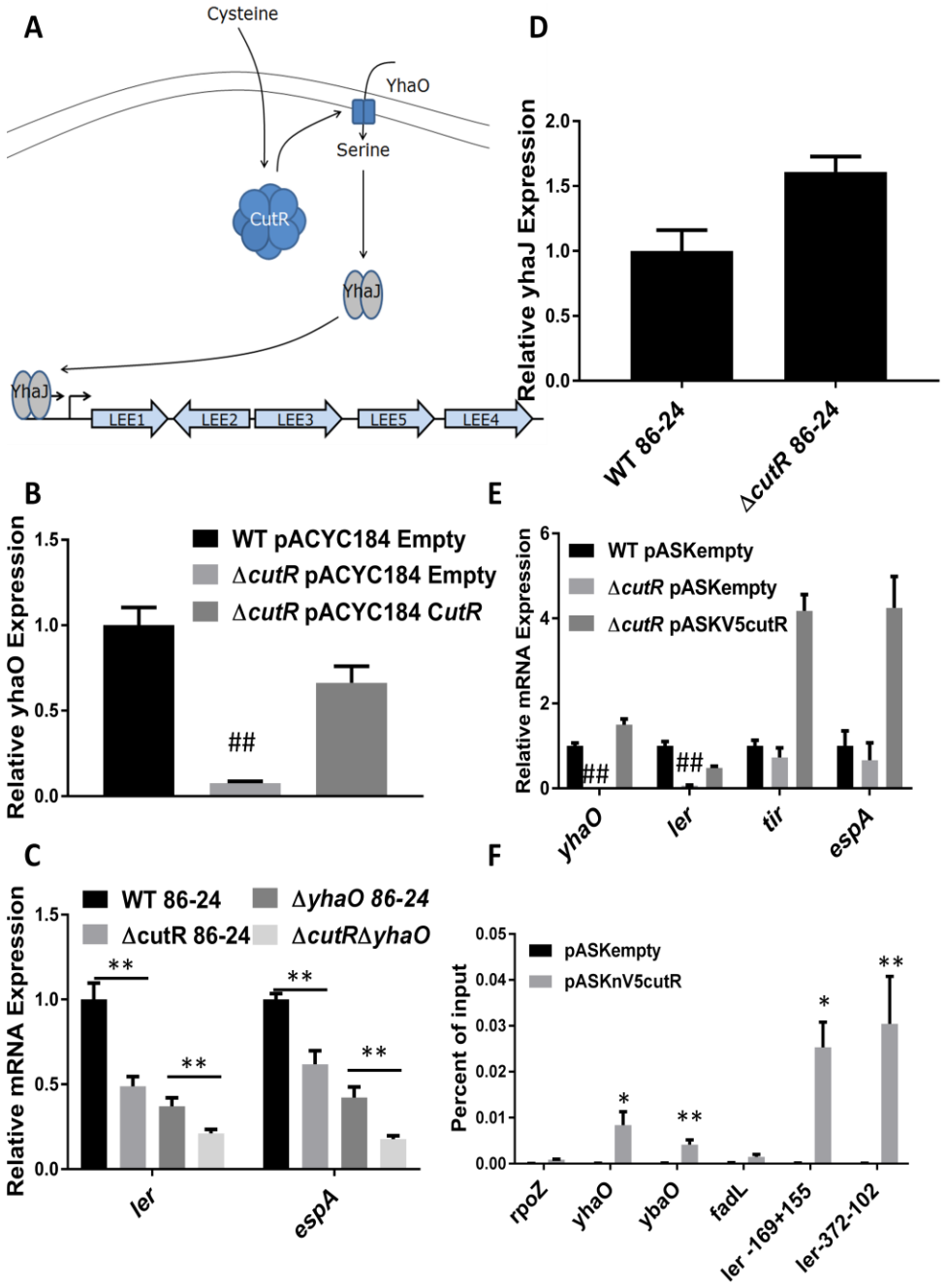

Figure 9. Exploring the mechanism of CutR-dependent LEE regulation
A) Schematic representing a putative mechanism of *cutR*-dependent LEE regulation. CutR positively regulates the expression of *yhaO*, a serine transporter that positively regulates LEE expression via the LysR-type transcription factor YhaJ. B) qRT-PCR of *yhaO* mRNA from WT or Δ*cutR* EHEC. C) QRT-PCR to

assess LEE expression from Δ*cutR*Δ*yhaO* doublemutant 86-24. D) qRT-PCR for *yhaJ* mRNA from WT or Δ*cutR* EHEC grown anaerobically in the presence of cysteine. E) Test by qRT-PCR for complementation of LEE transcriptional phenotype by N-terminally V5-tagged CutR during preparation of cells for ChIP. F) ChIP-qPCR results for empty vector control or N-terminally tagged CutR. Results are quantified as percentage of total lysed and sheared input DNA captured by immunoprecipitation. Probes are designed to amplify the promoter regions of *yhaO* (positive control), *ybaO* (*cutR* promoter), *rpoZ* (negative control), *fadL*, or overlapping fragments of the *ler* (*LEE1*) promoter, numbered from the proximal transcriptional start site. Statistics are calculated as *t*-tests unless otherwise stated; significance representations of * ($p < 0.05$), ** ($p < 0.01$), # ($p < 0.001$), ##($p < 0.0001$).

The CutR regulon has not been thoroughly explored; therefore we performed transcriptomic studies to evaluate differential gene regulation between Δ*cutR* and WT EHEC. These studies showed that 121 genes were up-regulated, and 227 genes were down-regulated in ΔcutR (Figures 10A and B, Appendices D and E). Notably, many of the upregulated (10%) and downregulated (21%) genes fall into a general category of transporters of metabolites, including fatty acids (*fadL*), polyamines (*ydcS*), glycine betaine (*yehY*), arginine (*artP*), and D/L-serine (*yhaO*). This suggests that CutR may have a broad function as a regulator of metabolite import. We confirmed the microarray results for a subset of genes via qRT-PCR. In agreement with previous reports, we observe that *yhaO* is the one of most strongly down-regulated genes in the *cutR* deletion mutant (Figure 9B, Appendix E).

Because there is YhaO independent CutR regulation of the LEE genes (Figure 4), we aimed to determine what other CutR-dependent processes may influence the LEE. We reasoned that because the original CutR-dependency of the LEE was observed in *E. coli* K-12, the mechanism of action must be at least partially conserved between K-12 and EHEC. Therefore, we decided to reutilize the LEE cosmid transformed Keio library to evaluate the importance of 304 differentially expressed non-essential K-12 genes on EspB expression. We observe that twelve qRT-PCR confirmed CutR-regulated genes (Figure 10E) are capable of

influencing EspB expression by at least five-fold. These data suggest that *cutR*-dependent LEE regulation may be multi-factorial.

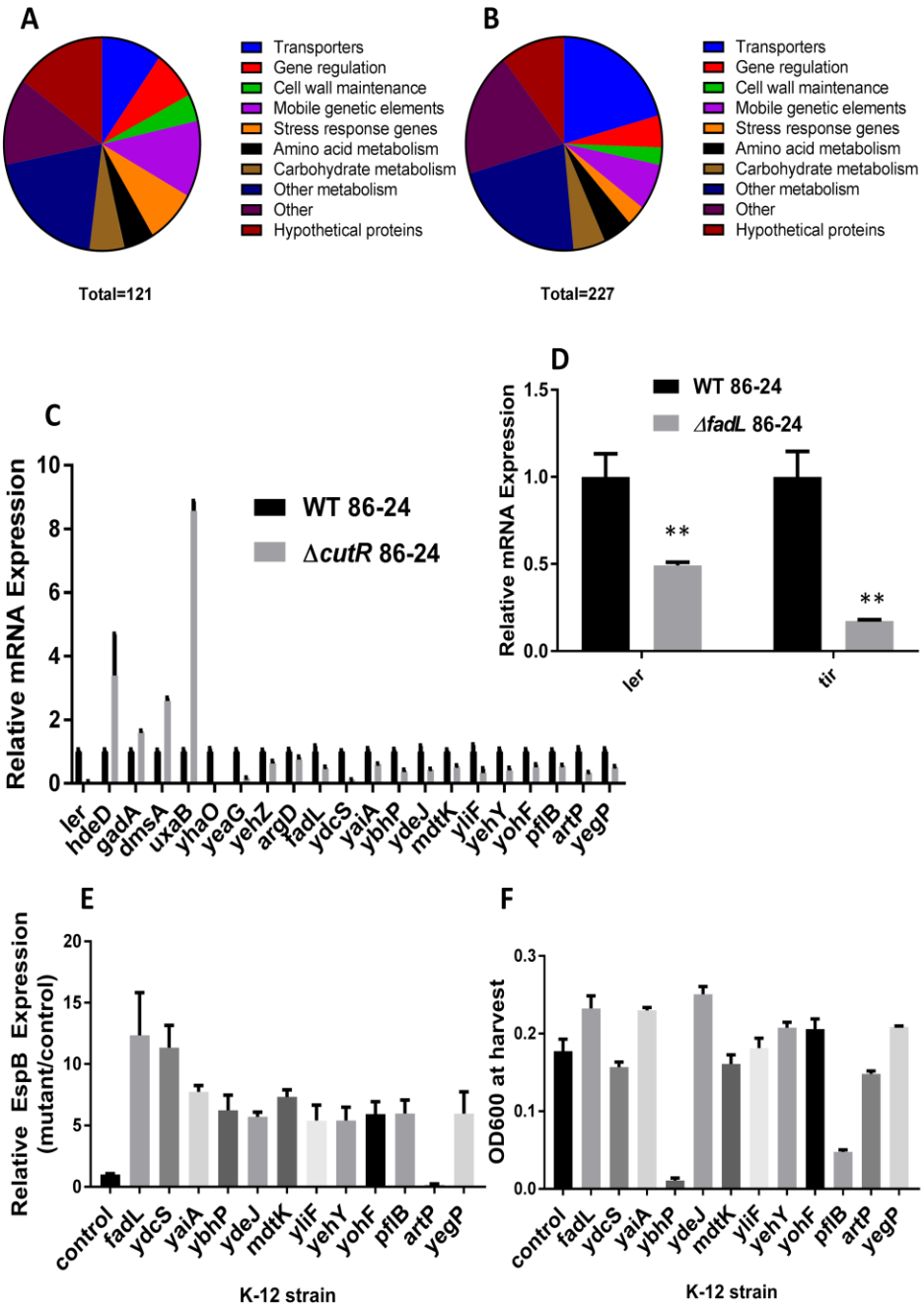

Figure 10. Evaluating the CutR regulon in EHEC
A-B) Summary of functions of genes found in microarray to be up-regulated (A) or down-regulated (B) in Δ*cutR* 86-24 relative to WT. See Appendices D and E. C) qRT-PCR of a subset of genes identified through

microarray as being differentially expressed in WT vs. Δ*cutR* 86-24. Only targets found to be statistically significant by *t*-test with p<0.05 are reported. D) qRT-PCR of *ler* and *tir* expression in WT or Δ*fadL* 86-24; t-test significance representations of ** (p<0.01). E) Relative expression of EspB from *cutR*-regulated target genes knocked out in BW25113 K-12 carrying pJAY1512. Statistical significance was calculated as ANOVA with Dunnett's post-hoc test from the broader screen, while only statistically significant results (p<0.01) greater than 5-fold are reported. F) OD600 at time of harvest for strains in (E). See Appendix F.

In these studies we observed that CutR activates expression of *fadL* (Figure 10C), which encodes a transporter for fatty acids. These fatty acids are converted to Acyl-CoA, which inhibits the transcription factor FadR (Figure 11G). In agreement with the *C. rodentium* data (Figure 7), FadR acts as a transcriptional repressor of the LEE genes (Figure 11A and B). These data are congruent with decreased transcription of the LEE *ler* and *tir* genes in the *fadL* mutant (Figure 10D). To determine whether FadR may serve as a direct regulator of the LEE, purified recombinant N-terminally His-tagged FadR protein (Figure 11D) was used on electrophoretic mobility shift assays (EMSAs) with overlapping fragments (Figure 11C) of the *LEE1* promoter. We observe that FadR is capable of interacting with the *fadL* promoter while not interacting with the kanamycin cassette of pRS551 (negative control). FadR interacts with *LEE1* fragments ranging from -967 to -693 bp and -102 tp -372 bp upstream of the proximal promoter transcription start site (Figure 11E). The *Citrobacter LEE1* regulatory region lacks the -967 to -693 bp region as an insertional element has rendered the region significantly shorter than that of EHEC. Therefore, we sought to determine whether FadR is capable of interacting with a more proximal region of *Citrobacter*'s *LEE1*. We find that FadR is capable of binding to a fragment of the *Citrobacter LEE1* region that is between -437 and -104 bp upstream of the transcriptional start site (Figure 11F).

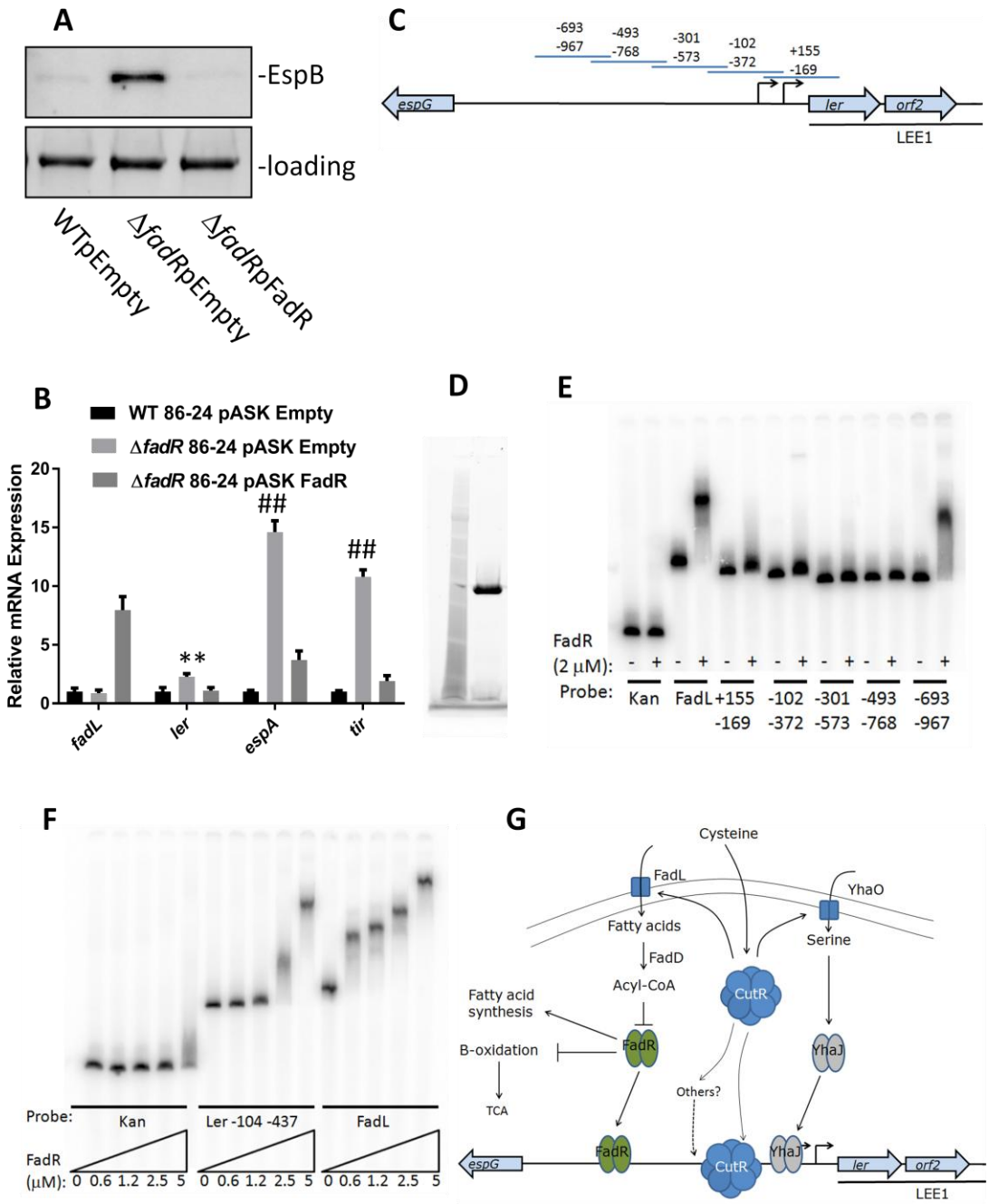

Figure 11. Evaluation of FadR as a LEE regulator

A) Western blotting for EspB from supernatants of WT or Δ*fadR* 86-24 EHEC complemented with pACYC184 empty vector or carrying a *fadR* containing cassette. B) qRT-PCR of LEE genes from WT or Δ*fadR* 86-24 EHEC. C) Diagram of EHEC *LEE1* promoter segments used for EMSA probes. Probes are designed to have approximately 80bp of overlap between segments, extending from 967 bp upstream of the proximal LEE1 transcription start site to 155bp downstream. D) SDS-PAGE of IMAC purified 6xHis-FadR (Mr ~ 29.1kDa) used for EMSA after dialysis and concentration. Left, marker. Right, recombinant protein. E) Electromobility shift assay by recombinant His-tagged FadR protein of radiolabelled DNA probes: kanamycin probe (negative control), *fadL* promoter probe (positive control), and segments of

the EHEC *LEE1* regulatory region numbered from the proximal transcriptional start site. F) EMSA of a *Citrobacter LEE1* promoter fragment extending from 437bp upstream to 104bp upstream of the transcriptional start site. G) Schematic of model of fadR- and cutR-dependent LEE regulation. The fatty acid metabolism regulator, FadR, is capable of binding to the LEE1 regulatory region to act as a repressor of Type III Secretion gene expression in EHEC. The cysteine utilization regulator, CutR, binds to LEE1 and facilitates expression of the LEE. CutR also controls the expression of other pathways that are capable of influencing LEE expression, including the fatty acid transport protein, FadL, and the serine transporter, YhaO. Statistics are calculated as t-tests unless otherwise stated; significance representations of * ($p < 0.05$), ** ($p < 0.01$), # ($p < 0.001$), ## ($p < 0.0001$).

Our findings link CutR-cysteine dependent regulation with YhaO-serine regulation to fatty acid metabolism (FadL and FadR) being converged and inter-connected to optimally regulate expression of the LEE virulence genes in EHEC and *C. rodentium*. These findings illustrate how the bacterial cells read the metabolic landscape of the gut environment to inform themselves on when, how, and where to deploy their virulence armamentarium.

DISCUSSION

Upon horizontal acquisition of a new genetic locus, an organism must control the expression of the genes within the locus. This may be accomplished by silencing gene expression, such as by the H-NS nucleoid-like protein (Navarre et al., 2006). However, to evolve a positive use for a set of acquired genes, the organism must incorporate the locus into the preexisting core genome regulatory architecture. Such incorporation may involve developing the capacity to govern regulators prepackaged within the acquired island. An example of such a system is the *LEE1* encoded Ler protein, which is capable of antagonizing the effects of H-NS, enabling the expression of the LEE operons (Bustamante et al., 2001). As *ler* is a lynchpin for the expression of the LEE, it is not surprising that it is under exquisite control by a large number of inputs. Our work represents a method of mapping the core genome components that

contribute to these inputs for the LEE, and by extension, to other virulence components of pathogenic *E. coli* strains. Specifically, we have utilized a K-12 knockout library transformed with a LEE encoding cosmid to study the conserved *E. coli* pathways that can influence the production of the T3SS translocon protein, EspB.

We have identified two transcription factors, CutR and FadR, which are known to regulate metabolism in *E. coli*, but had not yet been identified as contributing to regulation of virulence in EHEC, as key players in enteric virulence. CutR and FadR also intersect with the serine import pump YhaO to inform EHEC of the amino acid, redox and fatty acid availability within the gut environment. Our results reveal that FadR is a repressor of the LEE that is capable of directly interacting with the *LEE1* regulatory regions in EHEC and *C. rodentium*. CutR functions as an activator of LEE expression, while controlling a network of genes that influence the LEE. Importantly, our transcriptomics analysis for Δ*cutR* EHEC suggests that this transcription factor may be important for maintaining a network of metabolite transporters. This is interesting from a virulence perspective, as the importance of small molecules in governing LEE expression is being increasingly appreciated.

Our dataset reveals other yet unexplored pathways that may prove important for regulation of T3S in EHEC, comprising a complex cellular web with intersecting circuits that remain to be mapped in detail. The metabolic landscape of the gut is dramatically changed by the presence of certain members of the microbiota and upon enteric infection (Curtis et al., 2014). EHEC is a remarkably efficient pathogen, which is able to establish itself in the host

through a very small infectious dose. EHEC's proficiency to intersect metabolic, signaling, redox and oxygen sensing may be at the core of its prowess as a successful enteric pathogen.

CHAPTER FIVE

Discussion

My initial aim in this work was to create a system to rapidly identify *E. coli* core genome components that can influence LEE gene expression under a chosen *in vitro* growth condition. To accomplish this, I created a library of BW25113 K-12 strain deletion mutants transformed with a LEE carrying cosmid. I used this to screen for transcription factors that are capable of influencing the expression of a LEE-encoded type III secretion system component, EspB. Screening for EspB regulators in K-12 has generated a significant number of genes that could possibly regulate the LEE in EHEC (Appendix C; Figure 3C). As it is not possible to experimentally investigate every hit, my work has focused on validation of *cutR* and *fadR;* the results of these investigations are the topic of the previous chapter. However, in order to determine which genes might make the most promising candidates for follow-up studies, I will now review what is known about some of the remaining candidate genes and their potential relation to virulence regulation.

We observed three NagC-type transcription factors, *mak*, *yphH*, and *nanK*, as apparent activators of EspB production under our conditions. These proteins belong to the ROK (repressor, ORF, kinase) protein family that have N-terminal DNA binding domains and C-terminal sugar binding domains, which in some proteins has a kinase activity (Titgemeyer et al., 1994). The NagC protein has been described as a direct activator of *LEE1* expression in EDL933 (Le Bihan et al., 2017). NagC is a repressor of N-acetylglucosamine (NAG) catabolism that binds NAG-6 phosphate, derepressing transcription from the *nagE* and *nagBACD* promoters

(Plumbridge and Kolb, 1991). Bihan et al. indeed observe that treatment of EHEC with NAG is capable of diminishing LEE expression in a *nagC* dependent manner. Mak (manno(fructo)kinase) contributes to D-fructose utilization by phosphorylating the sugar to form B-D-fructose-6P (Sproul et al., 2001); *mak* is the most distantly related member of the ROK family and it is unclear what role *mak* has in gene regulation (Titgemeyer et al., 1994). YphH is an uncharacterized transcription factor (Brechemier-Baey et al., 2015). NanK encodes an N-acetylmannosamine kinase which contributes to the catabolism of N-acetylneuraminic acid (NANA) (Plumbridge and Vimr, 1999). It is unclear what role *mak*, *yphH*, or *nanK* may have in regulation of virulence in EHEC, but given the prominence of carbon source dependent LEE regulation (Carlson-Banning and Sperandio, 2016; Njoroge et al., 2012; Pacheco et al., 2012) this is worthy of further investigation. Interestingly, the NanK substrate, N-acetylmannosamine, is produced from the *nanA* mediated cleavage of NANA; NANA was recently shown to increase expression of *espB* (Carlson-Banning and Sperandio, 2016), however the mechanism is not known. It is possible that NANA exerts a phenotype upon the LEE in a *nanK* dependent manner.

The AraC family of transcription factors consists of an N-terminal ligand binding/dimerization domain and a C-terminal helix-turn-helix domain (Menon and Lee, 1990). The DNA binding activity of this family is governed by small molecule binding, as determined by the N-terminal domain. AraC, for example, binds to L-arabinose to induce the expression of the *araBAD* operon needed to feed arabinose into the pentose phosphate pathway of catabolism. The promoter of *araBAD* (pBAD) is adjacent to and in opposite orientation of the araC promoter and contains four upstream AraC binding sites: *araO2*, *araO1*, and two half sites, *araI1* and *araI2.* In the absence of L-arabinose, AraC is thought to bridge the *araO2* to *araI1* sites,

preventing transcription of *araBAD*. Upon binding to L-arabinose, an AraC dimer binds instead to araI1 and araI2 half sites, promoting transcription of araBAD (Bustos and Schleif, 1993; Hamilton and Lee, 1988). AraC represses its own transcription upon binding L-arabinose by binding *araO1*.

Four araC transcription factors were identified in our K-12 screen: *ydiP*, *eutR*, *gadX*, and *yijO*. GadX is a published LEE regulator with a complicated set of phenotypes. EutR is a known activator of the LEE, directly binding to *LEE1* to promote transcription, as previously discussed (Kendall et al., 2012). GadX appears to behave as a *LEE1* repressor, while *gadX*-deficient EHEC has diminished *LEE4* and *LEE5* expression, suggesting that it can activate expression of these transcripts; the mechanism for these phenotypes is unknown (Branchu et al., 2014).

The role of two AraC-type transcription factors found in our screen, *ydiP* and *yijO,* in gene regulation is completely undescribed. However, from genomic context, *ydiP* may be involved in fatty acid metabolism, as this gene is flanked by *ydiO* and the *ydiQRST* operon. While anaerobic β-oxidation equivalents of FadB and FadA exist, namely FadJ and FadI, respectively, there has not been described an anaerobic equivalent of FadE. FadE is a flavoprotein and acyl-CoA dehydrogenase that links the aerobic β-oxidation pathway to the electron transport chain, as reviewed previously. The *ydiQRST* operon is postulated to function as the flavoprotein complex in accordance with YdiO, a putative acyl-CoA dehydrogenase, to form an anaerobic equivalent to FadE (Campbell et al., 2003).

We observed eight two component system genes in our EspB production dataset: *atoS*, *atoC*, *evgS*, *uhpA*, *creB*, *yehU*, *yehT*, *qseE*. Two component systems represent a sensory

mechanism by which signals from the periplasmic space can be sensed and relayed to the cytoplasm to influence gene expression. The sensor of these systems is a histidine kinase located in the cytoplasmic membrane In *C. rodentium*, the histidine kinase QseE is required for full LEE gene expression (Moreira et al., 2016). However, *qseE*-deficient EHEC exhibits increased LEE expression (Njoroge and Sperandio, 2012). Our K-12 dataset suggests that *qseE* is an activator of EspB production, in our system, in agreement with the *C. rodentium* phenotype. In EHEC, QseE represses *rcsB*, which encodes an activator of *LEE1* (Reading et al., 2010; Tobe et al., 2005), suggesting that LEE activation in *qseE*-deficient EHEC is via RcsB disinhibition. Our K-12 dataset did not indicate that *rcsB* had a phenotype on the LEE, suggesting that this protein is not active under our tested conditions. This opens that possibility that an unknown mechanism for *qseE* to activate the LEE exists in C. rodentium and K-12, that distinquishes *C. rodentium* from EHEC.

EspB levels were reduced in both *atoS* and *atoC* knockouts. The *atoSC* containing locus is absent in EHEC, suggesting that the interaction with the LEE must be via a metabolic difference between EHEC and K-12. AtoSC is a positive regulatory two component system for the acetoacetate catabolism operon, *atoDABE*, in K-12, which has also been implicated in the activating production of the polymer PHB (Theodorou et al., 2006). Ubiquitous among bacterial species, PHB is a polymer of R-3-hydroxybutyrate, which in *E. col*i is composed of ~140 subunits per polymer and is distributed throughout the cell (Dai and Reusch, 2008; Huang and Reusch, 1996). Short fragments of PHB are thought to post-translationally modify proteins, including H-NS (Reusch et al., 2002). In screening to identify EspB regulators that were within the CutR regulon, we discovered that *cutR*-induced *ydcS* works as an apparent inhibitor of EspB

production. YdcS has been characterized as a periplasmic poly-3-hydroxybutyrate (PHB) synthase and a member of a putative ABC transporter (Dai and Reusch, 2008). While EspB production for *ydcS*-deficient and *atoSC*-deficient strains appears to be in contradiction with one another, as presumably all deficient strains would produce lower PHB, it is interesting that two systems capable of influencing PHB production were identified.

EvgS deletion diminished EspB production in K-12, however I did not witness a significant change from the *evgA* deficient strain, lacking the cognate response regulator. Similarly, the deficiency of the response regulator *uhpA* diminished EspB, but deficiency of the *uhpB* histidine kinase had no effect. I think that these findings probably represent false positives within the dataset.

CreB deficiency resulted in a diminishment of EspB production in K-12. CreB is the response regulator for the CreC histidine kinase. CreC deficiency resulted in a significant upregulation of EspB production, while not meeting our cutoff of a two fold change. The *creBC* TCS responds to nutrient availability in that it is capable of activating a *creD* reporter in response to short chain carbon sources, such as pyruvate (Cariss et al., 2008). Given the role of pyruvate as an inducer of the LEE (Carlson-Banning and Sperandio, 2016), further investigation of the role of *creBC* in LEE regulation would be interesting. The regulon of the CreBC TCS is incompletely known, however the system has been shown to regulate a variety of carbon utilization pathways that may contribute to LEE regulation (Avison et al., 2001).

The *yehUT* TCS was identified as a strong regulator of EspB, with production being low in *yehT* deficient K-12 and high in *yehU* deficient K-12. We have validated the phenotype of *yehU*

in EHEC. We did not witness a difference in virulence with animals infected with *yehU*-deficient *C. rodentium*, however. As I only wished to work with apparent LEE regulators that exhibited an *in vivo* virulence defect, I did not progress further with work on the YehUT system. However, the system is very intriguing in that YehU has been reported to be a high affinity receptor of pyruvate (Behr et al., 2017), which is a known LEE inducer (Carlson-Banning and Sperandio, 2016).

I witnessed a diminishment in EspB production for K-12 strains deficient in four antiterminator proteins: *ydfT*, *rof*, *rfaH*, and *nusB*. In general, these proteins play a role in gene regulation by preventing the termination of transcripts actively being polymerized. The strongest EspB decrease was seen for *nusB*. NusB encodes the only non-essential gene of the set of N utilization substances that is required for the function of lambda phage N antiterminators (Ghosh and Das, 1984). The lambda N protein, in conjunction with a set of host proteins including NusA, NusB, NusE, and NusG, bind to a nascent RNA and interact with RNA polymerase to prevent intrinsic transcription termination (Stagno et al., 2011). The Nus complex can interact with non-phage proteins, such as SuhB, to mediate antitermination of host transcripts (Singh et al., 2016). It is unclear what mechanism of action the antiterminator proteins may use to influence the LEE, as they could potentially affect the expression of many genes.

Surprisingly, antitoxin proteins represented 8 of the 51 undescribed putative LEE regulators: *yfjF*, *yafW*, *ybaQ*, *dinJ*, *yafN*, *ygfY*, *yfjO*, *ybbC*. These proteins are typically paired with a cognate toxin protein and together comprise the so called toxin-antitoxin (TA) systems.

These systems are thought to be associated with mobile genetic elements, such as plasmids, and constitute an addiction mechanism contributing to the maintenance of such elements. The antitoxins of type II TA systems often serve as autoregulators of TA system transcription (Li et al., 2008; Oberer et al., 2007), but other potential targets are largely unknown. TA system contribution to virulence has been described in Uropathogenic *E. coli*, where these loci contribute to in vivo colonization (Norton and Mulvey, 2012).

The GntR family of transcription factors consist of an N-terminal DNA binding HTH domain and a C-terminal ligand binding and oligomerization domain (Rigali et al., 2002). Four GntR proteins were identified as EspB regulators in K-12: *fadR*, *ydcR*, *lldR*, and *exuR*. This is interesting as there are no prior reports of this family of transcription factors contributing to virulence in EHEC. I have described the role of *fadR* in LEE regulation in a previous chapter. YdcR located adjacent to the *ydcS* gene, previously discussed, but otherwise little is known about the protein. LldR serves as an activator and repressor of the *lldPRD* operon. In the absence of L-lactate, LldR inhibits translation from this operons promoter, but upon L-lactate binding, LldR facilitates transcription of the *lldPRD* locus, which encodes a L-lactate permease and dehydrogenase (Aguilera et al., 2008). An *exuR*-deficient strain displayed a six-fold upregulation of EspB. The hexuronate utilization system is repressed by ExuR in the absence of the allosteric regulator and pathway metabolite D-galacturonate (Mata-Gilsinger et al., 1983; Tutukina et al., 2016). This is interesting as the hexuronates D-galacuronate and D-glucuronate have been demonstrated to strongly active LEE expression (Carlson-Banning and Sperandio, 2016). ExuR may provide the mechanism for this regulation; however the extent of the ExuR regulon outside of hexuronate metabolism has not been described.

We identified *gcvR* as a repressor of EspB production in K-12. GcvR is an accessory protein to the GcvA activator of the glycine cleavage system. The glycine cleavage system (GCS), *gcvTHP*, catalyzes the degradation of glycine to yield ammonium, CO_2, and a methyl group which is transferred to tetrahydrofolate to serve as a one-carbon donor for a variety of downstream applications. GcvR binds to GcvA in the absence of L-glycine to inhibit production of the GCS. When glycine levels are relatively high, GcvR binding to GcvA is inhibited by GcvR binding to glycine, allowing GcvA to activate *gcvTHP* transcription (Ghrist and Stauffer, 1995). Intriguingly, GCS regulation by GcvA is thought to happen in conjunction with Lrp (Lin et al., 1992; Stauffer and Stauffer, 1998), a known LEE activator. It is possible that regulation of the GCS represents an unknown mechanism for Lrp to govern the LEE.

In addition to our primary screen for transcription factors that could regulate EspB production in K-12, I reutilized the same method to investigate the contribution of the CutR regulon members to EspB production. In a *cutR* deficient K-12 strain harboring a LEE cosmid, there is an increase in total EspB protein levels. Microarray analysis of *cutR*-governed genes in EHEC revealed the regulon of this transcription factor. The non-essential regulon member deficient strains of K-12 were transformed with a LEE cosmid and EspB was evaluated for each strain (Appendix F; Figure 10E). We identified twelve strains that influenced EspB production in a manner that could not be explained by growth: *fadL*, *ydcS*, *yaiA*, *ybhP*, *ydeJ*, *mdtK*, *yliF*, *yehY*, *yohF*, *pflB*, *artP*, and *yegP*. Many of these genes are insufficiently researched and it is difficult to infer what role they might have in virulence regulation.

CutR is an activator of *pflB* expression. PflB is an anaerobic pyruvate formate-lyase that catalyzes the conversion of pyruvate to acetyl-coA and formate. Given our knowledge that pyruvate stimulates LEE expression (Carlson-Banning and Sperandio, 2016), it is interesting to consider that *cutR* may influence the LEE via regulation of metabolism, in addition to a direct effect on *LEE1*. We witnessed a diminishment of *fadL* expression in cutR-deficient EHEC. FadL, the long chain fatty acid transporter, allows for the import of long chain fatty acids, which can serve both as a carbon source through β-oxidation and as inhibitors of FadR activity. The fact that we witness an increase in EspB production in a *fadL*-deficient K-12 strain, suggests that the fatty acid uptake system may have a phenotype beyond what can be described by FadR regulation. Interestingly, we witness a diminishment of *ler* and *tir* expression in a *fadL*-deficient EHEC strain, in agreement with the repressor phenotype of FadR.

ArgR appeared in our original K-12 screen for transcription factors influencing the LEE as an apparent activator of EspB production. ArgR is the repressor of arginine biosynthesis and transport in E. coli that, upon binding to L-arginine as a corepressor, inhibits transcription (Caldara et al., 2006). CutR activated expression of *artP*, an ATP binding protein of an arginine ABC import system (Caldara et al., 2007; Wissenbach et al., 1995). In our system, ArtP was required for full expression of EspB in K-12, suggesting that exogenous L-arginine may be facilitating the positive effect of ArgR.

The current understanding of LEE regulation by the nutritional environment has highlighted the importance of sugars in regulating the virulence of EHEC (Carlson-Banning and Sperandio, 2016; Njoroge et al., 2012; Pacheco et al., 2012). My work has complements these

studies by suggesting that other nutrient sources, including fatty acids and amino acids, may be important influencers of LEE regulation in EHEC.

In general, I envision that our dataset and our methodology will be useful for researchers attempting to decipher elements of LEE regulation. We have provided a set of targets that may prove to be relevant to virulence of EHEC, upon further exploration. We chose to evaluate LEE expression via EspB production under anaerobic conditions; however one could imagine an experimental setup in which two conditions are tested against one another to identify the pathways which yield a differential result. A variety of metabolites are known to regulate the LEE without a currently understood mechanism. I propose that our approach can be usefully applied to evaluate the mechanism of such small molecules.

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APPENDIX A

Strains and plasmids used in this manuscript

Designation	Strain	Background (resistance)	Purpose	Reference
RP001	WT EHEC	86-24 (Str^R)	WT O157:H7 parent strain	Griffin et al., 1988
RP002	Δ*yehU* EHEC	86-24 (Str^R)	*yehU* deletion in 86-24	This study
RP003	Δ*fadR* EHEC	86-24 (Str^R)	*fadR* deletion in 86-24	This study
RP004	Δ*cutR* EHEC	86-24 (Str^R)	*cutR* deletion in 86-24	This study
RP005	Δ*yhaO* EHEC	86-24 (Str^R)	*yhaO* deletion in 86-24	This study
RP006	Δ*cutR*Δ*yhaO* EHEC	86-24 (Str^R)	*yhaO* deletion in ΔcutR 86-24	This study
RP007	Δ*fadL* EHEC	86-24 (Str^R)	*fadL* deletion in 86-24	This study
MMC1	Δ*espB* EHEC	86-24 (Str^R)	*espB* deletion in 86-24	Carlson-Banning et al., 2016
RP008	Δ*lacA* K-12	BW25113 (Kan^R)	Control strain for K-12 based screen	Baba et al., 2006
RP009	Δ*lacA* K-12	BW25113 ($Kan^R Cm^R$)	K-12 control strain carrying EHEC LEE containing cosmid	This study
RP010	NiCo21(DE3)		Protein expression strain	NEB, Ipswich, MA
RP011	NiCo21(DE3) pET28 FadR	NiCo21(DE3) (Kan^R)	FadR protein expression strain	This study
RP012	WT *C. rodentium*	DBS770 (Cm^R)	WT *C. rodentium* parent strain	Mallick et al., 2012
RP013	Δ*yehU C. rodentium*	DBS770 (Cm^R)	*yehU* deletion in DBS770	This study
RP014	Δ*fadR C. rodentium*	DBS770 (Cm^R)	*fadR* deletion in DBS770	This study
RP015	Δ*cutR C. rodentium*	DBS770 (Cm^R)	*cutR* deletion in DBS770	This study
RP016	WT 86-24 pACYC184 empty vector	86-24 ($Str^R Cm^R Tet^R$)	WT EHEC complementation control	This study
RP017	Δ*fadR* EHEC pACYC184 empty vector	86-24 ($Str^R Cm^R Tet^R$)	Δ*fadR* EHEC complementation control	This study
RP018	Δ*fadR* EHEC pACYC184 FadR	86-24 ($Str^R Tet^R$)	Δ*fadR* EHEC with *fadR* complemenation vector	This study
RP019	Δ*cutR* EHEC pACYC184 empty vector	86-24 ($Str^R Cm^R Tet^R$)	Δ*cutR* EHEC complementation control	This study
RP020	Δ*cutR* EHEC pACYC184 CutR	86-24 ($Str^R Tet^R$)	Δ*cutR* EHEC with *cutR* complemenation vector	This study
RP021	WT 86-24 pASKIBA32 empty vector	86-24 ($Str^R Amp^R$)	WT EHEC inducible complementation control	This study
RP022	Δ*fadR* EHEC pASKIBA32 empty vector	86-24 ($Str^R Amp^R$)	Δ*fadR* EHEC inducible complementation control	This study
RP023	Δ*fadR* EHEC pASKIBA32 V5FadR	86-24 ($Str^R Amp^R$)	Δ*fadR* EHEC inducible complementation V5-FadR	This study
RP024	Δ*cutR* EHEC pASKIBA32 empty vector	86-24 ($Str^R Amp^R$)	Δ*cutR* EHEC inducible complementation control	This study
RP025	Δ*cutR* EHEC pASKIBA32 V5CutR	86-24 ($Str^R Amp^R$)	Δ*cutR* EHEC inducible complementation V5-CutR	This study
RP026	WT DBS770 pACYC184 empty vector	DBS770 ($Cm^R Tet^R$)	WT DBS770 complementation control	This study
RP027	Δ*fadR C. rodentium* pACYC184 empty vector	DBS770 ($Cm^R Tet^R$)	Δ*fadR* DBS770 complementation control	This study
RP028	Δ*fadR C. rodentium* pACYC184 FadR	DBS770 ($Cm^R Tet^R$)	Δ*fadR* DBS770 with *fadR* complemenation vector	This study
RP029	Δ*cutR C. rodentium* pACYC184 empty vector	DBS770 ($Cm^R Tet^R$)	Δ*cutR* DBS770 complementation control	This study
RP030	Δ*cutR C. rodentium* pACYC184 CutR	DBS770 ($Cm^R Tet^R$)	Δ*cutR* DBS770 with *cutR* complemenation vector	This study
Designation	**Plasmid**	**Resistance**	**Purpose**	**Reference**
pRP001	pJAY1512	Cm^R	pCVD551 Cosmid containing LEE from EHEC strain 85-170	Elliott et al., 1999
pRP002	pACYC184 empty vector	$Cm^R Tet^R$	Parent complementation vector	NEB, Ipswich, MA
pRP003	pACYC184 FadR	Tet^R	FadR promoter and ORF cloned	This study

			into CmR cassette of pACYC184	
pRP004	pACYC184 CutR	Tet^R	CutR promoter and ORF cloned into CmR cassette of pACYC184	This study
pRP005	pASKIBA32 empty vector	Amp^R	Parent anhydrotetracycline inducible vector	IBA GmbH, Goettingen, Germany
pRP006	pASKIBA32 V5 CutR	Amp^R	Inducible N-terminally V5-tagged CutR expression vector	This study
pRP007	pASKIBA32 V5 FadR	Amp^R	Inducible N-terminally V5-tagged FadR expression vector	This study
pRP008	pET28 empty vector	Kan^R	Parent protein expression vector	EMD Millipore, Billerica, MA
pRP009	pET28 FadR	Kan^R	N-terminally 6His tagged FadR expression vector	This study

APPENDIX B

Primers used in preparation of this manuscript

Primer	Sequence	Purpose
ybaO_LR_F	GTTAGATAAAATTGACCGTAAGCTGCTGGCCTTACTACAGCAGGATTGCAC CCTCTCTTTGCGTGTAGGCTGGAGCTGCTTC	Primer to create ybaO(cutR) KO by lambda red in EHEC
ybaO_LR_R	CGATGGGTAAAGAAGTGGTGTATTTAATCTGTTCCATCGCGAAGCTGGAA GTGACGTCCGCATATGAATATCCTCCTT	Primer to create ybaO(cutR) KO by lambda red in EHEC
fadR_LR_F	GGAATAACCGCTTCCCTCCCGGGACTATTTTGCCCGCAGAACGTGAACTTT CAGAATTAATTGGTGTAGGCTGGAGCTGCTTC	Primer to create fadR KO by lambda red in EHEC
fadR_LR_R	CGGTGCCAAATCTCGCCACTCTCATGCCCATAGCGACGCACTGTTTCGTAC ACCTGATCGCATATGAATATCCTCCTT	Primer to create fadR KO by lambda red in EHEC
yehU_LR_F	GCTTCAGCAGATGTGCGTTTTTTTAGTCATTGCGTGGTTAATGAGTAAAAC GCCATTATTCATACCGGTGTAGGCTGGAGCTGCTTC	Primer to create yehU KO by lambda red in EHEC
yehU_LR_R	GGTGTAACTATCAGGCTCACAGGCGACGCTTATCCCATAGTCATCGCCAAA CCGTTCACGTAAACGCATATGAATATCCTCCTT	Primer to create yehU KO by lambda red in EHEC
yhaO_LR_F	GGAAATTGCATCGAATAAAGGCGTCATTGCAGACGCTTCGACCCCGGCGG GTCGTGCTGG GTGTAGGCTGGAGCTGCTTC	Primer to create yhaO KO by lambda red in EHEC
yhaO_LR_R	GCCAGGAACGGAGAAACACAAAGCAACAAACCAGTGACGATAATCAGGT ACAGAGACATCCC CATATGAATATCCTCCTT	Primer to create yhaO KO by lambda red in EHEC
fadL_LR_F	CGCAGTGGCACTTATCTCCACCCAGGCCTGGTCGGCAGGCTTTCAGTTAAA CGAATTTTCTTCCTCTGGC GTGTAGGCTGGAGCTGCTTC	Primer to create fadL KO by lambda red in EHEC
fadL_LR_R	CCTTCAGACTCGAACTGGTATGGGCCTTCGTTAATTTTCACGCTCTGACCGT GCATATAAGAAACACCAACGTCG CATATGAATATCCTCCTT	Primer to create fadL KO by lambda red in EHEC
citroYbaO_LR_F	ggtatgttagataaaattgaccgcaagctgctggcattactgcagcaggactgcaccctc GTGTAGGCTGGAGCTGCTTC	Primer to create ybaO(cutR) KO by lambda red in *C. rodentium*
citroYbaO_LR_R	ggagagttattcaatgggtaaagcggttgtgtacttaatctgttccatcgcaaagctcg CATATGAATATCCTCCTT	Primer to create ybaO(cutR) KO by lambda red in *C. rodentium*
citroFadR_LR_F	ggtcattaaggcgcagagcccggcgggtttcgcggaagagtacatcattgaaagtatctg GTGTAGGCTGGAGCTGCTTC	Primer to create fadR KO by lambda red in *C. rodentium*
citroFadR_LR_R	gaccctgaatggcgagatcgcccggcaggtttttctgcatccggtgccagatctcaccgc CATATGAATATCCTCCTT	Primer to create fadR KO by lambda red in *C. rodentium*
citroYehU_LR_F	gtacgagtttaatctggtgttgctgctgcttcagcaaatgtgcgtgtttctggttatcgc GTGTAGGCTGGAGCTGCTTC	Primer to create yehU KO by lambda red in *C. rodentium*
citroYehU_LR_R	gcgttctcctccagaggaagtcgtagggttattcgggtaaaacggtcaggttcgcaaac CATATGAATATCCTCCTT	Primer to create yehU KO by lambda red in *C. rodentium*
rpoZ_RTF	AGGACGCTGTAGAGAAAATTGG	QRT-PCR primer
rpoZ_RTR	GCGCGATTACAGTGGTTTTATC	QRT-PCR primer
espA_RTF	AGCTATTTGAGGAACTCGGTG	QRT-PCR primer
espA_RTR	CATCTTTTGTGCCGTGGTTG	QRT-PCR primer
tir_RTF	GAGGGAGTCAAATAGCGGTG	QRT-PCR primer
tir_RTR	ATCTGAACGAAGGCTGGAAG	QRT-PCR primer
eae_RTF	TGGGATGTTCAACGGTAAGTC	QRT-PCR primer
eae_RTR	TTTAACCTCAGCCCCATCAC	QRT-PCR primer
ler_RTF	CGAGAGCAGGAAGTTCAAAGTG	QRT-PCR primer
ler_RTR	ACACCTTTCGATGAGTTCCG	QRT-PCR primer
espP_RTF	ggtggtgcatacagtatcacagcc	QRT-PCR primer
espP_RTR	gtcgggatactgcaaaatcaggatgt	QRT-PCR primer
mhpR_RTF	TTGGCTGTCTGAATCTGGTG	QRT-PCR primer
mhpR_RTR	TCGATCTGTTTTGCTACCCG	QRT-PCR primer
moaA_RTF	AGCTCGACACCTTTCTGAAC	QRT-PCR primer
moaA_RTR	ACCAGAGATGTGATGCTTACG	QRT-PCR primer
yaiA_RTF	AACGAAACCGCCTTATCCTC	QRT-PCR primer
yaiA_RTR	TCGGATGTTCACTGATCAACG	QRT-PCR primer
ybaM_RTF	GCTGTCGATTTGATTGTGCTG	QRT-PCR primer
ybaM_RTR	GCCTCATCGCTCTGTAACTTC	QRT-PCR primer
ybhP_RTF	AATGGGTGAATGAGCTACCG	QRT-PCR primer
ybhP_RTR	GCCTGCACTTTTAACGGATG	QRT-PCR primer
mtfA_RTF	TTAGTCACTCTTGCCGAACG	QRT-PCR primer
mtfA_RTR	AACCATCCAGCCATTCCAG	QRT-PCR primer
ydeJ_RTF	GCAGGCTTTGTTACTTTCACC	QRT-PCR primer

ydeJ_RTR	ACTCACCGCAGAATATCGTTC	QRT-PCR primer
mdtK_RTF	CTAATGTTCGTGCTTCCAATGTC	QRT-PCR primer
mdtK_RTR	TTGCCATACAGACACCCAC	QRT-PCR primer
yliF_RTF	TGCGGGATCTACACTCAAATG	QRT-PCR primer
yliF_RTR	GTGGGATTTAGCGTGTTGATAAG	QRT-PCR primer
yehY_RTF	AATCGTTTAGTCTCGGGTGAAG	QRT-PCR primer
yehY_RTR	GAATTGCGCCAGAATGAGTG	QRT-PCR primer
yohF_RTF	AACTGGATCTCGGCAATCTG	QRT-PCR primer
yohF_RTR	CAAGAAACGGCACTTTGGTC	QRT-PCR primer
ccmH_RTF	AGGTTGGATTATGTTGGGTCG	QRT-PCR primer
ccmH_RTR	TTGGGATCAGATGAACGTGTC	QRT-PCR primer
yhaJ_RTF	CACCAAATTCACCAATGTCGG	QRT-PCR primer
yhaJ_RTR	AGTTTATCTGCGGCTTCCAG	QRT-PCR primer
pflB_RTF	TGAAACGTGCTCTTATCCCG	QRT-PCR primer
pflB_RTR	CCCTGGTTGTGAGTTTTACGG	QRT-PCR primer
psiF_RTF	AGAGAACCTTAACCCCACAAC	QRT-PCR primer
psiF_RTR	GTCTTACGAGCATCCCCTTTC	QRT-PCR primer
rimP_RTF	GTGACTCTGGTTCTCCGTATG	QRT-PCR primer
rimP_RTR	ACCGTCTACCGCTTTGATAAC	QRT-PCR primer
argO_RTF	TTCTTTGGTCTGGCTATTCTCG	QRT-PCR primer
argO_RTR	CATGACACATCCCACTACCAG	QRT-PCR primer
tdcC_RTF	CTCGTGGCTATCTTCAAATCTTTC	QRT-PCR primer
tdcC_RTR	AGTGTTCAGTTTACCCAGCG	QRT-PCR primer
gntT_RTF	TGATGGTTATTGCGGTGGG	QRT-PCR primer
gntT_RTR	CTCGCCGATAGTCAGGTTAAAG	QRT-PCR primer
yphD_RTF	GCGGAATGGGCTTGTTTATG	QRT-PCR primer
yphD_RTR	GCGGCTAATGAACACAAACAG	QRT-PCR primer
lysP_RTF	GCTTCCTGACCTCCATGTTTG	QRT-PCR primer
lysP_RTR	ATGTCGTGTCCCTGCAATAC	QRT-PCR primer
artP_RTF	TCACCAAAACACCCTCAGAC	QRT-PCR primer
artP_RTR	CCTGATCTTTACTCAACCCCAG	QRT-PCR primer
yegP_RTF	GAGCAGTGATAGTCAGTTCCG	QRT-PCR primer
yegP_RTR	CTTTTCCGCAGAGGCTTTTG	QRT-PCR primer
yiiF_RTF	TGAGGTGCAGCGTAATCTTC	QRT-PCR primer
yiiF_RTR	CACTGGTTCTTACTGTTTGCG	QRT-PCR primer
hdeD_RTF	TTGGTGTGCTGGATATCGTC	QRT-PCR primer
hdeD_RTR	CTTAACGAACAAACTGGCGAAG	QRT-PCR primer
gadA_RTF	ACCAAAGTACAGAACGCCTC	QRT-PCR primer
gadA_RTR	CGGATCTTCACCCTCTTTCAG	QRT-PCR primer
dmsA_RTF	ACCGCCTGAAATATCCGATG	QRT-PCR primer
dmsA_RTR	TCGTTGCCGTACTCTTTGATC	QRT-PCR primer
uxaB_RTF	GTATGACCAAGTTCCGTACCC	QRT-PCR primer
uxaB_RTR	TGTTTCTCCATTACGCTCACC	QRT-PCR primer
yhaO_RTF	CGAATAAAGGCGTCATTGCAG	QRT-PCR primer
yhaO_RTR	GATACTCATAATCACCCAGCCG	QRT-PCR primer
yeaG_RTF	TGTCGATGAAGGTATGAACGG	QRT-PCR primer
yeaG_RTR	CAGGACGTAGAACAGATGGAC	QRT-PCR primer
ydcS_RTF	GACTTCCGATGAGATGGTCAG	QRT-PCR primer
ydcS_RTR	GATCAAGCGTTTTCCAGTTGG	QRT-PCR primer
yjiY_RTF	ACTATTCAAGCACATACCCTGG	QRT-PCR primer
yjiY_RTR	CACCAGATACACCGACACAG	QRT-PCR primer
yehZ_RTF	CAGCAAGGTTACGAGAAAGTC	QRT-PCR primer
yehZ_RTR	CAGCAAGCGAAGTGAGTTTG	QRT-PCR primer
argD_RTF	TTTACTGTTAGTGCGGGTGG	QRT-PCR primer
argD_RTR	TGGATGGGTTCGACAATCAC	QRT-PCR primer
fadL_RTF	ACCCGGATGTAAATATCAGCG	QRT-PCR primer
fadL_RTR	GTTAGAGGTAATAGAAGCGCCC	QRT-PCR primer
ler_GS_-169_F	GTTGACATTTAATGATAATGTATTTTACACATTAG	Primer to amplify region -169bp upstream of LEE1 proximal promoter of EHEC
ler_GS_+155_R	CTCAATTACACTTTGAACTTCCTGCTCTCG	Primer to amplify region 155bp downstreamstream of LEE1 proximal promoter of EHEC
ler_GS_-372_F	CCTGTAACTCGAATTAAGTAGAGTATAGTG	Primer to amplify region -372bp upstream of LEE1

		proximal promoter of EHEC
ler_GS_-102_R	CTATCAAATTAGGACACATCTATTTCATCAAAC	Primer to amplify region -102bp upstream of LEE1 proximal promoter of EHEC
ler_GS_-573_F	GGTTACTGTTCAGCTATTTGTCCCTTGTTCC	Primer to amplify region -573bp upstream of LEE1 proximal promoter of EHEC
ler_GS_-301_R	GCGTTTCTCTTTATAAGATAGATCTCATTGC	Primer to amplify region -301bp upstream of LEE1 proximal promoter of EHEC
ler_GS_-768_F	GTCCCACCTTAGCGCTAAGCACTTTTGAATCAC	Primer to amplify region -768bp upstream of LEE1 proximal promoter of EHEC
ler_GS_-493_R	GACGAGTATATATATCTATTAGCTGTTTCCC	Primer to amplify region -493bp upstream of LEE1 proximal promoter of EHEC
ler_GS_-967_F	GGAAACTTATTTTGGCCTGTATATAATGTGATG	Primer to amplify region -967bp upstream of LEE1 proximal promoter of EHEC
ler_GS_-693_R	GTCCGTTTTACTTGGCATCAGATCATCGCCG	Primer to amplify region -693bp upstream of LEE1 proximal promoter of EHEC
fadL_GS_F	GGATATTTTACGCTTCCGGAAAGTGCTGC	Primer to amplify fadL promoter region from EHEC
fadL_GS_R	CTGGCTCATGACCATAACCTCAATG	Primer to amplify fadL promoter region from EHEC
kan GS F	CCGCGATTAAATTCCAACATGGATGCTG	Primer to amplify kanamycin cassette fragment of pRS551
kan GS R	GACCATCTCATCTGTAACATCATTGGCAACG	Primer to amplify kanamycin cassette fragment of pRS551
citLer_GS_+290_R	CTCAATTACAGTTTGAATCTCCTGCTC	Primer to amplify region 290bp downstream of LEE1 promoter of C. rodentium
citLer_GS_-29_F	GGATAATATATTTTACACATTATGTATCAG	Primer to amplify region -29bp upstream of LEE1 promoter of C. rodentium
citLer_GS_+76_R	GTAAGATAACGTCTTATCTATCAC	Primer to amplify region 76bp downstream of LEE1 promoter of C. rodentium
citLer_GS_-242_F	GGCCGGCAAATCGGGTACGCG	Primer to amplify region -242bp upstream of LEE1 promoter of C. rodentium
citLer_GS_-104_R	GGCAAAGCGGCATTAATTATTGTTTCTCC	Primer to amplify region -104bp upstream of LEE1 promoter of C. rodentium
citLer_GS_-437_F	GGGTGGAAATCGACAACAACATCGCAGAG	Primer to amplify region -437bp upstream of LEE1 promoter of C. rodentium
citLer_GS_-312_R	GATCAGCGAGTACAACACCGCCGC	Primer to amplify region -312bp upstream of LEE1 promoter of C. rodentium
citLer_GS_-618_F	GGATGTTCAGCAGAACAGCGTCTGGCG	Primer to amplify region -618bp upstream of LEE1 promoter of C. rodentium
citLer_GS_-492_R	CGCTTTTGCCGTATCCGAGTGACGCG	Primer to amplify region -492bp upstream of LEE1 promoter of C. rodentium
citLer_GS_-829_F	GAATCACCTGTCCGGTTACAGCGGAGTGC	Primer to amplify region -829bp upstream of LEE1 promoter of C. rodentium
promfadR_184_ScaI_gibs_F	gccctgccactcatcgcagtact GGAGATCTCCATGATGGTTTCCC	Primer to clone fadR complementation construct into pACYC184 by gibson cloning
promfadR_184_EcoRI_gibs_R	cctgatgaatgctcatccggaattc CAGCGTCGAGTTGCTGGAACG	Primer to clone fadR complementation construct into pACYC184 by gibson cloning
promybaO_184_ScaI_gibs_F	gccctgccactcatcgcagtact CTACCTTATTCCCCCGAATAACG	Primer to clone ybaO(cutR) complementation construct into pACYC184 by gibson cloning
promybaO_184_EcoRI_gibs_R	cctgatgaatgctcatccggaattc CGCGATAATGACAAGCAAGGCG	Primer to clone ybaO(cutR) complementation construct into pACYC184 by gibson cloning
fadR_28gibs_F_NdeI	Gccgcgcggcagccatatg GTCATTAAGGCGCAAAGCCC	Primer to clone fadR with N-terminal His tag into pET28 by gibson cloning
fadR_28gibs_R_BamHI	Cggagctcgaattcggatcc TTATCGCCCCTGAATGGC	Primer to clone fadR with N-terminal His tag into pET28 by gibson cloning
pASK_backbone_gibs_R	CATTTTTTGCCCTCGTTATCTAGATTTTTGTCG	Primer to amplify pASKIBA32 plasmid backbone for gibson cloning
pASK_backbone_gibs_F	TAAGCTTGACCTGTGAAGTGAAAAATGGC	Primer to amplify pASKIBA32 plasmid backbone for gibson cloning
pASK_nV5_ybaO_gibs_F	CTAGATAACGAGGGCAAAAAATG GGCAAACCGATTCCGAACCCGCTG	Primers to amplify N-terminally V5-tagged YbaO(CutR) and clone into pASKIBA32
pASK_nV5_ybaO_gibs_R	CACTTCACAGGTCAAGCTTA TTATTCGATGGGTAAAGAAGTGG	Primers to amplify N-terminally V5-tagged YbaO(CutR) and clone into pASKIBA32
pASK_nV5_fadR_gibs_R	CACTTCACAGGTCAAGCTTATTA TCGCCCCTGAATGGCTAAATCACC	Primers to gibson clone N-terminally V5-tagged FadR into pASKIBA32
pASK_nV5_fadR	GCCTGGATTCAACCGGATCC GTCATTAAGGCGCAAAGCCC	Primers to gibson clone N-terminally V5-tagged

_gibs_Fa		FadR into pASKIBA32
pASK_nV5_bb_ gibs_R	GGATCCGGTTGAATCCAGGC	Primers to gibson clone N-terminally V5-tagged FadR into pASKIBA32

APPENDIX C

ELISA results of K-12 mutant strains carrying LEE cosmid

ELISA results for EspB production by all pJAY1512 carrying Keio library K-12 deletion strains initially investigated. See Figure 3.

K-12 strain	Avg. Rel. EspB	Std. Dev. Rel. EspB	Dunnetts p value	Avg. OD600	Std. Dev. OD600	K-12 strain	Avg. Rel. EspB	Std. Dev. Rel. EspB	Dunnetts p value	Avg. OD600	Std. Dev. OD600
lacA	1.00	0.07		0.17	0.01	dksA	2.80	0.62	0.0001	0.12	0.01
ada	0.66	0.52	0.863	0.18	0.01	ygeH	0.27	0.16	0.0033	0.14	0.01
adiY	1.81	0.10	0.0023	0.15	0.04	yafN	0.23	0.40	0.0013	0.12	0.04
appY	1.36	0.27	0.7968	0.17	0.02	ymgB	0.59	0.37	0.4509	0.16	0.02
argR	0.01	0.29	0.0001	0.14	0.02	ydaS	1.78	0.33	0.001	0.10	0.02
asnC	0.85	0.21	0.999	0.16	0.03	ygfY	0.42	0.05	0.0055	0.17	0.01
betI	0.77	0.03	0.998	0.17	0.02	yggD	0.26	0.04	0.0001	0.18	0.01
bglG	0.70	0.16	0.9633	0.19	0.01	ygiV	0.66	0.12	0.4317	0.20	0.00
cadC	0.64	0.39	0.8037	0.19	0.02	yqjI	0.34	0.03	0.0007	0.16	0.02
cbl	0.48	0.18	0.2134	0.22	0.03	ygjM	0.59	0.12	0.1588	0.18	0.01
chbR	1.25	0.51	0.9914	0.15	0.02	yhcF	0.62	0.04	0.2418	0.22	0.02
crp	0.18	0.42	0.0018	0.14	0.02	yhiF	0.47	0.09	0.0149	0.20	0.02
csgD	0.65	0.40	0.8261	0.16	0.02	gadX	0.40	0.01	0.003	0.14	0.01
cynR	0.73	0.15	0.9818	0.19	0.01	yhjB	0.94	0.15	0.9995	0.13	0.03
cytR	1.21	0.33	0.9983	0.17	0.03	yiaG	0.89	0.20	0.999	0.17	0.01
deoR	1.59	0.34	0.0838	0.17	0.03	yiaJ	0.66	0.10	0.4238	0.17	0.02
dsdC	0.87	0.25	0.9991	0.18	0.02	yifB	0.97	0.17	0.9997	0.19	0.01
ebgR	1.04	0.28	0.9997	0.16	0.01	yihL	0.68	0.16	0.4946	0.18	0.01
envR	0.78	0.20	0.9982	0.14	0.00	yjeB	0.64	0.10	0.3223	0.18	0.01
envY	0.72	0.15	0.9813	0.20	0.03	ulaR	1.06	0.28	0.9996	0.16	0.01
fadR	LOD	LOD	LOD	0.10	0.02	ytfJ	0.49	0.03	0.0279	0.14	0.01
fis	0.10	0.16	0.0003	0.25	0.03	yjhI	0.91	0.19	0.9992	0.11	0.01
fnr	0.44	0.14	0.1241	0.21	0.04	yjiR	0.66	0.13	0.417	0.15	0.02
fucR	0.57	0.09	0.4766	0.19	0.04	yjjJ	0.91	0.24	0.9991	0.16	0.00
galR	0.92	0.15	0.9995	0.15	0.01	paaX	1.06	0.28	0.9995	0.18	0.00
galS	0.81	0.14	0.9986	0.15	0.02	osmE	1.80	0.28	0.0001	0.20	0.01
ihfB	0.63	0.26	0.7661	0.14	0.01	flhC	0.42	0.02	0.0055	0.17	0.02
hns	0.65	0.15	0.8107	0.17	0.03	rcsA	0.28	0.07	0.0001	0.15	0.00
hupA	0.61	0.09	0.6753	0.18	0.01	rfaH	0.44	0.02	0.0081	0.13	0.03
hupB	1.20	0.14	0.9984	0.22	0.04	cbpA	1.50	0.01	0.0335	0.19	0.01
argP	0.82	0.03	0.9987	0.13	0.02	dicC	0.59	0.10	0.1632	0.16	0.01
idnR	0.84	0.06	0.9988	0.17	0.02	putA	0.94	0.06	0.9994	0.19	0.01
ilvY	0.78	0.23	0.9982	0.17	0.01	glcC	0.59	0.04	0.1678	0.17	0.01
leuO	0.54	0.17	0.3675	0.17	0.02	allR	0.58	0.03	0.1429	0.13	0.01
lrp	LOD	LOD	LOD	0.11	0.00	sfsA	0.63	0.07	0.2881	0.17	0.01
lysR	0.80	0.03	0.9984	0.15	0.03	lacI	0.30	0.02	0.0002	0.15	0.01
malI	1.10	0.36	0.9993	0.15	0.02	kdgR	0.59	0.05	0.1586	0.18	0.01
melR	0.85	0.22	0.999	0.17	0.02	gutM	0.92	0.35	0.9993	0.15	0.02
metJ	0.96	0.28	0.9998	0.15	0.02	sfsB	0.40	0.04	0.0036	0.20	0.02
metR	0.88	0.16	0.9992	0.16	0.02	xylR	0.82	0.14	0.9921	0.15	0.00
mhpR	0.73	0.37	0.9821	0.16	0.01	lldR	0.38	0.03	0.0018	0.14	0.00
nac	0.67	0.24	0.904	0.16	0.01	ykgD	0.94	0.02	0.9995	0.17	0.02
nagC	1.18	0.32	0.9986	0.15	0.05	cueR	0.49	0.10	0.0241	0.19	0.01

nanR	1.24	0.28	0.9979	0.15	0.02	ybbS	0.76	0.02	0.9203	0.15	0.00
nhaR	1.01	0.22	0.9999	0.14	0.04	ycgE	1.09	0.21	0.9992	0.15	0.01
pdhR	1.07	0.14	0.9996	0.14	0.03	ycjW	0.57	0.16	0.1204	0.16	0.01
perR	1.09	0.10	0.9994	0.13	0.03	feaR	0.57	0.11	0.1248	0.18	0.01
phnF	1.23	0.30	0.9981	0.16	0.03	relB	1.13	0.30	0.9987	0.16	0.01
purR	0.97	0.36	0.9999	0.14	0.03	mlrA	0.53	0.01	0.0554	0.16	0.01
rhaR	1.07	0.15	0.9996	0.15	0.01	ygeV	0.45	0.01	0.0101	0.17	0.01
soxR	1.03	0.13	0.9999	0.14	0.02	ygiP	0.59	0.03	0.1592	0.15	0.00
soxS	1.80	0.02	0.0027	0.16	0.03	yhaJ	0.68	0.08	0.5393	0.13	0.01
hnsB	LOD	LOD	LOD	0.16	0.01	aaeR	0.56	0.03	0.0951	0.13	0.00
tdcA	0.94	0.14	0.9996	0.16	0.04	yidP	0.86	0.08	0.9986	0.15	0.00
treR	1.24	0.13	0.9919	0.12	0.02	phoU	0.48	0.06	0.0197	0.13	0.02
trpR	1.25	0.24	0.9914	0.17	0.01	yijO	0.33	0.05	0.0006	0.16	0.01
tyrR	0.98	0.14	0.9999	0.13	0.03	modE	1.06	0.10	0.9995	0.16	0.01
uidR	0.50	0.46	0.2469	0.16	0.01	caiF	0.82	0.08	0.9979	0.14	0.01
uxuR	1.24	0.07	0.9919	0.15	0.02	ybiH	1.37	1.62	0.9995	0.18	0.06
xapR	1.13	0.08	0.9991	0.17	0.01	pspF	0.44	0.96	0.9993	0.17	0.03
zntR	1.11	0.22	0.9993	0.17	0.00	fliZ	1.43	1.77	0.9994	0.18	0.03
yafC	1.14	0.14	0.999	0.18	0.00	pmrD	0.02	0.94	0.9984	0.19	0.02
ybdO	1.18	0.16	0.9986	0.17	0.00	gcvR	8.18	0.99	0.0001	0.13	0.04
ybeF	1.20	0.07	0.9985	0.17	0.01	csiR	1.45	0.33	0.9994	0.20	0.01
rcsC	0.88	0.52	0.9992	0.19	0.01	exuR	6.33	0.88	0.0001	0.17	0.01
fruR	0.96	0.09	0.9997	0.13	0.01	yrbA	4.28	0.91	0.0281	0.20	0.01
ihfA	0.77	0.21	0.998	0.12	0.00	rpiR	1.11	0.57	0.9999	0.19	0.05
rbsR	1.17	0.22	0.9987	0.17	0.01	nusB	LOD	LOD	LOD	0.12	0.04
sdiA	1.08	0.13	0.9995	0.15	0.00	dgsA	4.14	3.59	0.0433	0.19	0.04
srlR	1.09	0.25	0.9994	0.16	0.01	yegW	0.63	0.24	0.9995	0.18	0.04
phoB	1.16	0.28	0.9988	0.18	0.01	rtcR	3.61	1.18	0.1807	0.20	0.03
phoR	0.64	0.11	0.7822	0.17	0.00	arsR	3.90	1.43	0.0868	0.24	0.03
citA	0.50	0.12	0.2413	0.18	0.00	sgcR	1.32	0.25	0.9996	0.19	0.03
citB	0.58	0.19	0.5381	0.16	0.01	rob	1.73	0.45	0.999	0.20	0.01
kdpD	0.33	0.17	0.055	0.09	0.01	pspA	0.99	0.97	0.9999	0.17	0.03
torR	0.80	0.16	0.9985	0.14	0.01	pspB	0.93	0.15	0.9999	0.19	0.02
phoQ	0.33	0.12	0.0518	0.14	0.01	pspC	1.85	0.86	0.9987	0.20	0.01
phoP	0.43	0.33	0.1707	0.17	0.01	ogrK	1.45	1.94	0.9994	0.19	0.04
narL	0.57	0.33	0.5957	0.17	0.02	cspA	0.02	0.31	0.9984	0.17	0.03
narX	0.96	0.38	0.9997	0.19	0.01	yiiT	0.58	0.35	0.9994	0.18	0.02
rssB	1.42	0.18	0.6694	0.18	0.01	yhgF	3.17	0.50	0.4441	0.19	0.02
rstA	1.01	0.14	0.9999	0.18	0.00	iclR	0.12	0.41	0.9986	0.20	0.01
rstB	0.25	0.07	0.0172	0.13	0.03	yjjQ	0.37	0.61	0.9991	0.20	0.01
uvrY	0.29	0.57	0.0284	0.17	0.02	fur	LOD	LOD	LOD	0.19	0.01
yedV	1.02	0.13	0.9999	0.17	0.01	frvR	0.35	0.32	0.9991	0.18	0.01
baeS	0.81	0.19	0.9987	0.20	0.02	fabR	1.99	0.94	0.9984	0.17	0.00
narP	0.83	0.26	0.9989	0.18	0.01	torI	0.14	0.14	0.9987	0.20	0.03
rcsB	0.45	0.03	0.2103	0.10	0.01	nanK	LOD	LOD	LOD	0.19	0.01
atoS	0.19	0.27	0.0064	0.10	0.02	yiiE	0.31	0.44	0.999	0.22	0.01
atoC	0.20	0.17	0.0068	0.15	0.00	yihW	0.12	0.49	0.9986	0.22	0.00
evgA	0.47	0.42	0.2693	0.15	0.01	frlR	3.22	0.88	0.4065	0.21	0.01
evgS	0.17	0.04	0.0043	0.12	0.01	yheO	1.38	0.26	0.9995	0.20	0.01
narQ	0.67	0.10	0.9567	0.16	0.02	zur	0.27	0.40	0.999	0.16	0.02
yfhA	0.97	0.24	0.9999	0.18	0.02	ytfH	3.03	0.98	0.5666	0.15	0.01
barA	1.46	0.13	0.5098	0.15	0.02	yjhU	4.58	1.72	0.0102	0.20	0.01
qseB	0.84	0.10	0.999	0.09	0.01	dgoR	1.62	1.01	0.9991	0.19	0.01
qseC	0.71	0.17	0.9816	0.15	0.01	gntR	0.23	0.36	0.9988	0.17	0.02
arcB	0.24	0.32	0.0144	0.12	0.01	cdaR	0.68	0.30	0.9996	0.19	0.00
ompR	1.63	0.06	0.0914	0.13	0.01	ykgK	5.18	1.16	0.001	0.17	0.01

uhpB	1.07	0.21	0.9996	0.13	0.01	fimZ	0.77	0.55	0.9997	0.16	0.01
uhpA	0.19	0.22	0.0058	0.17	0.03	cusS	0.88	0.33	0.9999	0.17	0.02
glnG	1.64	0.33	0.0763	0.13	0.01	kdpE	0.94	0.53	0.9999	0.16	0.01
glnL	1.40	0.23	0.7456	0.12	0.02	ybjK	0.70	0.18	0.9996	0.18	0.01
cpxA	1.21	0.29	0.9985	0.11	0.02	ycfQ	0.69	0.44	0.9996	0.19	0.01
cpxR	0.79	0.49	0.9985	0.11	0.01	ymfT	5.01	2.31	0.002	0.19	0.01
zraS	0.59	0.59	0.6748	0.13	0.04	ydcI	4.06	1.04	0.0548	0.10	0.01
zraR	0.97	0.44	0.9998	0.13	0.01	marR	LOD	LOD	LOD	0.16	0.01
basS	0.48	0.24	0.2918	0.10	0.01	marA	0.05	0.24	0.9985	0.16	0.01
basR	1.01	0.26	0.9999	0.14	0.01	slyA	0.44	0.72	0.9993	0.18	0.01
dcuR	1.20	0.19	0.9986	0.16	0.02	gatR	LOD	LOD	LOD	0.17	0.02
dcuS	0.95	0.13	0.9997	0.13	0.01	yehT	LOD	LOD	LOD	0.18	0.02
creB	0.05	0.44	0.0005	0.13	0.02	yehU	7.63	6.52	0.0001	0.15	0.00
creC	1.92	0.10	0.0009	0.12	0.01	ypdA	0.19	0.49	0.9988	0.12	0.05
arcA	0.58	0.22	0.6591	0.17	0.00	yphH	LOD	LOD	LOD	0.13	0.04
cusR	0.89	0.22	0.9993	0.16	0.01	yfhK	LOD	LOD	LOD	0.15	0.03
envZ	0.48	0.25	0.2803	0.13	0.02	yfjO	LOD	LOD	LOD	0.14	0.04
hha	2.19	0.29	0.0001	0.11	0.03	yqeH	1.56	0.59	0.9993	0.18	0.01
fecR	1.15	0.18	0.9991	0.14	0.01	ygeK	2.37	0.97	0.998	0.19	0.01
yqeI	0.45	0.08	0.2108	0.13	0.01	ygfI	4.89	1.30	0.0279	0.15	0.01
ybhD	0.67	0.21	0.953	0.13	0.04	yliH	3.79	1.76	0.3172	0.21	0.00
yeaT	0.22	0.40	0.0105	0.11	0.04	yagL	3.81	0.86	0.3042	0.20	0.01
yeiE	0.68	0.20	0.962	0.11	0.00	malT	2.16	0.33	0.9984	0.20	0.02
yiaU	0.73	0.23	0.9913	0.13	0.03	glpR	2.63	1.41	0.9804	0.19	0.00
yidZ	0.25	0.38	0.0165	0.13	0.03	ydhB	0.59	0.77	0.9996	0.17	0.01
yjiE	0.63	0.28	0.839	0.15	0.01	yidF	1.81	0.62	0.999	0.19	0.01
ynfL	0.53	0.30	0.4409	0.13	0.02	yhfZ	4.39	0.98	0.0963	0.17	0.05
prpR	0.04	0.23	0.0004	0.15	0.02	yafY	1.33	0.94	0.9996	0.21	0.01
hyfR	0.71	0.05	0.9822	0.11	0.02	ycdC	1.87	0.45	0.999	0.19	0.02
agaR	1.15	0.13	0.999	0.09	0.00	ycjZ	2.31	0.25	0.9981	0.10	0.02
sohA	0.46	0.17	0.2444	0.12	0.00	ydeO	3.23	1.30	0.6909	0.16	0.01
yeiL	0.76	0.08	0.9981	0.14	0.00	yncC	2.46	1.07	0.9914	0.17	0.01
ydgT	1.26	0.02	0.9919	0.14	0.01	yagI	3.42	0.50	0.5474	0.17	0.01
ydcQ	0.35	0.29	0.0637	0.08	0.01	ybaJ	3.58	0.18	0.4414	0.22	0.03
yaiV	0.25	0.06	0.0165	0.13	0.03	ycaQ	2.34	0.41	0.998	0.17	0.04
ybiI	0.79	0.33	0.9985	0.12	0.02	ymfN	2.18	0.28	0.9984	0.17	0.02
ybjN	0.40	0.14	0.0359	0.16	0.02	yeeU	3.14	0.57	0.7585	0.16	0.02
yfjF	LOD	LOD	LOD	0.20	0.01	yegI	2.47	0.15	0.9913	0.16	0.02
ygcP	0.80	0.14	0.9982	0.19	0.01	yfjS	3.66	0.47	0.3899	0.18	0.02
yafW	LOD	LOD	LOD	0.20	0.00	yfjZ	1.11	0.37	0.9999	0.19	0.03
yfaX	0.55	0.14	0.2881	0.18	0.01	lrhA	2.20	0.45	0.9983	0.17	0.01
frmR	0.97	0.45	0.9998	0.17	0.02	araC	3.40	0.15	0.5669	0.16	0.02
yohL	0.20	0.22	0.0007	0.15	0.04	mngR	3.06	0.40	0.8115	0.18	0.01
rdgC	1.69	0.31	0.0066	0.17	0.02	gcvA	2.32	0.17	0.9981	0.17	0.01
ybaQ	0.19	0.12	0.0006	0.21	0.05	rhaS	2.29	0.87	0.9982	0.15	0.03
yccR	0.43	0.03	0.062	0.21	0.01	yfeR	1.63	0.61	0.9993	0.17	0.00
yagP	0.47	0.27	0.1023	0.22	0.03	yhgG	1.90	0.60	0.9989	0.19	0.01
abgR	0.26	0.08	0.0024	0.17	0.03	gadE	2.57	0.29	0.9823	0.18	0.01
hcaR	0.67	0.15	0.7946	0.14	0.03	gadW	1.81	0.62	0.999	0.19	0.01
mak	LOD	LOD	LOD	0.16	0.01	flhD	3.12	1.06	0.7727	0.19	0.01
ycfX	0.72	0.28	0.9491	0.18	0.06	hycA	2.54	0.29	0.983	0.18	0.02
yeiI	0.42	0.23	0.0537	0.18	0.00	fhlA	3.62	1.28	0.4151	0.16	0.03
ypdB	1.54	0.18	0.0865	0.16	0.01	mtlR	2.42	0.46	0.9919	0.20	0.02
ypdC	1.29	0.44	0.9292	0.16	0.01	yidL	3.16	1.32	0.7431	0.19	0.02
yfeT	0.29	0.22	0.0043	0.16	0.03	acrR	3.21	1.38	0.7072	0.21	0.00
yfhH	0.55	0.26	0.272	0.18	0.03	nikR	2.09	0.75	0.9986	0.19	0.02

ygaV	0.43	0.39	0.0564	0.19	0.04	cspE	1.23	0.42	0.9997	0.18	0.01
ycgF	0.28	0.16	0.0036	0.18	0.00	aphA	2.62	0.58	0.9809	0.17	0.02
puuR	1.36	0.10	0.6391	0.19	0.01	yiiF	2.19	0.35	0.9983	0.19	0.02
ydcN	1.05	0.09	0.9997	0.17	0.02	yieP	3.69	0.75	0.3733	0.20	0.00
ydcR	LOD	LOD	LOD	0.16	0.03	yjdC	2.52	1.05	0.9836	0.18	0.02
ydiP	0.33	0.12	0.0097	0.15	0.03	yjjM	1.52	0.58	0.9994	0.21	0.01
yebK	0.81	0.05	0.9983	0.20	0.01	bolA	1.51	0.09	0.9994	0.18	0.01
mprA	0.77	0.06	0.9919	0.22	0.00	yfdN	2.93	1.98	0.8918	0.17	0.02
ydfT	0.23	0.10	0.0013	0.18	0.01	tdcR	2.51	1.12	0.9839	0.15	0.02
sgrR	0.74	0.03	0.981	0.18	0.01	hdfR	5.19	2.07	0.0119	0.16	0.04
yahA	0.50	0.21	0.1577	0.15	0.02	ybfE	3.28	1.50	0.653	0.17	0.02
ybaX	1.11	0.43	0.9993	0.15	0.03	yddM	2.83	0.14	0.9334	0.19	0.02
mntR	1.33	0.26	0.7853	0.21	0.01	yeeY	2.23	0.49	0.9983	0.16	0.03
rcsD	0.73	0.18	0.9644	0.18	0.02	norR	2.45	0.90	0.9916	0.19	0.02
yfeG	0.10	0.13	0.0001	0.17	0.01	yghO	2.39	0.97	0.9979	0.18	0.01
yfiE	0.70	0.19	0.9198	0.09	0.00	yqhC	2.24	1.20	0.9983	0.16	0.03
yfjR	0.94	0.28	0.9996	0.16	0.02	bglJ	4.68	1.55	0.0481	0.18	0.01
ygbI	0.70	0.11	0.896	0.15	0.01	cysB	3.78	1.29	0.3172	0.19	0.01
rof	0.25	0.11	0.0022	0.17	0.02	abrB	1.65	0.18	0.9993	0.19	0.01
iscR	0.48	0.20	0.1265	0.19	0.01	torS	2.79	0.56	0.9489	0.21	0.03
dinJ	LOD	LOD	LOD	0.13	0.01	oxyR	1.37	0.59	0.9996	0.19	0.01
yahB	0.37	0.11	0.0218	0.13	0.03	hipB	0.84	0.43	0.9998	0.18	0.01
ybaO	2.21	0.20	0.0001	0.14	0.04	yhjC	2.08	0.38	0.9986	0.15	0.02
ybcM	0.98	0.29	0.9999	0.17	0.04	dhaR	3.54	0.81	0.467	0.17	0.00
ycaN	0.88	0.10	0.9991	0.18	0.02	yedW	2.92	0.74	0.8969	0.18	0.01
yciT	2.22	0.32	0.0001	0.11	0.00	baeR	4.15	1.00	0.161	0.19	0.01
ydeW	1.15	0.10	0.9988	0.18	0.01	ascG	1.86	0.50	0.999	0.17	0.01
yneJ	0.58	0.17	0.3946	0.11	0.01	ydhM	2.75	1.91	0.9613	0.19	0.01
ydfH	0.85	0.22	0.9988	0.14	0.04	yibA	0.64	0.43	0.9993	0.17	0.01
ydjF	0.79	0.28	0.9982	0.14	0.03	yhhH	0.23	0.26	0.9979	0.18	0.00
yeaM	0.63	0.11	0.6089	0.16	0.02	ybbC	LOD	LOD	LOD	0.12	0.02
djlA	LOD	LOD	LOD	0.15	0.02						

APPENDIX D

Microarray results of genes increased by two fold in Δ*cutR* EHEC

Gene	Affymetrix Probe	Fold Change	Description
yiiF	1765608_at	43.9	CopG family putative transcriptional regulator
flu	1760040_at	29.1	phase-variable bipartite outer membrane fluffing protein
intK	1767538_at	24.5	pseudogene
yhcE	1759255_at	16.2	pseudogene
yehC	1768963_s_at	14.6	fimbrial chaperone
yegJ	1767406_at	10.8	hypothetical protein
yggC	1765103_at	7.8	protein with nucleoside triphosphate hydrolase domain
yjhC	1768996_at	7.6	putative oxidoreductase
aceB	1762343_s_at	6.9	malate synthase
ygjQ	1766826_s_at	6.4	DUF218 superfamily
yi82	1762957_s_at	6.1	IS186 transposase
waaS	1766029_at	5.8	lipopolysaccharide core biosynthesis protein
hdeD	1765786_s_at	5.3	acid-resistance membrane protein
atoD	1768251_s_at	4.6	acetyl-CoA:acetoacetyl-CoA transferase subunit alpha
upp	1764921_s_at	4.4	uracil phosphoribosyltransferase
yaiY	1768358_s_at	4.2	inner membrane protein, DUF2755 family
gadA	1768498_s_at	4.0	glutamate decarboxylase
hdeA	1765321_s_at	4.0	acid-resistance protein
dmsA	1765899_s_at	4.0	dimethyl sulfoxide reductase, anaerobic
uxaB	1767064_s_at	4.0	altronate oxidoreductase
aceA	1763981_s_at	3.9	isocitrate lyase
flxA	1761538_at	3.9	hypothetical protein
ECs4949	1764138_at	3.8	hypothetical protein
Z4864	1762244_s_at	3.8	beta-hydroxydecanoyl-ACP dehydrase
yggA	1768598_s_at	3.8	arginine exporter protein
metF	1768562_s_at	3.7	5,10-methylenetetrahydrofolate reductase
yzgL	1765193_at	3.7	pseudogene
dsdC	1767140_at	3.7	D-serine dehydratase transcriptional activator
narI	1762880_s_at	3.6	nitrate reductase 1, cytochrome b(NR), gamma subunit
narJ	1761649_s_at	3.6	nitrate reductase 1, delta subunit
ycjQ	1760206_s_at	3.5	Zn-dependent NAD(P)-binding oxidoreductase
jcfJ	1763404_s_at	3.4	hypothetical protein
gntK	1768650_s_at	3.4	gluconate kinase
moaA	1765186_s_at	3.3	molybdenum cofactor biosynthesis protein A
yhhI	1759954_at	3.1	predicted transposase
rygB	1761411_s_at	3.0	small RNA
Z0258	1761993_s_at	2.8	component of the type VI protein secretion system
hdeB	1759829_s_at	2.8	acid-resistance protein
kdpA	1762473_s_at	2.8	potassium-transporting ATPase subunit A
srlB	1764665_s_at	2.8	PTS system glucitol/sorbitol-specific transporter
ygeK	1761613_s_at	2.8	hypothetical protein
iclR	1766089_s_at	2.7	transcription factor
yjbH	1764066_s_at	2.7	putative porin
gntT	1766810_s_at	2.7	gluconate permease
yiaL	1767602_at	2.7	hypothetical protein
wecE	1766616_s_at	2.7	TDP-4-oxo-6-deoxy-D-glucose transaminase
qseE	1760218_s_at	2.7	sensor kinase
ykfE	1768146_s_at	2.7	inhibitor of c-type lysozyme, periplasmic
ypeC	1760812_s_at	2.6	DUF2502 family putative periplasmic protein

cysG	1765149_s_at	2.6	siroheme synthase
Z2146	1766728_x_at	2.6	lom protein of prophage CP-933O
ygeY	1765003_s_at	2.6	putative peptidase
yggF	1762222_s_at	2.6	fructose 1,6 bisphosphatase isozyme
folC	1769213_s_at	2.6	bifunctional folylpolyglutamate synthase/ dihydrofolate synthase
lysA	1761847_s_at	2.5	diaminopimelate decarboxylase
dmsB	1766933_s_at	2.5	anaerobic dimethyl sulfoxide reductase
moaB	1765982_s_at	2.4	molybdenum cofactor biosynthesis protein B
Z3316	1761650_s_at	2.4	tail component of prophage CP-933X
malT	1769204_at	2.4	DNA-binding transcriptional activator for the mal regulon
yidK	1765969_s_at	2.4	putative transporter
Z1456	1763065_at	2.4	hypothetical protein O-island 45
hofC	1761645_s_at	2.4	type-IV pilus assembly protein
ypfG	1767520_s_at	2.3	hypothetical protein
yhiM	1768136_s_at	2.3	acid resistance protein, inner membrane
yfjT	1765394_at	2.3	hypothetical protein
ECs5363	1765056_s_at	2.3	hypothetical protein
mdlA	1760972_s_at	2.3	multidrug transporter membraneATP-binding component
entH	1761866_s_at	2.3	enterobactin synthesis proofreading thioesterase
mdlB	1763681_s_at	2.3	multidrug transporter membraneATP-binding component
ycjS	1767412_s_at	2.3	D-galactose 1-dehydrogenase
fliP	1767430_s_at	2.3	flagellar biosynthesis protein
phnO	1759980_s_at	2.3	aminoalkylphosphonate N-acetyltransferase
napB	1763464_s_at	2.3	citrate reductase cytochrome C subunit
Z2087	1764524_s_at	2.3	hypothetical protein
uxuA	1763776_s_at	2.3	mannonate dehydratase
Z2115	1765405_s_at	2.3	phage terminase
narH	1763499_s_at	2.2	nitrate reductase 1, beta (Fe-S) subunit
yegU	1762399_s_at	2.2	ADP-ribosylglycohydrolase family protein
gadB	1760545_s_at	2.2	glutamate decarboxylase
fliF	1763750_s_at	2.2	flagellar MS-ring protein
emrB	1762168_s_at	2.2	multidrug efflux system protein
ecpR	1759211_s_at	2.2	luxR type transcriptional regulator of ecp operon
degS	1769096_s_at	2.2	serine endoprotease
Z4046	1762535_s_at	2.2	3-octaprenyl-4-hydroxybenzoate decarboxylase
Z4982	1762428_at	2.2	small toxic polypeptide
Z0249	1759564_s_at	2.2	component of the type VI protein secretion system
ybaM	1760935_s_at	2.2	hypothetical protein
yfcI	1763000_s_at	2.2	hypothetical protein
acpS	1768712_s_at	2.2	holo-[acyl-carrier-protein] synthase 1
cpxP	1764983_s_at	2.2	inhibitor of the cpx response
yfcP	1769126_at	2.2	putative fimbrial-like adhesin protein
recB	1766590_s_at	2.2	exonuclease V subunit beta
yadS	1762915_s_at	2.2	UPF0126 family inner membrane
yphG	1762666_s_at	2.1	hypothetical protein
wzx	1762953_s_at	2.1	O antigen flippase
Z2390	1759286_s_at	2.1	hypothetical protein
htpX	1760965_s_at	2.1	putative endopeptidase
lspA	1765959_s_at	2.1	lipoprotein signal peptidase
ypfH	1765694_s_at	2.1	palmitoyl-CoA esterase activity
citF	1765926_s_at	2.1	citrate lyase
recF	1764113_s_at	2.1	gap repair protein
ECs1644	1760442_s_at	2.1	phage minor tail protein
metN	1768746_s_at	2.1	DL-methionine transporter ATP-binding subunit
cysK	1762560_s_at	2.1	cysteine synthase A
metE	1766722_s_at	2.1	5-methyltetrahydropteroyltriglutamate/homocysteine S-

			methyltransferase
aldH	1764174_s_at	2.1	gamma-glutamyl-gamma-aminobutyraldehyde dehydrogenase
Z3451	1761270_s_at	2.1	subunit of heme lyase
ygaA	1767077_s_at	2.0	anaerobic nitric oxide reductase transcriptional regulator
cysP	1759777_s_at	2.0	thiosulfate transporter subunit
thiS	1767226_s_at	2.0	immediate sulfur donor in thiazole formation
moaD	1768589_s_at	2.0	molybdopterin synthase small subunit
yqeK	1760655_s_at	2.0	hypothetical protein
Z1460	1767713_s_at	2.0	S-adenosylmethionine-dependent methyltransferase, O-island 45
ypfG	1762051_s_at	2.0	DUF1176 family protein
wbdP	1760999_s_at	2.0	glycosyl transferase family protein
frvR	1765658_at	2.0	putative frv operon regulator
ybjQ	1764725_s_at	2.0	UPF0145 family protein
chiP	1761785_s_at	2.0	chitoporin
Z2507	1762602_s_at	2.0	multidrug-efflux transport protein
omrA	1763092_at	2.0	sRNA
Z2251	1762507_s_at	2.0	flavin reductase (DIM6/NTAB) family

APPENDIX E

Microarray results of genes decreased by two fold in Δ*cutR* EHEC

Gene	Affymetrix Probe	Fold Change	Description
yhaO	1760781_s_at	-18.5	serine transport protein
yhaM	1763323_s_at	-13.8	L-serine dehydratase
yghW	1767183_s_at	-11.1	hypothetical protein
ECs1529	1762729_at	-8.6	hypothetical protein
Z4462	1764952_s_at	-8.5	L-cysteine desulfidase
modD	1760482_s_at	-8.4	molybdenum transport protein
yjgB	1765890_at	-7.7	putative alcohol dehydrogenase
modA	1764243_s_at	-6.9	periplasmic binding component of the molybdate ABC transporter
Z1842	1761503_s_at	-6.8	primase pseudogene
ycaK	1765581_at	-6.5	hypothetical protein
fliA	1763490_s_at	-6.1	flagellar biosynthesis sigma factor
yeaG	1762658_s_at	-6.0	PrkA family of serine protein kinases
thiD	1768959_s_at	-5.7	hydroxy-methylpyrimidine kinase and hydroxy-phosphomethylpyrimidine kinase
ydcS	1764131_s_at	-5.5	predicted spermidine/putrescine ABC transporter
ecnB	1766745_s_at	-5.3	entericidin B
mdtA	1762462_s_at	-5.2	multidrug efflux system, subunit A
yjiY	1769169_s_at	-5.0	carbon starvation protein
astD	1766809_s_at	-4.8	succinylglutamic semialdehyde dehydrogenase
yehZ	1762722_s_at	-4.8	transport system permease
astC	1761406_s_at	-4.7	succinylornithine transaminase
glpT	1761983_s_at	-4.6	sn-glycerol-3-phosphate transporter
yihS	1766032_at	-4.6	D-mannose isomerase
rplD	1761917_s_at	-4.5	50S ribosomal protein L4
yphD	1759670_s_at	-4.5	transport system permease
Z1481	1762787_s_at	-4.4	sensor kinase O-island 45
rpsR	1762305_s_at	-4.4	30S ribosomal protein S18
yidB	1764387_s_at	-4.3	hypothetical protein
Z1963	1759610_s_at	-4.3	S-adenosylmethionine-dependent methyltransferase
blc	1766770_s_at	-4.3	outer membrane lipoprotein (lipocalin)
acnB	1768073_at	-4.2	bifunctional aconitate hydratase 2
astA	1760622_s_at	-4.2	arginine succinyltransferase
yehY	1762149_s_at	-4.2	membrane component of ABC superfamily
Z1965	1762429_s_at	-4.2	iron ABC transporter permease
Z1966	1762649_s_at	-4.1	iron ABC transporter permease
ycgB	1765610_s_at	-3.9	SpoVR family protein
hyaA	1765453_s_at	-3.8	hydrogenase-1 small subunit
tdcE	1759176_s_at	-3.7	pyruvate formate-lyase 4/2-ketobutyrate formate-lyase
astE	1760113_s_at	-3.7	succinylglutamate desuccinylase
fdoG	1761290_s_at	-3.6	formate dehydrogenase-O major subunit
yahB	1759100_s_at	-3.6	putative DNA-bindng transcriptional regulator
modB	1763211_s_at	-3.6	molybdate ABC transporter permease
hyaB	1768184_s_at	-3.6	hydrogenase 1 large subunit
yciX	1764922_s_at	-3.5	hypothetical protein
pykF	1766913_s_at	-3.5	pyruvate kinase
ydcJ	1765345_s_at	-3.5	putative metalloenzyme
aldB	1765157_s_at	-3.5	aldehyde dehydrogenase
putA	1766150_s_at	-3.4	trifunctional transcriptional regulator/proline dehydrogenase/pyrroline-5-carboxylate dehydrogenase

ymgE	1761638_s_at	-3.4	transglycosylase associated protein
ybaS	1759740_s_at	-3.4	glutaminase
ugpB	1763611_s_at	-3.4	glycerol-3-phosphate transporter periplasmic binding protein
fadL	1760611_s_at	-3.3	long-chain fatty acid outer membrane transporter
ytfR	1766470_s_at	-3.2	putative sugar transporter subunit: ATP-binding component of ABC superfamily
yegS	1768412_s_at	-3.2	phosphatidylglycerol kinase, metal-dependent
yeaV	1760424_s_at	-3.2	putative transporter
ybgA	1768055_s_at	-3.2	conserved protein, DUF1722 family
dkgA	1764019_s_at	-3.1	2,5-diketo-D-gluconate reductase A
yedP	1762965_s_at	-3.1	mannosyl-3-phosphoglycerate phosphatase
yeaQ	1765823_s_at	-3.1	conserved protein, UPF0410 family
wrbA	1761735_s_at	-3.1	NAD(P)H:quinone oxidoreductase
pflB	1767207_s_at	-3.1	pyruvate formate lyase
cyoA	1769018_s_at	-3.1	cytochrome o ubiquinol oxidase subunit II
tdcC	1761588_s_at	-3.0	L-threonine/L-serine transporter
proP	1764343_s_at	-3.0	proline/glycine betaine transporter
ygjG	1764660_s_at	-3.0	putrescine:2-oxoglutaric acid aminotransferase
yhiL	1768691_at	-3.0	pseudogene
Z1772	1760122_s_at	-2.9	rha family transcription factor
yhfG	1767488_s_at	-2.9	antitoxin to fic toxin
acs	1761238_s_at	-2.9	acetyl-CoA synthase
Z1964	1767905_s_at	-2.9	iron ABC transporter permease
yciR	1762888_s_at	-2.9	cyclic-di-GMP phosphodiesterase
psiF	1761076_s_at	-2.9	hypothetical protein
ycaC	1759749_s_at	-2.9	putative hydrolase, isochorismatase family
ydiF	1768141_s_at	-2.9	hypothetical protein
Z3625	1763907_s_at	-2.9	sucrose-6 phosphate hydrolase
ysgA	1766239_s_at	-2.8	carboxymethylenebutenolidase
tdcD	1768255_s_at	-2.8	propionate kinase/acetate kinase C, anaerobic
elaB	1760724_s_at	-2.8	putative membrane-anchored DUF883 family ribosome-binding protein
rnc	1759102_s_at	-2.8	Rnase III
yhcO	1764073_s_at	-2.8	barnase inhibitor
yeaH	1759650_s_at	-2.8	UPF0229 family protein
ECs4956	1762659_at	-2.8	hypothetical protein
ureA	1763746_s_at	-2.8	urease
oppA	1764969_s_at	-2.8	oligopeptide ABC transporter periplasmic binding protein
guaA	1763034_s_at	-2.8	GMP synthetase
rimP	1763922_s_at	-2.8	ribosome maturation factor for 30S subunits
glnP	1766676_s_at	-2.8	glutamine ABC transporter permease
yedK	1759787_s_at	-2.8	hypothetical protein
Z5139	1763252_s_at	-2.7	LEE orf2
sapA	1762238_s_at	-2.7	antimicrobial peptide transport ABC transporter
tdcD	1767841_s_at	-2.7	propionate kinase/acetate kinase C, anaerobic
glnQ	1759461_s_at	-2.7	glutamine ABC transporter ATP-binding protein
dppA	1759395_s_at	-2.7	periplasmic binding component of the dipeptide ABC transporter
lsrB	1759256_s_at	-2.7	periplasmic-binding component of ABC superfamily
tolR	1767600_s_at	-2.7	colicin uptake protein TolR
ytfS	1759937_s_at	-2.7	putative sugar transporter subunit: ATP-binding component of ABC superfamily
yoaA	1763085_at	-2.7	conserved protein with nucleoside triphosphate hydrolase domain
poxB	1763037_s_at	-2.7	pyruvate dehydrogenase
yegP	1759909_s_at	-2.7	conserved protein, UPF0339 family
malK	1760041_s_at	-2.7	maltose ABC transportor ATPase
artP	1762904_s_at	-2.6	arginine transporter ATP-binding protein
adhE	1767238_s_at	-2.6	fused acetaldehyde-CoA dehydrogenase/iron-dependent alcohol dehydrogenase/pyruvate-formate lyase deactivase
ygaM	1762690_s_at	-2.6	hypothetical protein

tdcB	1760555_s_at	-2.6	L-threonine dehydratase
fic	1767487_s_at	-2.6	toxin, yhfG antitoxin system
tdcF	1760738_s_at	-2.6	putative reactive intermediate deaminase
tnaB	1763204_s_at	-2.6	tryptophan permease
Z1452	1761186_s_at	-2.6	hypothetical protein, O-island 45
appC	1767792_s_at	-2.6	cytochrome bd-II oxidase
dctR	1763257_s_at	-2.6	transcription factor
nrdD	1759912_s_at	-2.6	anaerobic ribonucleoside-triphosphate reductase
yebV	1768481_s_at	-2.6	hypothetical protein
ygiH	1760077_s_at	-2.5	putative glycerol-3-phosphate acyltransferase
yadN	1763419_s_at	-2.5	fimbrial-like protein
fdnI	1760891_s_at	-2.5	formate dehydrogenase-N subunit gamma
potE	1767922_s_at	-2.5	putrescine/proton symporter: putrescine/ornithine antiporter
yjdI	1769100_s_at	-2.5	putative 4Fe-4S mono-cluster protein
ydeJ	1765113_s_at	-2.5	competence damage-inducible protein A
rpoA	1767563_s_at	-2.5	DNA-directed RNA polymerase subunit alpha
Z1207	1769229_s_at	-2.5	hypothetical protein
yhaQ	1767162_s_at	-2.5	L-serine deaminase
Z0953	1763387_s_at	-2.5	phage recombinantion protein, O-island 36
ymiA	1761738_s_at	-2.4	hypothetical protein
dosC	1761786_s_at	-2.4	diguanylate cyclase
nrdA	1764659_s_at	-2.4	ribonucleoside-diphosphate reductase 1
pgaA	1769025_s_at	-2.4	poly-beta-1,6-N-acetyl-D-glucosamine export protein
fdoH	1762501_s_at	-2.4	formate dehydrogenase-O, iron-sulfur subunit
proV	1760798_s_at	-2.4	high-affinity transport system for the osmoprotectant glycine betaine
ydcT	1765189_s_at	-2.4	ATP binding component of ABC transport system for spermidine/putrescine
ymcA	1762456_s_at	-2.4	putative O-antigen capsule production periplasmic protein
ECs2630	1763075_at	-2.4	hypothetical protein
treA	1759084_s_at	-2.4	periplasmic trehalase
rpsD	1765207_s_at	-2.4	30S ribosomal protein S4
sdhC	1767518_s_at	-2.4	succinate dehydrogenase
parE	1764016_s_at	-2.4	DNA topoisomerase subunit
pta	1766550_s_at	-2.4	phosphate acetyltransferase
yjdJ	1768134_s_at	-2.4	putative acyltransferase with acyl-CoA N-acyltransferase domain
putP	1763553_s_at	-2.4	proline:sodium symporter
yhbP	1764098_s_at	-2.4	UPF0306 family protein
fadE	1761891_at	-2.4	acyl coenzyme A dehydrogenase
sibA	1764769_at	-2.4	sRNA
mdtK	1763022_s_at	-2.4	multidrug efflux system transporter
ptrB	1761413_s_at	-2.3	protease II
rbbA	1762645_s_at	-2.3	ABC transporter ATP-binding protein
Z0870	1763522_at	-2.3	putative fimbrial gene
Z4858	1759642_s_at	-2.3	hypothetical protein
ybaT	1766093_s_at	-2.3	putative amino acid/amine transporter of the APC superfamily
puuE	1764428_s_at	-2.3	4-aminobutyrate aminotransferase
lysP	1762857_s_at	-2.3	lysine transporter
cyoB	1759374_s_at	-2.3	cytochrome bo terminal oxidase complex
cydB	1765588_s_at	-2.3	cytochrome d terminal oxidase
yjdC	1760748_s_at	-2.3	tetR family transcription factor
ECs1293	1769156_s_at	-2.3	hypothetical protein
psuT	1767840_at	-2.3	putative nucleoside transporter
	1759662_s_at	-2.3	Intergenic region between yfjZ-ypjF and ypjA
dsbG	1762886_s_at	-2.3	thiol:disulfide interchange protein, periplasmic
ldtE	1767891_s_at	-2.3	murein L,D-transpeptidase
ydcJ	1759558_s_at	-2.3	putative metalloenzyme
ECs1074	1764302_s_at	-2.3	replication protein

dcuR	1762536_s_at	-2.3	response regulator
otsB	1762741_s_at	-2.3	trehalose-6-phosphate phosphatase
rpoD	1765431_s_at	-2.3	RNA polymerase, sigma 70 (sigma D) factor
Z4695	1763833_s_at	-2.2	bacterioferritin
yceK	1767925_s_at	-2.2	outer membrane integrity lipoprotein
nuoC	1763897_s_at	-2.2	NADH:ubiquinone oxidoreductase
yjaH	1762371_s_at	-2.2	DUF1481 family putative lipoprotein
yhbO	1764792_s_at	-2.2	stress responsive protease
gatD	1767932_s_at	-2.2	galactitol-1-phosphate dehydrogenase
yecH	1761022_s_at	-2.2	DUF2492 family protein
hipA	1766342_s_at	-2.2	toxin with hipB antitoxin
Z2563	1767744_at	-2.2	putative transposase
gcd	1762774_s_at	-2.2	glucose dehydrogenase
Z0315	1769001_s_at	-2.2	phage tail fibre assembly protein
ytfQ	1765105_s_at	-2.2	periplasmic binding component of a predicted sugar ABC transporter
dosC	1764000_s_at	-2.2	diguanylate cyclase
ler	1761795_s_at	-2.2	transcription factor
mglC	1761896_s_at	-2.2	integral membrane component of the galactose ABC transporter
ymdC	1761915_s_at	-2.2	cardiolipin synthase
yicO	1761807_s_at	-2.2	putative adenine permease
phnB	1768688_s_at	-2.2	metalloprotein superfamily protein
rplQ	1765986_s_at	-2.2	50S ribosomal subunit protein L17
potF	1759866_s_at	-2.2	putrescine ABC transporter
caiB	1766861_at	-2.2	crotonobetainyl CoA:carnitine CoA transferase
ugpA	1760060_s_at	-2.2	glycerol-3-phosphate transporter
sdhB	1769260_s_at	-2.1	succinate dehydrogenase, FeS subunit
yghX	1759500_s_at	-2.1	pseudogene
tusC	1766523_s_at	-2.1	mnm(5)-s(2)U34-tRNA synthesis 2-thiolation protein
rho	1765454_s_at	-2.1	rho termination factor
rpmJ	1763137_s_at	-2.1	50S ribosomal protein L36
ackA	1765234_s_at	-2.1	acetate kinase A and propionate kinase 2
yaiA	1764470_s_at	-2.1	hypothetical protein
lptD	1760068_s_at	-2.1	organic solvent tolerance protein
ybhP	1767041_s_at	-2.1	endo/exonuclease/phosphatase family PFAM PF03372
Z5137	1762756_s_at	-2.1	LEE orf4
speF	1759406_s_at	-2.1	ornithine decarboxylase
rpsS	1761856_s_at	-2.1	30S ribosomal protein S19
rplW	1762454_s_at	-2.1	50S ribosomal protein L23
mysB	1764777_s_at	-2.1	secY/secA suppressor protein
Z0949	1766160_s_at	-2.1	primase, O-island 36
Z3260	1765793_s_at	-2.1	fructose-bisphosphate aldolase
ECs5422	1759369_at	-2.1	hypothetical protein
Z1847	1765657_s_at	-2.1	capsid protein of prophage CP-933C
yiaG	1764713_s_at	-2.1	xre family transcription factor
hycB	1764203_s_at	-2.1	small subunit of hydrogenase-3
yhjR	1766976_s_at	-2.1	DUF2629 family protein
yodD	1768573_s_at	-2.1	hypothetical protein
yohF	1761377_s_at	-2.1	putative acetoin dehydrogenase
nuoH	1760147_s_at	-2.1	NADH dehydrogenase subunit
plsB	1764259_s_at	-2.0	glycerol-3-phosphate acyltransferase
afuA	1762261_s_at	-2.0	ATP-binding component of ABC ferric cation transporter
pgaC	1767261_s_at	-2.0	poly-beta-1,6-N-acetyl-D-glucosamine synthase
cspG	1763772_s_at	-2.0	cold shock protein
nac	1766591_s_at	-2.0	lysR type txn factor
ybdK	1760063_s_at	-2.0	gamma-glutamyl:cysteine ligase
fdoI	1760958_s_at	-2.0	formate dehydrogenase

Z2039	1760357_s_at	-2.0	regulator of cell division encoded by prophage CP-933O
ECs4325	1769040_s_at	-2.0	O-methyltransferase
yjcH	1767573_s_at	-2.0	DUF485 family inner membrane protein
mhpR	1768538_s_at	-2.0	iclR type transcription factor
Z1489	1765615_s_at	-2.0	outer membrane protein, O-island 45
yfcG	1762727_s_at	-2.0	glutathione S-transferase
araH	1761969_s_at	-2.0	L-arabinose transporter permease
Z3125	1760081_s_at	-2.0	hypothetical protein, O-island 79
osmY	1767463_s_at	-2.0	putative ABC transporter
yliF	1760159_s_at	-2.0	membrane-anchored diguanylate cyclase
erfK	1766673_s_at	-2.0	L,D-transpeptidase
rpsH	1768794_s_at	-2.0	30S ribosomal protein S8
rplF	1766400_s_at	-2.0	50S ribosomal protein L6

APPENDIX F

ELISA results for K-12 mutants of genes regulated by CutR

ELISA results for EspB production by pJAY1512 carrying Keio library K-12 deletion strains of genes found to be within the CutR regulon. See Figure 10, Appendix D, and Appendix E.

K-12 strain	Avg. Rel. EspB	Std. Dev. Rel. EspB	Dunnetts p value	Avg. OD600	Std. Dev. OD600	K-12 strain	Avg. Rel. EspB	Std. Dev. Rel. EspB	Dunnetts p value	Avg. OD600	Std. Dev. OD600
lacA	1.00	0.08		0.18	0.02	jiP	1.29	0.29	0.8903	0.18	0.02
ybaO	6.84	0.22	0.0001	0.22	0.01	ivy	1.61	0.15	0.0102	0.17	0.02
dsdC	2.12	0.74	0.9825	0.25	0.02	ybgA	1.06	0.25	0.9996	0.19	0.02
mhpR	6.28	0.19	0.0001	0.22	0.01	yadN	1.22	0.18	0.9828	0.22	0.03
nac	1.82	1.14	0.9985	0.28	0.03	yahB	0.95	0.04	0.9996	0.17	0.02
acnB	3.95	2.57	0.0153	0.24	0.01	glsA	1.48	0.17	0.1149	0.15	0.02
cyoB	1.93	0.40	0.9982	0.16	0.00	ycaK	0.95	0.06	0.9996	0.16	0.00
cyoA	2.62	1.05	0.6753	0.18	0.01	gfcD	0.79	0.19	0.9916	0.18	0.01
sdhC	3.72	1.55	0.0367	0.22	0.03	lsrB	0.76	0.30	0.9794	0.16	0.03
sdhB	3.15	0.32	0.2188	0.20	0.01	flxA	0.60	0.05	0.315	0.21	0.02
pflB	5.97	1.11	0.0001	0.05	0.00	yhaM	1.69	0.15	0.0021	0.05	0.02
cbdA	1.39	0.67	0.9994	0.27	0.03	yfjT	1.02	0.07	0.9999	0.22	0.01
adhE	0.88	0.52	0.9998	0.07	0.01	blc	1.19	0.20	0.9981	0.20	0.01
pykF	1.48	0.62	0.9993	0.24	0.01	yeaG	0.83	0.10	0.9983	0.16	0.02
nuoH	0.60	0.33	0.9994	0.17	0.01	cysG	0.56	0.13	0.1902	0.17	0.01
tktB	0.99	0.32	0.9999	0.17	0.01	ycjQ	0.83	0.09	0.9983	0.17	0.00
aceB	1.24	0.15	0.9997	0.18	0.00	elaB	0.82	0.08	0.9982	0.17	0.01
aceA	2.73	0.83	0.5664	0.20	0.02	argO	0.33	0.12	0.003	0.18	0.01
evgS	3.78	1.04	0.0296	0.21	0.01	yggC	0.72	0.06	0.8791	0.20	0.02
dcuR	4.50	0.31	0.0014	0.25	0.02	yggF	0.84	0.10	0.9985	0.21	0.02
treA	2.00	0.40	0.9979	0.24	0.02	yghW	0.62	0.06	0.396	0.20	0.01
oppA	2.73	0.87	0.5635	0.21	0.02	ygiV	0.76	0.06	0.979	0.15	0.01
pta	LOD	LOD	LOD	0.07	0.01	plsY	0.80	0.17	0.998	0.08	0.01
poxB	1.12	0.44	0.9998	0.17	0.02	ygjQ	0.91	0.13	0.9993	0.17	0.01
cydB	LOD	LOD	LOD	0.02	0.01	yhbP	0.63	0.28	0.4544	0.05	0.02
moaA	7.80	1.77	0.0001	0.20	0.01	yhcO	0.84	0.09	0.9985	0.17	0.01
ackA	LOD	LOD	LOD	0.04	0.01	yheM	1.47	0.29	0.1246	0.14	0.03
pgaA	3.67	0.79	0.0439	0.23	0.02	yhfG	0.62	0.05	0.4263	0.20	0.01
fadL	12.33	3.49	0.0001	0.23	0.02	dctR	1.01	0.11	0.9999	0.18	0.01
ygeK	3.88	2.04	0.0208	0.20	0.01	hdeA	0.66	0.03	0.5762	0.17	0.02
gcd	0.81	0.30	0.9997	0.16	0.01	hdeD	0.78	0.13	0.9833	0.15	0.00
yoaA	1.50	0.83	0.9992	0.16	0.02	yhjR	0.82	0.15	0.9983	0.16	0.00
htpX	3.19	0.38	0.2003	0.18	0.04	yiaG	0.75	0.05	0.9611	0.17	0.01
ptrB	1.22	0.57	0.9997	0.19	0.01	yidK	0.61	0.05	0.3858	0.20	0.01
ygeY	2.08	0.66	0.9841	0.25	0.02	wecD	0.53	0.26	0.128	0.12	0.03
yfcP	1.23	0.53	0.9997	0.22	0.03	yjaH	0.98	0.04	0.9999	0.22	0.04
ypeC	1.60	0.20	0.999	0.26	0.03	yjbH	0.80	0.09	0.998	0.20	0.03
ypfG	1.64	0.46	0.9989	0.23	0.01	yjcH	0.58	0.10	0.2535	0.15	0.01
ygaM	3.73	0.50	0.0365	0.15	0.00	mdtN	0.98	0.18	0.9999	0.06	0.01
yecH	0.64	0.24	0.9994	0.18	0.02	yjdI	0.93	0.21	0.9994	0.17	0.00
tfaQ	3.63	1.11	0.0505	0.18	0.02	yjdJ	0.93	0.22	0.9994	0.08	0.01
yaiY	2.57	0.91	0.7285	0.25	0.02	ytfQ	0.96	0.21	0.9997	0.20	0.01
yaiA	7.73	0.52	0.0001	0.23	0.00	hofC	1.21	0.44	0.9916	0.19	0.01

ybaM	5.29	2.71	0.0001	0.23	0.02	metN	1.09	0.25	0.9993	0.22	0.01
chiP	1.61	0.70	0.999	0.25	0.01	mdlA	1.25	0.38	0.9599	0.19	0.03
ybhP	6.24	1.25	0.0001	0.01	0.00	ybaT	0.60	0.10	0.3174	0.16	0.01
ycgB	0.08	0.17	0.9982	0.15	0.02	wrbA	0.62	0.06	0.4043	0.16	0.02
ydcJ	0.10	0.18	0.9983	0.15	0.00	trpD	0.53	0.04	0.127	0.18	0.01
yedP	1.43	0.97	0.9993	0.18	0.01	puuE	0.81	0.04	0.998	0.17	0.02
mtfA	6.20	0.93	0.0001	0.19	0.01	astE	0.89	0.07	0.9991	0.19	0.01
iscX	0.71	0.32	0.9996	0.24	0.01	astA	1.03	0.04	0.9998	0.18	0.01
sufE	0.80	0.22	0.9997	0.21	0.01	wcaM	0.67	0.05	0.6504	0.20	0.00
elyC	3.86	0.99	0.0226	0.29	0.06	cysM	0.85	0.14	0.9986	0.18	0.00
yedK	0.92	0.11	0.9999	0.24	0.01	cysE	1.74	0.36	0.0006	0.16	0.01
yegU	2.48	1.19	0.8102	0.16	0.01	cysK	0.64	0.08	0.5151	0.15	0.01
psuT	3.30	1.26	0.1478	0.16	0.00	lysA	0.66	0.14	0.6142	0.17	0.01
yadS	1.62	0.47	0.999	0.19	0.00	metE	0.74	0.10	0.9442	0.17	0.01
ybdK	1.95	1.57	0.9981	0.20	0.02	moaB	0.87	0.07	0.9989	0.20	0.00
ydeJ	5.70	0.38	0.0001	0.25	0.01	moaD	1.22	0.82	0.9827	0.10	0.05
ycjS	4.17	0.60	0.0062	0.26	0.01	gshA	0.87	0.11	0.999	0.20	0.01
ybjQ	3.54	1.07	0.0692	0.26	0.04	hipA	0.51	0.07	0.0966	0.18	0.02
ydiF	4.57	0.59	0.001	0.23	0.03	fliF	0.67	0.07	0.6711	0.17	0.03
eamB	4.96	0.97	0.0002	0.18	0.01	fliP	0.78	0.15	0.9837	0.14	0.02
entH	0.35	0.21	0.9989	0.16	0.02	wzxB	0.61	0.04	0.3736	0.18	0.01
ydcS	11.34	1.82	0.0001	0.16	0.01	waaS	0.88	0.21	0.999	0.17	0.01
yegS	4.29	0.67	0.0037	0.19	0.01	dicB	0.99	0.12	0.9999	0.18	0.00
osmF	3.31	1.06	0.1407	0.23	0.02	otsB	1.30	0.58	0.8041	0.17	0.01
yfcG	1.91	0.63	0.9982	0.21	0.01	fic	0.95	0.14	0.9996	0.21	0.01
pgaC	1.45	0.54	0.9993	0.19	0.00	dppA	0.92	0.26	0.9994	0.18	0.01
yddH	2.05	0.32	0.9914	0.23	0.00	speF	0.68	0.03	0.6962	0.13	0.01
gmr	1.49	0.34	0.9992	0.17	0.01	putA	0.94	0.07	0.9996	0.15	0.01
mdtK	7.32	0.59	0.0001	0.16	0.01	gadB	1.06	0.02	0.9996	0.17	0.01
ybhA	4.91	0.96	0.0002	0.18	0.01	cysI	1.00	0.14	0.9999	0.15	0.03
yliF	5.42	1.25	0.0001	0.18	0.01	cysJ	1.11	0.05	0.9991	0.14	0.02
ycaC	2.11	0.88	0.983	0.20	0.01	gadA	0.80	0.06	0.998	0.18	0.01
yehY	5.41	1.08	0.0001	0.21	0.01	metF	1.32	0.35	0.7124	0.19	0.03
yohF	5.92	1.02	0.0001	0.21	0.01	uxuA	0.80	0.13	0.998	0.21	0.01
csdA	2.17	0.63	0.9807	0.21	0.00	psiF	3.65	0.94	0.0001	0.24	0.02
eutC	1.34	0.32	0.6875	0.19	0.02	mdlB	2.31	0.48	0.2442	0.24	0.02
wecE	0.93	0.36	0.9995	0.23	0.03	citF	2.12	0.31	0.4849	0.24	0.01
acs	1.29	0.55	0.8945	0.13	0.05	dmsA	1.89	0.37	0.8606	0.22	0.01
malZ	1.29	0.11	0.8758	0.24	0.02	yciX	1.67	0.65	0.9846	0.17	0.01
hyaA	0.50	0.05	0.1173	0.17	0.02	astD	0.18	0.12	0.9309	0.18	0.01
hyaB	0.76	0.22	0.9806	0.12	0.03	yeaV	3.06	0.42	0.0034	0.21	0.02
ymgE	0.61	0.24	0.428	0.12	0.01	dcyD	1.60	0.56	0.9981	0.21	0.01
narJ	1.02	0.31	0.9999	0.18	0.01	yoeB	3.33	0.39	0.0004	0.26	0.01
narI	0.89	0.29	0.9991	0.22	0.01	bcr	3.25	1.13	0.0008	0.21	0.02
puuC	0.97	0.20	0.9998	0.20	0.00	napB	3.22	0.53	0.001	0.27	0.05
fdnI	0.71	0.18	0.8842	0.23	0.01	yghQ	1.91	0.33	0.821	0.22	0.00
uxaB	0.83	0.04	0.9985	0.24	0.01	yghX	0.56	0.17	0.9987	0.15	0.01
gatD	0.89	0.04	0.9991	0.16	0.01	dkgA	1.91	0.72	0.8278	0.18	0.02
ccmH	LOD	LOD	LOD	0.14	0.01	patA	0.84	0.34	0.9996	0.19	0.01
atoD	0.67	0.05	0.7199	0.18	0.01	yhaO	0.64	0.60	0.999	0.18	0.00
glpB	0.84	0.26	0.9986	0.16	0.02	rimP	7.23	1.21	0.0001	0.19	0.01
atpE	LOD	LOD	LOD	0.01	0.00	yihS	1.02	0.61	0.9999	0.23	0.02
fdoI	0.82	0.08	0.9983	0.20	0.01	adeQ	1.96	0.47	0.7478	0.22	0.03
fdoG	1.24	0.25	0.9805	0.21	0.03	bcsC	0.68	0.59	0.9991	0.21	0.02
recB	0.26	0.24	0.0011	0.15	0.02	hdeB	0.69	0.12	0.9992	0.14	0.01
nrdD	0.65	0.30	0.6206	0.14	0.03	rbbA	1.25	0.20	0.9994	0.16	0.01

hsdR	0.55	0.16	0.2164	0.15	0.01	yjfF	2.63	0.67	0.0519	0.20	0.02
yjdN	0.42	0.09	0.0299	0.18	0.01	yjhC	3.79	0.12	0.0001	0.18	0.01
potE	0.88	0.07	0.9991	0.17	0.01	yjiY	0.94	0.14	0.9999	0.21	0.01
kdpA	1.15	0.08	0.9988	0.19	0.01	fadE	2.45	0.63	0.1325	0.27	0.00
modB	1.70	0.10	0.0027	0.13	0.01	ecpR	3.18	0.18	0.0014	0.22	0.02
glnQ	0.70	0.34	0.8491	0.23	0.02	clsC	0.75	0.09	0.9994	0.22	0.01
glnP	0.72	0.13	0.9189	0.21	0.01	yceK	0.06	0.27	0.7785	0.14	0.01
potF	0.62	0.05	0.4856	0.16	0.02	dosC	0.54	0.11	0.9987	0.17	0.01
artP	0.15	0.09	0.0001	0.15	0.00	eamA	2.17	0.42	0.4199	0.18	0.00
putP	0.80	0.16	0.998	0.16	0.01	yebV	1.42	0.99	0.9988	0.19	0.01
sapA	0.80	0.15	0.9981	0.18	0.01	yodD	1.30	0.29	0.9992	0.21	0.01
ydcT	0.90	0.17	0.9992	0.20	0.00	mdtA	0.70	0.34	0.9992	0.22	0.01
mglC	1.09	0.23	0.9994	0.22	0.01	yegP	5.97	1.77	0.0001	0.21	0.00
lysP	0.02	0.12	0.0001	0.20	0.01	yphG	0.92	0.07	0.9998	0.21	0.01
malK	0.53	0.17	0.1683	0.22	0.01	qseE	0.88	0.64	0.9997	0.14	0.01
proP	0.58	0.03	0.3077	0.16	0.01	slrQ	1.42	0.19	0.9988	0.17	0.00
fecE	0.95	0.05	0.9996	0.14	0.01	ygcG	2.68	0.23	0.0385	0.18	0.00
fecC	1.08	0.38	0.9994	0.16	0.01	yhbO	1.22	0.43	0.9994	0.20	0.02
cysZ	2.26	0.26	0.0001	0.18	0.01	yhhI	1.41	0.29	0.9988	0.21	0.01
cysP	0.97	0.16	0.9998	0.18	0.00	modA	6.22	1.13	0.0001	0.21	0.01
yphD	1.61	0.03	0.018	0.18	0.02	ymiA	1.04	0.15	0.9999	0.24	0.00
proV	0.82	0.13	0.9983	0.21	0.01	recF	1.44	0.43	0.9987	0.21	0.01
srlB	1.01	0.20	0.9999	0.19	0.00	yehC	0.09	0.38	0.8249	0.13	0.02
gntT	0.39	0.11	0.0175	0.16	0.01	ycfJ	2.02	0.50	0.6577	0.18	0.01
ugpA	0.77	0.13	0.9827	0.17	0.00	ldtE	0.92	0.23	0.9998	0.19	0.02
ugpB	0.88	0.12	0.9991	0.18	0.01	yeaQ	1.10	0.14	0.9998	0.21	0.01
ilvI	1.04	0.14	0.9997	0.18	0.01	yeaH	1.66	0.24	0.9847	0.23	0.01
afuC	1.55	0.25	0.0545	0.22	0.01	cbtA	2.57	0.41	0.0716	0.26	0.01
dsbG	0.51	0.12	0.1219	0.19	0.02	yegJ	1.41	0.17	0.9989	0.24	0.01
msyB	1.31	0.26	0.8251	0.21	0.02	yqeK	1.27	0.18	0.9993	0.21	0.02
upp	1.33	0.13	0.7326	0.21	0.00	yzgL	1.15	0.54	0.9997	0.16	0.02
tdcD	1.32	0.20	0.7441	0.14	0.01	yiaL	1.51	0.44	0.9985	0.17	0.00
rpmJ	0.05	0.13	0.0001	0.01	0.01	astC	0.50	0.07	0.9985	0.18	0.01
rho	0.81	0.09	0.9983	0.16	0.01	phnO	1.14	0.20	0.9997	0.19	0.01
tolR	0.96	0.16	0.9997	0.15	0.00	dmsB	1.40	0.21	0.9989	0.21	0.01
cspG	0.51	0.14	0.1247	0.20	0.01	narH	1.45	0.35	0.9987	0.23	0.01
pspE	0.42	0.07	0.0296	0.19	0.00	hycB	1.46	0.35	0.9986	0.24	0.01
sufS	0.84	0.27	0.9986	0.20	0.01	fdoH	2.35	0.16	0.2125	0.21	0.01
emrB	0.66	0.09	0.6777	0.21	0.01	caiB	1.05	0.98	0.9999	0.15	0.01
aldB	1.19	0.06	0.9983	0.16	0.03	guaA	LOD	LOD	LOD	-0.01	0.01
yidB	0.90	0.08	0.9993	0.15	0.01	kdpB	3.29	1.08	0.0006	0.19	0.00
metL	0.61	0.18	0.4429	-0.02	0.00	glpT	2.62	1.16	0.0545	0.13	0.02
thiS	1.01	0.02	0.9999	0.17	0.02	tdcE	1.75	0.78	0.9776	0.21	0.01
iclR	1.35	0.30	0.6133	0.19	0.03	yiiF	6.95	1.88	0.0001	0.22	0.01
ecnB	0.59	0.28	0.3579	0.17	0.02	yhiM	3.79	0.43	0.0001	0.26	0.04
fecD	0.98	0.15	0.9999	0.21	0.01	yjdC	2.52	0.49	0.0919	0.21	0.00
osmY	1.23	0.24	0.9841	0.19	0.02	ahr	2.89	1.05	0.0105	0.16	0.02
thiD	1.35	0.15	0.5991	0.14	0.02	tnaB	2.21	0.33	0.3575	0.15	0.01
iscS	0.35	0.18	0.0083	0.02	0.02	nuoC	1.86	0.23	0.8864	0.20	0.01
tdcC	2.27	0.20	0.0001	0.14	0.03	norR	2.07	0.36	0.5744	0.21	0.02
tdcB	1.40	0.38	0.3821	0.16	0.01	ysgA	1.34	0.15	0.9991	0.19	0.01
yhcE	1.10	0.13	0.9993	0.18	0.01	cysB	6.17	0.64	0.0001	0.20	0.01
yhiL	1.21	0.33	0.9979	0.16	0.02	intK	4.72	0.91	0.0001	0.21	0.02
frvR	1.00	0.10	0.9999	0.20	0.01	ypfH	3.22	0.91	0.001	0.22	0.04