EHEC UTILIZES TWO-COMPONENT SYSTEMS TO MODULATE EXPRESSION OF THE MAJOR FLAGELLAR SUBUNIT PROTEIN, FLIC, IN RESPONSE TO HOST INTESTINAL CUES

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EHEC utilizes two-component systems to modulate expression of the major flagellar subunit protein, FliC, in response to host intestinal cues

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Abstract

Enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 is a food- and water-borne human enteric pathogen that infects human hosts. In order to colonize the host, EHEC uses many virulence factors including flagella to reach its site of colonization in the distal colon. Expression of flagella can be modulated in response to microenvironmental conditions within the host, sensed by two-component systems (TCS). We have demonstrated through immunoblot and motility assays that small intestinal short-chain fatty acid (SCFA) mixes upregulate flagella expression while large intestinal mixes downregulate expression in WT EHEC. We have also shown that three specific TCSs in EHEC are necessary for establishment of the SCFA-induced WT phenotype. Our results suggest that the ArcAB and RcsBC TCSs positively modulate flagella expression in response to small intestinal-like environmental conditions, while the BarA/UvrY TCS negatively modulates flagella expression in response to large intestinal-like environmental cues.

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List of Abbreviations:

GI	Gastrointestinal
EPEC	Enteropathogenic *Escherichia coli*
ETEC	Enterotoxigenic *Escherichia coli*
EAEC	Enteroaggregative *Escherichia coli*
EIEC	Enteroinvasive *Escherichia coli*
EHEC	Enterohemorrhagic *Escherichia coli*
HUS	Hemolytic Uremic Syndrome
SCFA	Short chain fatty acid
Stx	Shiga toxin
T3SS	Type III secretion system
A/E	Attaching and effacing
LEE	Locus of Enterocyte Effacement
PAI	Pathogenicity islands
Esp	*E. coli* secreted protein
Nle	Non-LEE-encoded
Gb3	Glycosphingolipid globotriosylceramide
HAP	Hook-associated protein
TCS	Two-component system
HK	Histidine kinase
RR	Response regulator
Hpt	His-containing phosphotransferase
ABC	ATP-binding cassette
PTS	Phosphotransferase system
IM	Inner membrane
WT	Wild-type
LB	Luria Bertani
DMEM	Dulbecco's Modified Eagle Medium
LG	Low Glucose

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1.0 Introduction:

1.1 Overview

Escherichia coli is a gram-negative facultative anaerobic bacterium found in the mammalian gastrointestinal (GI) tract, specifically colonizing the host intestine.[1] *E. coli* can survive outside of the gut in both aquatic and terrestrial environments for extended periods of time.[2] *E. coli* and the family *Enterobacteriaceae* is a model organism because of its intensive study since its discovery in 1885.[3] *E. coli* has a diverse number of strains that all possess unique characteristics fixing them to different environmental niches.[4] The *E. coli* genome is approximately 4.2-6.0 Megabases, composed of approximately 4-6,000 genes.[4] These genes allow *E. coli* to respond and adapt to the hosts gastrointestinal microenvironments. There are strains of *E. coli* that are non-pathogenic, yet there are other strains that can cause significant diseases within the mammalian host. These strains are known to be enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E.* coli (EIEC) and enterohemorrhagic *E. coli* (EHEC).[5]

Enterohemorrhagic *Escherichia coli* (EHEC) is a pathogen that is a leading cause of bloody diarrhea that can lead to hemorrhagic colitis and in rare cases, hemolytic uremic syndrome (HUS), a life-threatening complication.[6] The serotype specifically related to this study, O157:H7, is associated with the highest rates of outbreaks and severe disease development in North America.[6] The O157 serotype is not the only one that poses a significant health risk. There are non-O157 serotypes that can still lead to the development of HUS, but these serotypes are less prevalent in North America and are found with higher frequency in Latin America and Europe.[6]

EHEC infections typically begins with cramping and vomiting, progressing to watery diarrhea followed by bloody diarrhea (hemorrhagic colitis).[6] In 5-7% depending of infected individuals, the infection progresses to hemolytic uremic syndrome (HUS) depending on factors such as age, antibiotic use, and environmental factors. HUS is characterized by microangiopathic hemolytic anemia and thrombocytopenia.[5,6, 63]

Transmission of EHEC typically occurs when the host ingests contaminated food or water.[5] Infections can also occur from direct contact with contaminated sources, infected persons or animals.[7] EHEC has a very low infectious dose of <100 bacteria to cause an infection.[6] This could be due to EHEC's ability to survive exposure to stressful microenvironments during passage from the mouth to the site of colonization in the large intestine.[8] These microenvironmental stresses include but are not limited to acid stress at pH values between 2-6, bile stress, microbial flora metabolites like SCFA (short chain fatty acids), host hormones, host defense peptides, and varying oxygen concentrations.[6]

Treatment for an EHEC infection is typically limited to rehydration and palliative care, as antibiotic use is not recommended due to complications with HUS and Shiga toxin production.[9] The fact that EHEC infections are so hard to treat demonstrates that more research must be done to prevent the initial infection. This can be accomplished through the study of EHEC and its pathogenicity and virulence factors associated with infection.

1.2 Virulence Factors of EHEC O157:H7

1.2.1 Locus of Enterocyte Effacement Pathogenicity Island (LEE) and the Type III Secretion System (T3SS)

To better understand EHECs pathogenicity we must first understand the basics of EHEC's virulence factors and how they all contribute to infection. EHEC employs multiple virulence factors during infection of the human host. These include flagella, fimbrial and nonfimbrial adhesins, shiga toxin (Stx), and the type III secretion system (T3SS).[6] When EHEC is ingested by humans, it travels through the GI tract to the large intestine where it binds, on the host epithelial cells.[10] Here it forms attaching and effacing (A/E) lesions employing virulence factors, like actin pedestals encoded by the Locus of Enterocyte Effacement Pathogenicity Island (LEE).[10] Pathogenicity islands (PAIs) like the LEE found in pathogenic *E. coli* are large sections of chromosomal DNA that play essential roles in the pathogenicity of the associated bacteria. The ability of EHEC to induce A/E lesions, for tight binding to host cells, is encoded within LEE. LEE 1,2, and 3, and LEE 4 encodes the *E. coli* secreted proteins EspA, EspB, EspD, and EspF.[64] The LEE, highly regulated by other virulence factors and microenvironmental cues,

encodes for the T3SS that works as a syringe to translocate bacterial proteins into the host epithelial cells cytoplasm directly.[6,10] When EHEC binds to the host epithelium, the T3SS is employed and it then injects the bacterial effector proteins into the hosts cells.[10] The T3SS injects Tir (translocated intimin receptor) into the host epithelial cells to serve as the receptor for intimin, an outer membrane protein of EHEC. EHEC also binds to host cell proteins, integrin and nucleolin as another means of adhesion.[11,12] The T3SS also injects Esp (*E. coli* secreted proteins), into the host cell while the Esp proteins EspA,B and D are structural proteins for the T3SS itself.[13] The other LEE-encoded effector proteins include EspF, G, H and Map (mitochondrial associated proteins), which all aid in the disruption of host cell signaling.[13,14] The T3SS also allows for the delivery of Nle (Non-LEE-encoded) effectors. Mills et al., showed that the effectors, LEE-encoded and non-LEE-encoded, participate in overlapping roles to optimize colonization and infection.[14]

Similar to the T3SS, *E. coli* employs a system known as T3bSS for its flagellar systems.[65] The different components of the flagellum are secreted by the T3bSS. These include the components of the hook, the filament, the hook-filament junction protein, and the filament cap protein.[65] Although structurally similar, the two T3SS differ in function the former being utilized in attachment and colonization while the latter is utilized in motility leading to tight regulation of virulence factors including those to mediate colonization and those that mediate infection.

1.2.2 Shiga Toxins

One of the main virulence factors associated with EHEC pathogenesis is the Shiga-like toxin. The first documentation of the cytotoxic effect of the Shiga toxin used African green monkey kidney epithelial cells, or Vero cells, in 1977.[15] This effect was confirmed in 1983 when a clinical EHEC O157:H7 infection resulted in HUS.[16] Further studies revealed that EHEC can contain one or two Shiga-like toxins (Stx1 and Stx2).[17]

The Shiga toxins of EHEC are like those found in *Shigella dysenteriae*. Stx1 shows a 98% sequence homology to *S. dysenteriae* and Stx2 has approximately 55% amino acid identity to Stx1.[18] Most Stxs are encoded by lambda-like bacteriophages, and transcribed from a promoter of late lambda phage lysis genes , which in turn links the expression of the toxin to the lytic

function allowing for the release of the toxin.[64]

Stxs consist of two subunits, a toxic moiety (A) and a binding receptor (B). Between Stx1 and Stx2 there is only a one amino acid difference that occurs in Stx2 on subunit A, other than this small difference in amino acid sequences the two toxins share the same cellular receptor (Gb3), and both utilize the same mechanism in vitro.[64]

During initial infection, the secreted toxins B subunit binds to the Gb3 (glycosphingolipid globotriosylceramide) receptors on the surface of the cells lining the intestinal walls.[20] Once internalized by the host endosome, the A subunit is cleaved from the toxin, allowing for the cleavage of adenine from the hosts rRNA, thereby arresting protein synthesis.[64] Stx has also been shown to cause apoptosis in epithelial cells and renal cells. There is data that suggests that the Gb3 receptor is not the only receptor of Shiga toxins.[21,22] The Shiga toxins target the Gb3 receptors on podocytes, and other tubular epithelial kidney cells, thus causing kidney damage which can lead to HUS in ~1-5% of the known EHEC infections.[18] Stxs have also been shown to trigger monocytes to produce pro-inflammatory cytokines through an unknown receptor.[64]

The production of Stxs distinguishes EHEC from similar pathogens like EPEC and leads to more serious infections within the human host due to the abundance of Gb3 receptors compared to host reservoirs like cattle. Stx1 is less potent than Stx2 in mice and humans, as demonstrated by a lethal dose 400- fold greater than that of Stx2.[19]

1.2.3 Fimbriae and Non-Fimbrial Adhesins

Fimbriae are thread-like structures protruding from the basal body of the bacteria and provide multiple functions including mediating host attachment.[64] EHEC itself contains 16 fimbrial loci, yet little is known about their contributions to virulence.[64] EHEC O157 contains a 16 bp deletion within the *fimA* region causing a loss of type 1 fimbriae production found in other pathogenic *E. coli* strains.[64] EHEC also contains a host of non-fimbrial adhesins responsible for mediating the host-pathogen interaction associated with binding to the intestinal epithelial cells (40). The main non-fimbrial adhesins associated with EHEC are intimin, a LEE-encoded protein that binds to translocated *tir*.

1.2.4 Flagella

Although all aforementioned virulence factors are implicated in the establishment of an EHEC infection the specific focus in our study is flagella expression and bacterial motility.

EHEC flagella are used in motility within the host GI tract. Typically, flagella can be expressed in different forms: monotrichous (single flagella), or lophotrichous (multiple flagella). This expression can be polar (expressed at one pole), amphitrichous (expressed at both poles), laterally, or peritrichous (expressed all over). *E. coli* have peritrichous expression of one flagellar system with the formation of a 'ponytail' when swimming occurs.[23]

1.2.4.1 Regulation of Flagella

Flagella are complicated structures made of multiple proteins, with more than 40 genes involved in its assembly and function.[40,100] Motility gene expression in EHEC is mainly regulated by the flagella master regulator FlhDC. The *flhDC* operon is the Class 1 transcription unit within the system. FlhDC expression and activity is regulated on multiple levels allowing for the tight regulation of flagella expression depending on nutritional, environmental, and growth-phase signals.[40] Therefore, FlhDC is necessary for the activation of transcription of all regulatory and structural components of the flagellar machinery.[40]

FlhDC directly activates Class 2 promoters which are transcribed by RNA polymerase that contains σ^{70}.[40] Activation of the RNA polymerase depends on contact between FlhDC and the carboxy terminal domain of the α-subunit, however the exact mechanism by which this occurs has not yet been elucidated.[101] There are seven major FlhDC-dependant operons; *flgAMN*, *flgBCDEFGHIJ*, *flhBAE*, *fliAZY*, *fliE*, *fliFGHIJK*, and *fliLMNOPQR,* which each encode different structural components and regulatory factors necessary for flagellar assembly and function.[102] The genes *fliA* and *flgM* encode an alternate σ factor and the cognate anti-σ factor respectively, that are implicated in the regulation of early-stage flagella gene expression and late-stage flagellar gene expression.[103] When FliA and FlgM are in the cytoplasm, the two bind which in turn prevents interaction with RNA polymerase leading to a repression of FliA-

dependant transcription.[103] Yet once the basal body of the flagella is assembled and the secretion apparatus, FlgM is exported allowing for FliA-dependant transcription of Class 3 flagellar genes to initiate.[103] FliA is capable of driving transcription of Class 3 operons including one specific to this study *fliC*. The operons encode flagellar products required in the late-stage of flagella assembly including the flagella subunit (flagellin).[103]

1.2.4.2 Structure of Flagella

The structure of a flagellum in *E. coli* consists of a long-capped filament that is attached to a hook via Hook-associated proteins (HAPs) FlgK and FlgL.[23] The length of the flagella filament averages between 5 – 10 μm long, and this changes based on growth conditions, and stressors placed on the bacteria.[24] The hook of the flagellum is attached to the basal body located within the cell wall[23] (Figure 1). The basal body is made up of an L-ring located in the outer membrane, a P-ring within the peptidoglycan layer, an MS ring within the inner membrane, and a C-ring found in the cytoplasm.[25] The rod holding the hook passes through the P and L rings to connect to the MS-ring and a secretin.[23] The secretin is part of the flagellar T3SS found in the C and MS rings, with the C portion serving as an attachment site for substrates of secretion.[23] The MS-ring is composed of FliF, and connects via the C-ring to the motor/stator complex.[26]

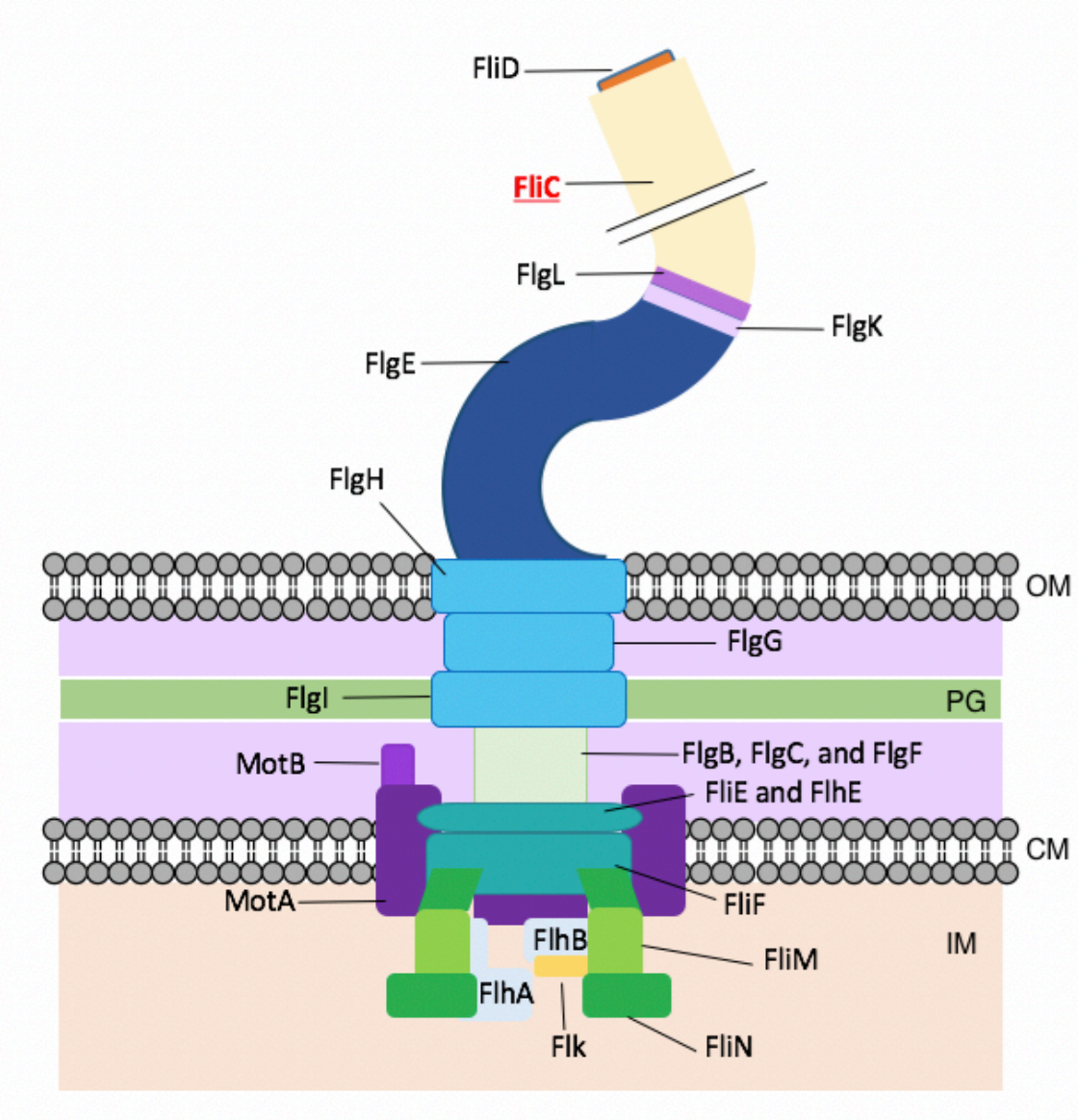

Figure 1: A bacterial flagellum with the basal body anchored to the inner membrane (IM).

The basal body functions as both an anchor and motor, with the hook (FlgE) functioning as a joint, and the filament (FliC) acting as the propeller. CM, cytoplasmic membrane, PG, peptidoglycan, OM, outer membrane.[25]

1.2.4.3 Flagellin

Flagellin is the major part of the flagella filament. In *E. coli,* the flagellin protein is also called FliC.[27] The flagellin structure is similar to an elaborate hair pin, in which the flagellins fold back onto themselves with the terminal ends being associated with one another.[28] The terminal ends contain ridged helical D0 and D1 domains which are within the flagella filament.[28] They are essential for polymerisation, therefore making them necessary for motility.[28,29] The central region of the flagellin contains D2 and D3 structural domains that form the surface portion of the flagella filament (Figure 2).[27,28, 66]

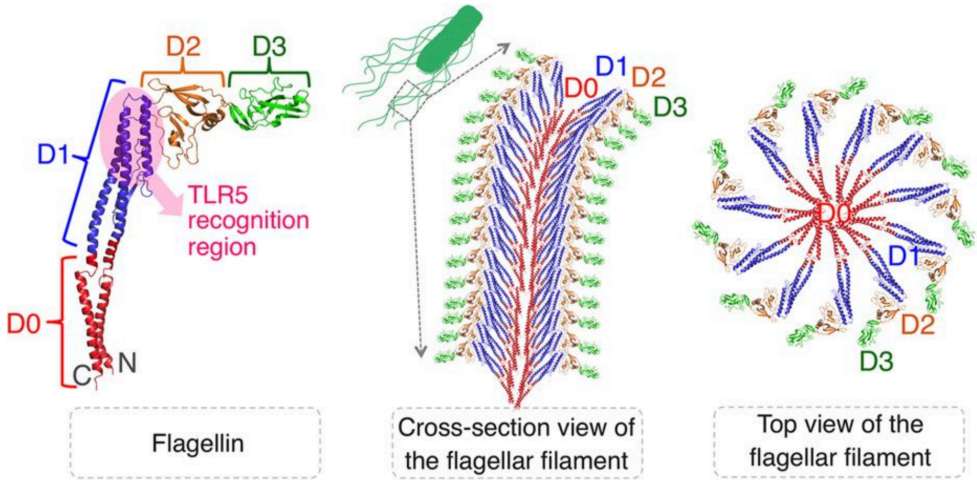

Figure 2: Flagellin assembled into the flagellar filament.

The flagellar filament is composed of repeating FliC subunits (Left). The terminal ends contain D0 and D1 domains for polymerization of subunits into the final whip portion of the appendage (Middle and Right).[66]

1.2.4.4 Flagella Assembly

The regulation of flagella is hierarchical. There are three sets of co-ordinately regulated genes that are expressed in different phases.[34] The master regulator, *flhDC*, is the first gene expressed. The *flhDC* promoter uses environmental stimuli, such as nutrient availabilities, oxidative stress, small molecules, and osmolarity to control *flhDC* expression via sensing by two-component systems.[35] *flhDC* expression is tightly regulated due to the high overall energetic cost of the assembly and use of the flagella systems.[37] Flagella expression can be coordinately regulated

with other surface factors. For example, *E. coli* flagella are inversely regulated with the T3SS,[38] and type-1 fimbriae.[39]

The expression of *flhDC* is necessary to produce flagella because it drives the transcription of genes at the mid-phase.[35] The mid-phase genes encode specific proteins for the hook and basal body, HAPs, the associated T3S chaperones, flagellar sigma factor (σ28), the anti-sigma factor, a regulator of hook length and FliK.[35] These proteins aid in the regulation of late-phase genes, and associated protein excretion.[35] Genes that are under late-phase, controlled by the mid-phase genes, encode the filament, T3S chaperones, HAPs, motor, and chemotaxis proteins.[35] HAPs, FlgK, FlgL and FliD are secreted in this order, but first overall, as they are regulated by mid-phase.[40] There are also specific chaperones for FlgK, FlgL, FliD, and FliC, that aid in the regulation of protein secretion in the late-phase.[40] FliD drives the formation of filaments by increasing the efficiency of polymerization of the FliC subunit.[41] The FliD allows flagellin to refold to make contact with adjacent FliC units.[41] The result is a step-by step twisting of FliD at the end of a filament as it grows subunit by subunit.[41]

1.2.4.5 Swimming Motility

The rotation of the flagella, like that of a propeller, is used to push the *E. coli* through liquid in a process termed swimming motility. There are other types of motility; gliding motility, that does not use flagella, and swarming motility that does use flagella but occurs on solid surfaces.[30] The rigidity of the flagella filaments and their left-handed helical structures are important factors in developing the forward swimming motion in *E. coli*.[31] The flagella filaments must be resistant to shearing and this is accomplished by forming the flexible filaments, and this flexibility allows the peritrichous flagella to bundle on the 'rear' of the moving bacteria.[32] This allows the *E. coli* to swim in a straight line, often called 'running'.

E. coli use chemotaxis, the process of cells moving along a chemical gradient, to orient itself within the environment, in doing so it has the ability to move more than just bi-directionally. To do so, *E. coli* alters the conformation of FliG, which changes the direction of the rotation of the flagella.[33] This change causes the flagella to spread from the bundle 'ponytail' structure, in turn twisting the *E. coli* allowing it to swim away in a new straight line.[33] Chemotaxis plays a role in the motility of *E. coli* and EHEC specifically. The phosphorylation of the chemotaxis protein

CheY, which binds to FliM, can control the direction of the *E. coli* swimming by causing the aforementioned downstream effects of FliG.[32]

1.3 Environmental cues stimulating EHEC virulence

1.3.1 Environmental cues EHEC encounters within the human host

Before reaching the site of colonization in the distal colon, EHEC must first pass through the host GI-tract to reach that site. As a result, EHEC must survive all the different microenvironmental conditions found within the human host. After ingestion, EHEC must first survive passage through the stomach where it encounters low pH environments. To combat these environments EHEC has critical acid resistance systems to protect against this environement.[91] After passing through the stomach EHEC enters the small intestine where pH levels increase but a host of other environmental factors are present such as: bile, microbiota metabolites, host hormones, and decreasing oxygen conditions.[91] After passage through the ileum (O_2 ~32mmHg), EHEC encounters the colon where it encounters very low oxygen conditions (O_2 ~0.5mmHg), host hormones and higher levels of microbiota metabolites, as microbiota levels are higher in the colon compared to the ileum.[91,95]

1.3.2 Chemical gradients that stimulate chemotaxis in EHEC within the GI Tract

There are many chemical gradients within the GI tract. In this study, however a focus is placed on the decreasing oxygen concentration gradient through the GI tract,[42] the increasing SCFA concentration gradient,[26,43] and the bicarbonate levels.

The oxygen concentration gradient decreases in relative oxygen concentration from the proximal to the distal GI tract and increases from the intestinal lumen towards the epithelial cells.[42,46] The SCFA concentration increases from the small to large intestine, with relative concentrations ranging from 20 to 40mM in the small intestine and 70 to 200mM in the large intestine.[43,44] We have previously shown that small intestinal like-mixes of SCFA upregulate motility and FliC expression in EHEC O157:H7.[43] The bicarbonate gradient is thought to decrease from the small intestine to the large intestine. Bicarbonate is quickly released in the small intestine during passage from the stomach to the small intestine as the low stomach pH needs to be neutralized to

a pH 6 to continue passage through the GI tract so to not cause damage.[45] There has also been evidence to show that bicarbonate is used by the host to neutralize by-products of short-chain fatty acid production.[88] Another bicarbonate gradient exists along mucosal barrier in which the host epithelial cells utilize $NaHCO_3$ to protect against the lower pH of the gastrointestinal tract by secreting sodium bicarbonate into the mucous.[96] Bicarbonate has been reported to activate RcsB which in turn negatively regulates biofilm formation, while also coordinating LEE activation with a repression of *flhDC* the master regulator of flagella expression.[62] There are also bicarbonate gradients associated with the intestinal epithelial cells with increasing concentrations towards the epithelial cells.[45,46]

1.3.3 Media components in bacterial culture

Growth media used to culture samples in laboratory studies are not simple mixes. They contain components such as inorganic salts, amino acids, vitamins, glucose, pH indicators, and buffer systems.[47] Previous studies have reported that media composition can influence virulence factor expression.[14,87,91] Comparisons between growth in minimal media and LB can contribute to differential results for EHEC virulence factor expression. For example, in two studies comparing SCFA stress on EHEC flagella expression similar results were found, indicating upregulation of FliC under low [SCFA] or small intestinal SCFA mixes and decreasing under high [SCFA] or large intestinal mixes but the methods differed in what media was used and what method of culturing was used.[36,43] The similar results in the two studies could be caused by the virulence factors being activated by different media components signalling a similar response within the bacteria. For example, LEE-encoded genes were expressed at low levels in a LB culture but at higher levels when cultured in DMEM with bicarbonate.[47] Although few studies have been done on direct comparisons between rich and minimal media on EHEC virulence factor expression, we propose that the media components will alter the expression of the virulence factors based on the culture conditions and media used in study.

1.3.4 Short-Chain Fatty Acids (SCFAs)

Short-chain fatty acids (SCFA) are saturated aliphatic organic acids with carbon chains less than six carbons long.[47] SCFA are produced as a by-product of the fermentation process of dietary fibre by the commensal gut microbiota.[47] The SCFA found in the human GI tract tend to be a mixture

of acetate, butyrate, and propionate with the individual mixtures varying by person based on, genetics, their diet, or the microbial composition within the GI tract.[49]

1.3.4.1 Production of SCFAs

The structure of SCFAs is similar to a carboxylic acid with an aliphatic tail less than six carbons. SCFAs are found in the liver through host metabolic pathways but the major site of production is within the colon through the fermentation of dietary fibre by commensal gut microbiota. SCFAs are shown to be absent in the colon in germ-free mice.[67] There are three main SCFA generated by the fermentation process, they are acetate (C2), propionate (C3), and butyrate (C4) (Figure 3). These SCFAs are produced in varying concentrations throughout the gastrointestinal tract with typically lower levels being found in the small intestine (20-40mM) and higher concentrations found in the large intestine (160-200mM).[68] Acetate is found at a higher ratio compared to propionate and butyrate in a 10:1 ratio, although this ratio varies depending on the host microbiota, diet, and genotype.[69] SCFAs are produced by the host's commensal gut microbiota via fermentation of dietary fibre, but there are a set of complex enzymatic pathways associated with different bacterial species. The most common pathway is the glycolytic pathway utilized by most commensal bacteria, yet some Bifidobacteria are able to utilize the pentose phosphate pathway.[70] Different bacterial species are able to produce specific SCFAs. For example, Firmicutes are acetate producers. Bacteroidetes are the major producers of butyrate.[70]

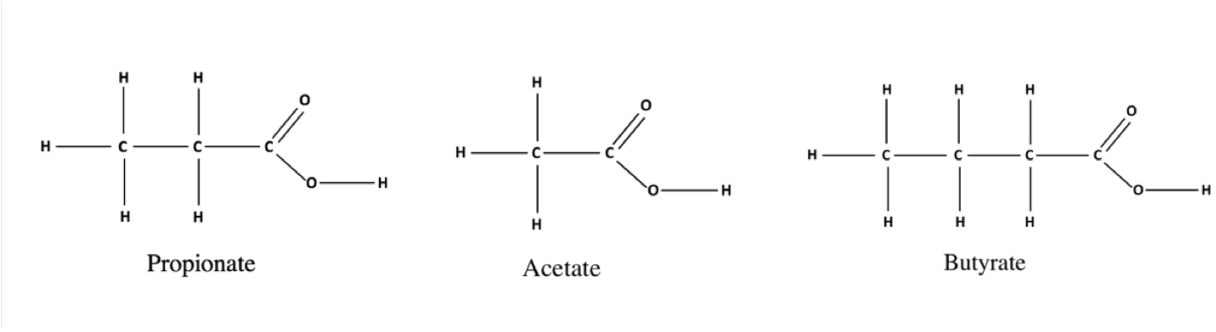

Figure 3: The structures of three main SCFAs generated by human host commensal microbiota

The interaction between the commensal gut microbiota also drives the production and degradation of SCFAs within the system causing fluctuations depending on the host's microbiome. For example, butyrate and propionate may be degraded into the smaller two carbon chain acetate by sulfate- or nitrate-reducing acetogenic bacteria such as *Acetobacterium, Acetogenium, Eubacterium*, and C*lostridium* species.[71] These interactions can also involve the production of SCFAs together as demonstrated by *Bacteroides thetaiotaomicron* and *Eubacterium rectale* where the acetate produced by *B. thetaiotaomicron* acts as a precursor for the production of butyrate by *E. rectale*.[72]

The expression of protein transporters in the microbiota has also been shown to link to the availability of SCFA.[73] The ATP- binding cassette (ABC) transporters in *Bifidobacterium longum* are required for the uptake and transport of fructose, required for acetate production.[74] Another transporter, phosphotransferase system (PTS), is able to transport carbohydrates which can be metabolized to produce SCFAs.[75] Therefore, the complex interactions within the microbiota may have some control over the levels of SCFAs in the GI tract.

For EHEC to be able to survive all the environments discussed, it has to orient itself within the host. It does so by sensing the environment utilizing a two-component signal transduction system. After the bacteria is able to sense the environment, it can upregulate or down regulate production of genes necessary for survival and passage to the site of colonization.

1.4 Bacterial Two- Component Signal Transduction Systems

A two-component regulatory system (TCS) is a signal transduction system utilized by the bacteria to sense external stimuli and react accordingly through regulatory pathways and adapt to the environments.[48] TCSs are very important to organisms that are constantly exposed to differing environmental surroundings such as pathogenic bacteria. Infection of host tissue by pathogenic bacteria involves being exposed to a range of different microenvironments, therefore researching the functions of these TCSs and how they respond to the host's microenvironments will allow for the better understanding of the bacteria. Although there are other types of regulatory proteins and systems within a bacterium, the focus of this project is within the TCS.[65]

A typical TCS is composed of two proteins, the membrane bound histidine kinase (HK) and the cytoplasmic response regulator (RR). The HK is a transmembrane receptor that provides sensory function and amino acid sequences that encode ATP binding domains responsible for the autophosphorylation of the histidine residue.[49] The phosphorylated form of the HK then donates the phosphoryl group to an aspartate residue found on the cytoplasmic RR generating the response. Hybrid kinases can also exist where the his- and asp- domains are utilized in the phosphotransfer, both found on the HK, where the phosphotransfer from HK to RR activates the RR and generates the response signal. Conserved amino acid domains found across multiple bacterial species are also found in the RR. The RR also contain non-conserved domains; species specific sequences, that are utilized as an effector to provide specific outputs.[50]

An unorthodox type of TCS exists which involves a multi-component phosphorelay system.[50] Upon activation of the unorthodox TCS, a hybrid kinase is activated and donates a phosphoryl group to a receiver Asp residue on the hybrid kinase. After transfer to the C-terminus -asp residue on the hybrid kinase, the phosphoryl group is transferred to the His- residue of the His-containing phosphotransferase protein (HPt). This group is then shuttled to the Asp- binding domain on the RR. It is also noted that the HPt do not possess any phosphatase or kinase activities like some HK. Therefore, it is thought to be an intermediary step to allow for tight regulation of the downstream effects of these TCS.[66]

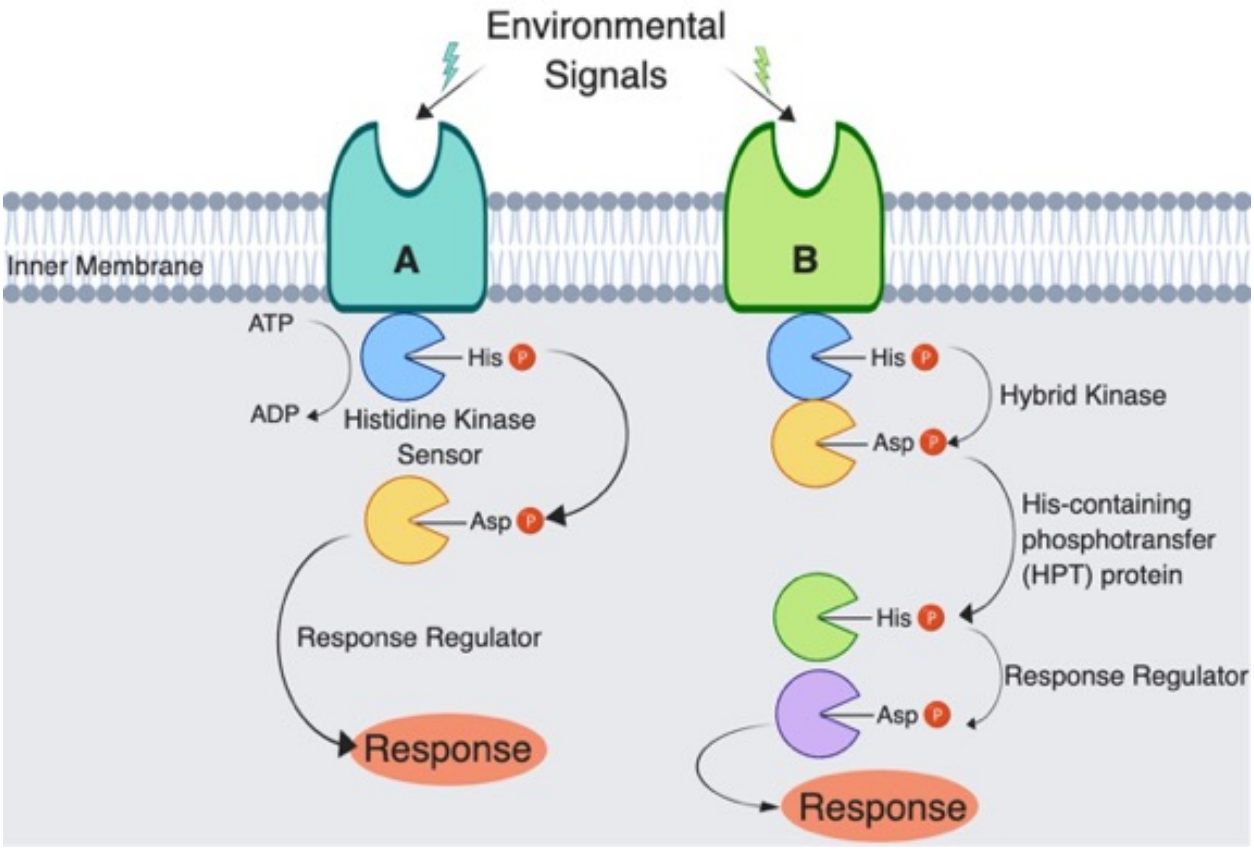

Figure 4: Bacterial Two-component signal transduction systems (TCS) bound to the inner membrane (IM)

A typical (A) and unorthodox (B) TCS found in bacterial species depicting the phosphoryl transfer from the IM bound HK to the cytoplasmic RR.

Escherichia coli contains 25 typical HKs and 5 unorthodox ones, as well as 32 RRs.[51] EHEC O157:H7 also contains an additional fucose sensing TCS.[53] There are multiple TCS in *E. coli* associated with flagella expression with some being positive regulators and others being negative regulators.[52] In previous work in our laboratory, we confirmed that *E. coli* K12 demonstrated the same SCFA-induced flagella expression and motility phenotype as that of WT EHEC.[44] This permitted us to take advantage of a set of readily available TCS mutants in K12 to evaluate the roles of these TCS in the SCFA-induced flagellar phenotype. In that study, a set of 7 TCS that had been previously reported to affect flagellar expression[44] were selected and evaluated against WT *E. coli* K12 for flagella expression/motility after treatment with either 30mM SCFA or 172mM SCFA. Results were assessed by comparing the mutant responses to WT where WT exhibits a motility phenotype of upregulation motility under the small intestinal SCFA mix (30mM SCFA) compared to large intestinal SCFA mix (172mM) (Fig 5).[44]

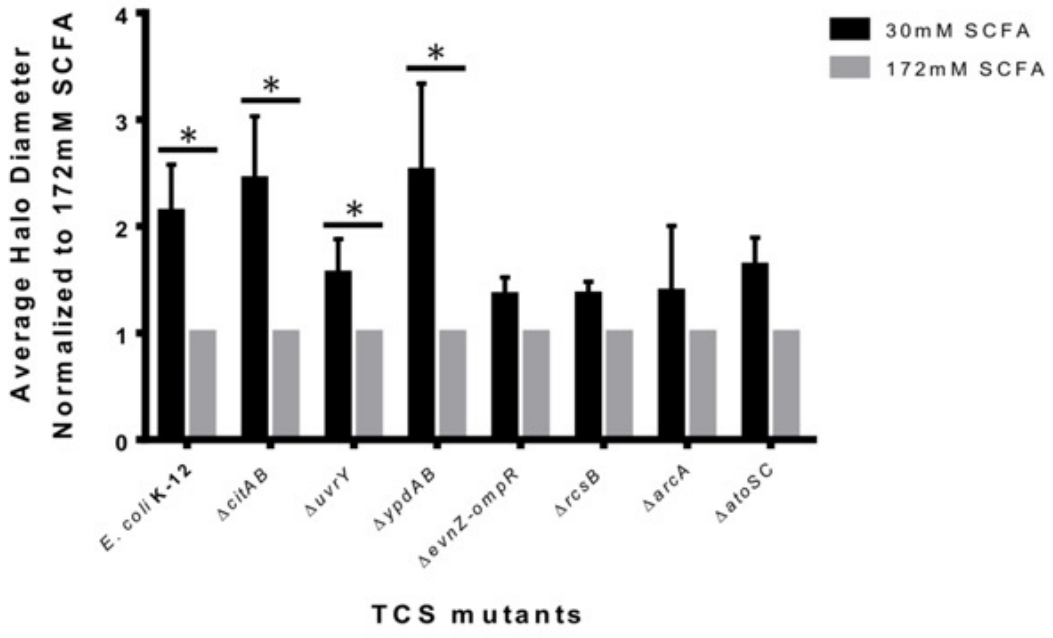

Figure 5: Motility as measured by halo diameter of *E. coli* K-12 WT and TCS mutants in response to each of 30mM and 172mM SCFA mix treatments.
Average Halo diameters are normalized to the 172mM SCFA treatment. * = significant differences in motility between the two SCFA mix treatments in LB media, for that specific TCS.[44]

The results from Kim (2016) show the distinctive motility phenotype for WT K12 where there is significantly increased motility under 30mM SCFA relative to 172mM SCFA (results are normalized to 172mM SCFA). Of the seven TCS studied, the SCFA-induced motility phenotype was lost in 4 TCS mutants (*envZ-ompR, rcsB, arcA, atoSC*) and there was an abrogated motility phenotype for *uvrY* mutant. These results were confirmed with western blot for flagellar expression.[44] Based on this study, we were now particularly interested in examining the SCFA-induced flagellar phenotypes of the RR of three of these mutants, *rcsB, arcA* and *uvrY* in EHEC for the following reasons.

1.4.1 RcsC (HK) / RcsB (RR)

RcsBC is an unorthodox phosphorelay system involving the protein RcsD as the HPt that transfers the phosphoryl group from RcsC to RcsB.[53] Upon sensing of stimulus, thought to be through an accessory protein RcsF, the hybrid kinase RcsC undergoes an ATP dependant His-Asp- autophosphorylation event.[109] The phosphoryl group is then transferred to a His- domain on RcsD where the phosphoryl group is then transmitted to the Asp- residue on RcsB generating the response.[109] This TCS regulates expression of *osmC* an osmoregulated gene, *ftsAZ* cell division genes, *cps* genes responsible for capsular polysaccharide synthesis, and the RNA gene *rprA* that leads to the modulation of genes involved in response to the starvation stress.[54] A study previously showed that Δ*rcsB* upregulates expression of numerous flagellar genes, showing that it is a negative flagellar regulator.[55] Another study showed that RcsBC senses sodium bicarbonate, resulting in the negative regulation of flagellar proteins.[47] Since we are interested in understanding the impact of this TCS in response to SCFA combined with bicarbonate in minimal media, we selected this mutant in EHEC for this study.

Figure 6: Summary of the regulation of *fliC* by the RcsBC TCS

RcsC directly activates through phosphorylation events the RcsD Hpt leading to activation of the RcsB RR then negatively regulates the flagella master regulator *flhDC* via binding to the RcsAB box on the FlhDC promoter.[99] FlhDC being the master regulator controls the class-2 flagella gene *fliA* (a RNA polymerase sigma factor that controls expression of flagella-related genes).[12] As a class-2 flagella gene *fliA,* has direct positive control over FliC expression leading to polymerization of the FliC subunit.[41]

1.4.2 ArcB (HK) / ArcA (RR)

ArcAB is a well characterized, classical TCS that modulates expression of genes in response to oxygen deficiency.[56] ArcB is a tripartite sensor kinase that undergoes a phosphorelay event when in anaerobic conditions.[108] The ArcAB TCS has primarily been implicated in anaerobic growth it has also been shown to be involved in chromosomal replication, stress response, the aging of bacteria, and resistance to H_2O_2.[107] The ArcAB TCS modulates motility through the repression of the sRNA (small RNA) *arcZ. arcZ* binds to *flhDC* leading to a downregulation of flagellar genes.[98,12] In previous studies, a loss of flagellar gene expression was reported in the Δ*arcA* mutant, suggesting that ArcA is a positive regulator of flagella expression.[55] Another study showed that in Δ*arcA* mutant, FliA expression is inhibited, thereby negatively affecting motility.[56] Given our interest in understanding the impact of SCFAs in conjunction with varying oxygen levels relevant to the small and large intestine on EHEC flagella expression, this TCS is of interest to us.

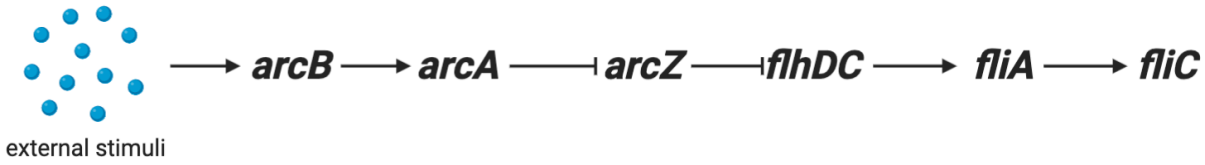

Figure 7: Summary of the regulation of *fliC* by the ArcAB TCS

ArcB directly activates *arcA* expression leading to repression of sRNA *arcZ.*[98]*acrZ* then negatively regulates the flagella master regulator *flhDC* which controls the class-2 flagella gene *fliA* (a RNA polymerase sigma factor that controls expression of flagella-related genes).[12] As a class-2 flagella gene *fliA,* has direct positive control over FliC expression leading to polymerization of the FliC subunit.[41]

1.4.3 BarA (HK) / UvrY (RR)

BarA/UvrY is a classical TCS but it contains a hybrid BarA and UvrY where the genes are not located on the same operon.[57] BarA is a tripartite sensor that undergoes a His- Asp- His- transfer before phosphorylating the UvrY RR.[59,108] This system protects against hydrogen peroxide mediated stress via RpoS, which synthesizes *E. coli's* major catalase.[57] This TCS has also been shown to sense SCFAs including acetate, propionate, and valerate and it is able to do so using UvrY alone.[58] The BarA/UvrY TCS has also been implicated in the expression of carbon storage regulation system which is required for bacterial long-term survival.[108] Disruption of *uvrY* has been reported to increase numerous flagellar genes.[55] That study concluded that *uvrY* is a negative regulator of flagella through CsrB.[55] It was proposed that UvrY phosphorylation induces CsrB production, which in turn inhibits CsrA which is required for switching between glycolytic and gluconeogenic carbon sources.[59] CsrA has been shown to directly regulate *flhDC* expression which is required for flagella production.[60] Since BarA/UvrY has been shown to be involved in direct SCFA sensing, this makes this TCS in EHEC of particular interest to us.[10]

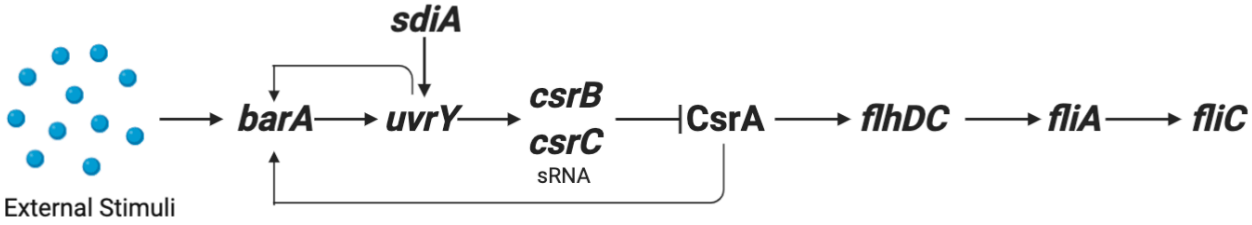

Figure 8: Summary of the regulation of *fliC* by the BarA/UvrY TCS

BarA and UvrY can be activated alone, but an autoregulation loop of UvrY activation of *barA* exists.[99] UvrY then directly activates *csrB* and *csrC* transcription which bind to CsrA and sequesters its function.[99] CsrA post-transcriptionally activates *flhDC* which activates the class-2 flagella gene *fliA* (a RNA polymerase sigma factor that controls expression of flagella-related genes).[12,99] As a class-2 flagella gene *fliA,* has direct positive control over FliC expression leading to polymerization of the FliC subunit.[41] *sdiA* encodes a LuxR-like protein that binds DNA and is involved in bacterial quorum sensing and cell division genes.[104]

2.0 Rationale

Short chain fatty acid mixes that are representative of the small and large human intestines have been shown to modulate flagella expression in *E. coli* and enterohemorrhagic *E. coli.*[43,44] In *E. coli*, three two-component regulatory systems ArcAB, RcsBC, and BarA/UvrY have been reported to play a role in modulating flagella expression and motility in response to these short chain fatty acid mixes.[44] However, no study has examined the role of these TCSs in regulating expression of flagella in enterohemorrhagic *E.coli* in response to these short chain fatty acid mixes. There is also no published evidence of how flagella expression may also be modulated by these TCSs in response to differential conditions found within the human GI tract including: oxygen concentration, nutrient availability, and metabolites present.

2.1 Hypotheses and Objectives

Overall Hypothesis: Short chain fatty acids serve as an environmental cue to modulate EHEC flagellar expression and motility, with small intestinal like mixes and large intestinal like mixes having contrasting expression profiles. The SCFAs are sensed by several TCSs such that different TCSs are responding to combinations of SCFAs in concert with different environmental conditions representative of the different microenvironments within the host GI tract.

Hypothesis 1: Flagella expression in EHEC is modulated by microenvironmental cues within the host intestinal tract including SCFAs, oxygen levels, bicarbonate concentrations and media composition.

Objective 1:

Assess flagella expression in WT EHEC in response to 30mM SCFA mix in combination with varying levels of oxygen, bicarbonate and media composition. Experimental design must ensure that only one variable is being tested at one time. In each case, appropriate controls including osmolarity controls for SCFA (30mM NaCl), media controls are included.

Hypothesis 2: In K12, ArcAB positively regulates flagella expression under low oxygen and anaerobic conditions and plays a role in short chain fatty acid induction of flagella expression.

EHEC is expected to show a similar phenotype to K-12. Therefore, there will be a significant difference in flagella expression between the Δ*arcA* isogenic mutant and wild-type EHEC when they are introduced to combinations of conditions representative of intestinal microenvironments. A complemented strain should restore WT phenotype and a strain that overexpresses *arcA* should enhance the phenotype seen in WT.

Objective 2:

2.1. Create isogenic mutant of *arcA* in EHEC 86-24 using allelic exchange protocol.
2.2. Create an *arcA* complemented strain in a low copy plasmid in the mutant background and an overexpressing strain using a pBAD inducible promoter expressed inside WT EHEC.
2.3. Compare FliC expression, induced by SCFA, in wild type EHEC, the isogenic mutant, complemented strain, and the over-expressing strain under different oxygen conditions.
2.4. Compare FliC expression, induced by SCFA, in wild type EHEC, the isogenic mutant, complemented strain, and the over-expressing strain, under different nutritional conditions.
2.5 Compare swimming motility in the presence of differing SCFA concentrations between wild type EHEC, the isogenic mutant, complemented strain, and the over-expressing strain

Hypothesis 3: In K-12, RcsBC negatively regulate flagella expression under starvation like conditions. EHEC is expected to show a similar phenotype to K-12. Therefore, there will be a significant difference in flagella expression between the Δ*rcsB* isogenic mutant and wild-type EHEC when they are introduced to combinations of conditions representative of intestinal microenvironments. A complemented strain should restore WT phenotype and a strain that overexpresses *rcsB* should enhance the phenotype seen in WT.

Objective 3:

3.1. Create isogenic mutant of *rcsB* in EHEC 86-24 using allelic exchange protocol.
3.2. Create a *rcsB* complemented strain in a low copy plasmid in the mutant background and an overexpressing strain using a pBAD inducible promoter expressed inside WT EHEC.
3.3. Compare FliC expression, induced by SCFA, in wild type EHEC, the isogenic mutant, complemented strain, and the over-expressing strain under different oxygen conditions.

3.4. Compare FliC expression, induced by SCFA, in wild type EHEC, the isogenic mutant, complemented strain, and the over-expressing strain under different nutritional conditions.
3.5. Compare FliC expression, induced by SCFA, in wild type EHEC, the isogenic mutant, complemented strain, and the over-expressing strain under different bicarbonate levels.
3.6 Compare swimming motility in the presence of differing SCFA concentrations between wild type EHEC, the isogenic mutant, complemented strain, and the over-expressing strain

Hypothesis 4: In K-12, BarA/UvrY negatively regulate flagella expression through CsrB under SCFA stress. EHEC is expected to show a similar phenotype to K-12. Therefore, there will be a significant difference in flagella expression between the Δ*uvrY* isogenic mutant and wild-type EHEC when they are introduced to combinations of conditions representative of intestinal microenvironments. A complemented strain should restore WT phenotype and a strain that overexpresses *uvrY* should enhance the phenotype seen in WT.

Objective 4:
4.1. Create isogenic mutant of *uvrY* in EHEC 86-24 using allelic exchange protocol.
4.2. Create an *uvrY* complemented strain in a low copy plasmid in the mutant background and an overexpressing strain using a pBAD inducible promoter expressed inside WT EHEC.
4.3.3. Compare FliC expression, induced by SCFA, in wild type EHEC, the isogenic mutant, complemented strain, and the over-expressing strain under different oxygen conditions.
4.4. Compare FliC expression, induced by SCFA, in wild type EHEC, the isogenic mutant, complemented strain, and the over-expressing strain under different nutritional conditions.
4.5 Compare swimming motility in the presence of differing SCFA concentrations between wild type EHEC, the isogenic mutant, complemented strain, and the over-expressing strain

3.0 Materials and Methods

3.1 Bacterial Culture

All strains used in this study were maintained in bacterial glycerol stocks stored in -80°C. When required the strains were streaked out onto LB agar (Bacto™ Tryptone) with the appropriate antibiotics (Table 1) prior to use to isolate single colonies. Single colonies were then inoculated into LB Broth containing the appropriate antibiotics and were incubated in 37°C shaking for 12 – 16 hours.

3.2 Construction of *rcsB, arcA,* and *uvrY* mutants utilizing the allelic exchange protocol

To construct all mutants, primers were designed based on the EHEC isolate EDL933 since it has 98-99% sequence homology to all genes of interest. This is the strain with the highest homology to EHEC 86-24 that has been sequenced. Utilizing Primer3 software, and the EDL933 genome, a forward and reverse primer were created 500bp upstream and downstream, P1 and P4 respectively and a PCR was run to amplify the gene of interest from an already constructed RR mutant in E.*coli* K-12. This PCR was column cleaned and T-tailed using Taq polymerase. The T-tailed samples were column cleaned and cloned into pCR2.1 – TOPO and transformed into DH5α cells. These newly transformed cells were plated on plates with the appropriate antibiotic and X-gal to perform a blue and white screen. White clones were selected and a mini-prep was done to allow for digestion by EcoRI then analyzed by agarose gel electrophoresis. If an insert was present, they were sent for sanger sequencing (The Centre for Applied Genomics (TCAG), Toronto). After sequence confirmation using Benchling alignment software, subcloning into pRE112-Gm was performed with a control of vector only control ligation. The transformants were then plated onto the appropriate antibiotic plates. The clones were then digested for the appropriate insert. Following cloning into the suicide vector pRE112-Gm biparental mating and counter selection is performed. The clones of the recipient strains are selected and confirmed using primers 1 and 4 and sanger sequencing.

3.3 Construction of *rcsB, arcA*, and *uvrY* overexpression and complemented EHEC strains in pBAD-Gr and pWSK129

Utilizing benchling primer designing software, and the EDL933 genome, forward and reverse primers were designed with overlaps at the start and end of each gene(Table 2). An EcoRI cut-site was designed in the forward primer with a palindrome sequence at the 5' of the primer. An HindIII cutsite was designed in the reverse primer with a palindrome sequence at the 5' of the primer. gDNA (genomic DNA) from WT EHEC O157:H7 was amplified for the genes of interest using the aforementioned primers. The PCR products were column purified and ligated at a 3:1 insert: plasmid concentration, into a pre-double digested pBAD-Gr or pWSK129 plasmid. An outgrowth stage at 16℃ for 12-16 hours followed the ligation. The ligated plasmid was then transformed into chemically competent DH5α. The clones are then digested for the appropriate insert with verification by DNA gel. After confirmation a mini-prep of the plasmid was completed and the final plasmid was electroporated into WT EHEC generating the final overexpression strain. The pWSK129 plasmid was transformed into the EHEC TCS mutant backgrounds to generate the final complemented strains.

Table 1: List of Strains and plasmids

Strains	Description	Reference
EHEC 86-24	*stx1, stx2;* serotype O157:H7 isolate from patient	62
EHEC 86-24 Δ*arcA*	In frame *arcA* mutant disrupted with Km^R cassette	This study
EHEC 86-24 Δ*rcsB*	In frame *rcsB* mutant disrupted with Km^R cassette	This study
EHEC 86-24 Δ*uvrY*	In frame *uvrY* mutant disrupted with Km^R cassette	This study
EHEC 86-24 pBADGr::*arcA*	Overexpression strain: WT 86-24 harbouring pBADGr with *arcA* from 86-24	This study
EHEC 86-24 pBADGr::*rcsB*	Overexpression strain: WT 86-24 harbouring pBADGr with *rcsB* from 86-24	This study
EHEC 86-24 pBADGr::*uvrY*	Overexpression strain: WT 86-24 harbouring pBADGr with *uvrY* from 86-24	This study
EHEC 86-24 Δ*arcA*:pWSK129::*arcA*	EHEC 86-24 Δ*arcA* mutant harbouring pWSK129 with complemented *arcA* from 86-24	This study
EHEC 86-24 Δ*rcsB*:pWSK129::*rcsB*	EHEC 86-24 Δ*rcsB* mutant harbouring pWSK129 with complemented *rcsB* from 86-24	This study
EHEC 86-24 Δ*uvrY*:pWSK129::*uvrY*	EHEC 86-24 Δ*uvrY* mutant harbouring pWSK129 with complemented *uvrY* from 86-24	This study
DH5α	General cloning and storage of plasmids, blue/white screening.	Invitrogen
S17-1λ*pir*	*E. coli* containing phage lambda lysogens	105
Plasmids		
pCR 2.1-TOPO	Contains Amp^R and Km^R and the *LacZ*α	Invitrogen
pRE112-Gm	Suicide vector for allelic exchange Gm^R	105
pWSK129	Low-copy plasmid for complementation containing Km^R and *LacZ*α	105
pBADGr	Arabinose-inducible complementation vector Gm^R	Lab Stock

Table 2: List of Primers

Primer	Sequence 5' → 3'	Reference
arcA_F_P1	aaagcgccgtttttttgacggtggtaaag	This study
acrA_R_P4	ggcaatttaggtagcaaacatgcagacccc	This study
rcsB_F_P1	gccatgctaaatctggtacccggcaagcag	This study
rcsB_R_P4	cgacgctgacgcgtcttatctggcctactt	This study
uvrY_F_P1	tttttaaaaacgcttttgcgtcaaactgat	This study
uvrY_R_P4	ttcctttgatcaacgttctacttgttgatg	This study
arcA_comp_F	catcatgaattcgccgattaatct	This study
arcA_comp_R	tactacaagcttagcaaacatgca	This study
rcsB_comp_F	catcatgaattcatgaacaatatg	This study
rcsB_comp_R	tactacaagcttttagtctttatc	This study
uvrY_comp_F	catcatgaattctcactgacttga	This study
uvrY_comp_R	tactacaagcttttgatcaacgtt	This study

3.4 SCFA treatment of EHEC

After overnight growth all EHEC strains OD's were set in subcultures based on the corresponding treatment. The subcultures were treated in either a 30mM SCFA treatment, or a 172mM SCFA treatment per Lackraj *et al*., 2016.[43,44] For the 30mM SCFA treatment, the overnight LB broth was resuspended in either low or high glucose DMEM (1g/L glucose and 4.5g/L glucose respectively, with L-glutamine, sodium pyruvate and sodium bicarbonate buffer) or LB broth all of which contain 100mM MOPS (morpholonepropanesulfonic acid, pH 6.7) as a pH buffer.. The OD_{600} of the sample was set to 0.05. The cultures were then stressed with 30mM SCFA mixture (25mM sodium acetate, 2.5mM sodium butyrate, and 2.5mM sodium propionate) and then incubated at 37 ℃ in shaking conditions, static conditions and statically with 5% CO_2 to an OD_{600} of approximately 1.0 in the late log phase. The 172mM SCFA mixture (95mM sodium acetate, 17mM sodium butyrate, and 60mM sodium propionate),[43] is added in a similar nature to the 30mM SCFA conditions. There is an extended lag phase in EHEC incubated in 172 mM SCFA, therefore the LB broth overnight was re-suspended in either low or high glucose DMEM (1 g/L glucose and 4.5 g/L glucose respectively) or LB broth, all containing 100mM MOPS (pH 6.7) to a starting OD_{600} of 0.2. The samples were incubated at 37 ℃ in shaking conditions, static conditions and statically with 5% CO_2 for 2 h before the addition of 172mM SCFA mixture. The samples were put back in incubation and grown to a late log phase (OD_{600} of 1.0). The samples were then spun down at 3500 rpm for 10 minutes and frozen in -80 ℃ freezer until the samples were prepared for western blot.

3.5 Immunoblot analysis of EHEC for FliC expression

To determine the expression of the FliC (H7) within the samples, immunoblot analysis was used per Lackraj *et al*., 2016[43,44]. Following the SCFA stress, bacterial cultures were centrifuged at 3500 rpm for 10 min at 4 ℃, washed twice with 1x PBS (137 mM NaCl, 10 mM Na_2HPO_4, 2.7 mM KCl, and 1.8 mM KH_2PO_4) and resuspended in a 1x SDS sample loading buffer [60 mM Tris/HCl (pH6.8), 10% glycerol, 2% SDS, 1.25% β-mercaptoethanol, and 0.01% bromophenol blue].[43] Samples were run on a 10% SDS-PAGE and transferred onto a Immobilon-P PVDF membrane [0.45 μm (EMD Millipore)]. The membranes were blocked at room temperature on a

shaking table for 1 h in a 1% tris buffered saline with Tween20 (TBST) and bovine serum albumin (BSA). The membranes were incubated overnight in 4°C with TBST + BSA and either anti-DnaK (1 : 10 000 dilution; Enzo Life Sciences) or anti-H7 (1 : 1 000 dilution, Denka-Seiken).[43] The primary antibodies were washed off using TBST followed by incubation at room temperature with a horseradish peroxidase-conjugated secondary antibody for 1 h. The membranes were visualized using enhanced chemiluminescent (ECL) detection and data was analyzed through Image Lab™Software (BioRad). The software was used to first correct for background noise. Next it was used to normalize band intensities between the anti-DnaK loading control and anti-H7. These normalized intensities were then used to generate a ratio of normalized intensity of H7:DnaK

3.6 Soft-agar Motility Assays

The impact of SCFA treatments on EHEC and the isogenic TCS mutant's motility was assessed by soft agar motility assay utilizing 0.25% Bacto™ Tryptone agar plates supplemented with SCFA mixtures or corresponding NaCl osmolarity controls. Following NaCl or SCFA treatment, 2μL of the subculture was spotted onto the corresponding SCFA or NaCl soft-agar plate. After incubation at 37°C for 12 h the diameters of the motility halos were measured.

3.7 Statistical Analysis

All the experiments were conducted with a minimum of three biological replicates. All error bars are presented with the standard errors of the means. Two-way ANOVA utilizing Tukey's multiple-comparison test was used to determine the differences between groups in immunoblot and motility analysis conducted with EHEC O157:H7 strain 86-24. The P value of <0.05 was considered to be significant for all tests.

4.0 Results

4.1 WT EHEC flagella expression is modulated by small intestinal SCFA mixes and media components

Flagella protein expression was analyzed through immunoblot to determine if there were differences in expression between typical laboratory culture conditions of EHEC. In LB media under shaking conditions, there was no difference between the 30mM SCFA mix and the NaCl control. In static conditions, expression of FliC was significantly upregulated compared to shaking conditions and the corresponding NaCl control. In static + 5% CO_2 conditions, FliC expression was significantly upregulated compared to the shaking condition and the corresponding NaCl control (Figure 9A)

In low glucose (LG) DMEM, considered to be more representative of the host intestinal environment, a different trend was observed. Under shaking conditions, FliC expression was significantly upregulated in the 30mM SCFA mixes compared to its NaCl control. This result was consistent across all oxygen conditions. There was also significant FliC expression in the 30mM SCFA shaking condition compared to static conditions regardless of supplementation with CO_2 (Figure 9B).

With the differences in expression trends across oxygen conditions from DMEM to LB further investigation into which media components caused this differential were explored. Supplementation of LB components into complete LG DMEM, showed that tryptone and NaCl had no effect on the FliC expression trend (data not shown). However, supplementation of yeast extract showed that overall expression trends of FliC in WT EHEC cultured with LG DMEM and yeast extract changed to resemble that of complete LB, with an overall increase of FliC expression across all oxygen conditions (Figure 9C).

When comparing across all three media types, the data suggests that DMEM, a more minimal media than LB and considered to mimick the starvation-like stress of the host's intestinal lumen, elicits higher FliC expression than complete LB media, both alone and when supplemented with

yeast extract. This is why the results of the TCS studies are mainly focused primarily on the DMEM culture conditions.

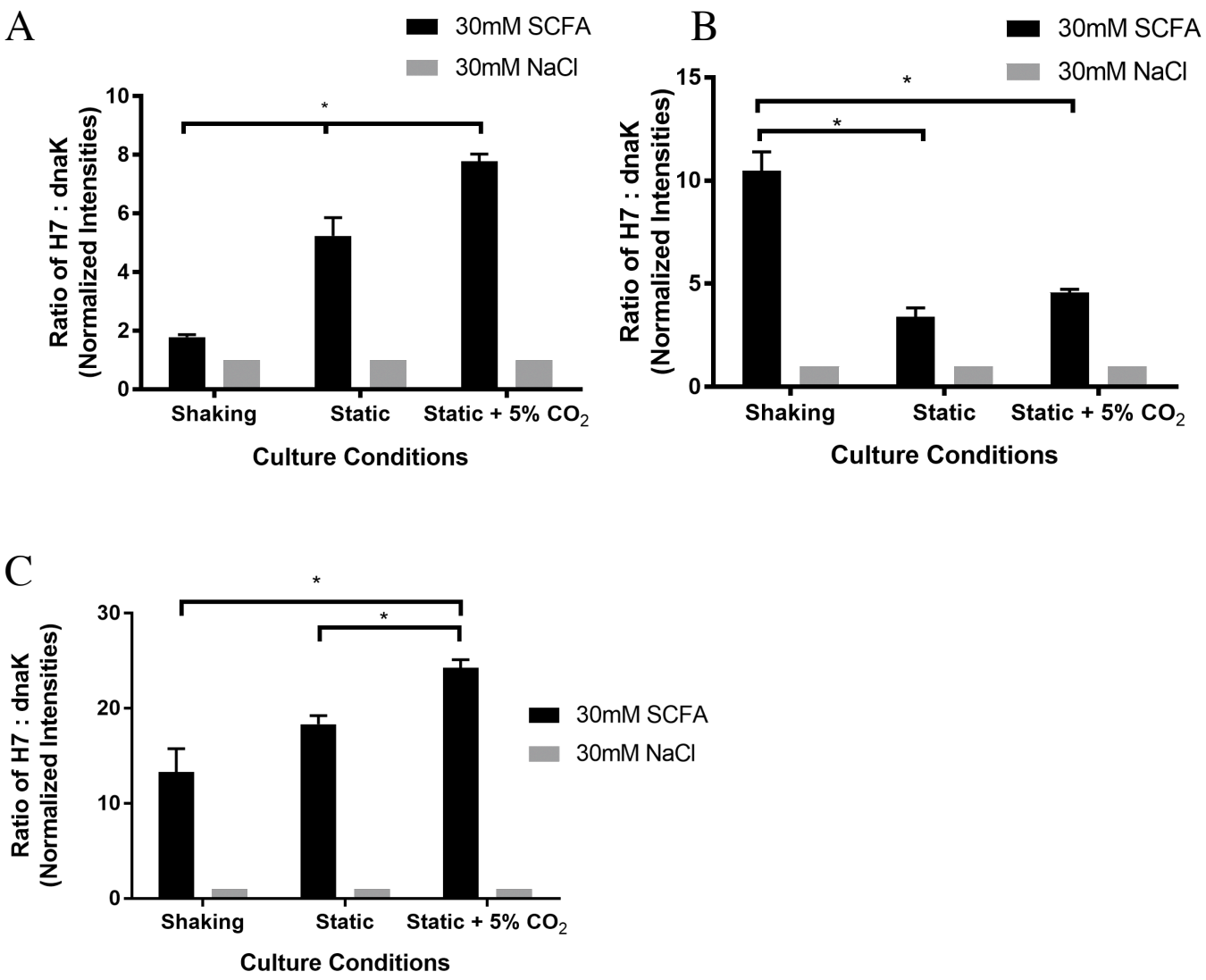

Figure 9: Immunoblot analysis of H7 FliC expression of EHEC O157:H7 in (A) LB (B) DMEM (C) DMEM supplemented with yeast extract treated with small intestinal SCFA mixes.

EHEC 86-24 was cultured in different medias with 30mM SCFA mixes or NaCl osmolarity controls for 6 hours at 37˚C in either shaking, static, or static +5% CO_2 conditions. The samples were prepared for immunoblot and visualized with rabbit poly-clonal flagella anti-H7 and donkey anti-rabbit HRP conjugated IgG. FliC expression was determined through the normalization to anti-dnaK loading controls. Error bars indicate the mean ± the standard error of the mean, *$p<0.01$

4.2 FliC regulation by ArcAB, RcsBC, and BarA/UvrY

The next three sections namely 4.2.1 – 4.2.3 focus on flagella expression and motility assay results for each of the RR. In each section, flagella expression using immunoblot assay with anti-FliC is compared across WT, isogenic RR mutant, complemented mutant and overexpression RR strain. Data are presented contrasting culture in 30mM SCFA mix versus 172mM SCFA mix in each of low glucose DMEM in static +5% CO_2 conditions (low O_2 DMEM) and low glucose DMEM shaking conditions (high O_2 DMEM). Data for results in LB static + 5% CO_2 and LB shaking culture conditions are provided in the supplementary figures (Appendix I). Growth curves of all strains were generated in every culture condition and the growth inhibition under 172 mM SCFA was accounted for in all experiments (Data not shown).

Motility assays are provided comparing culture in 30mM SCFA mixes versus 172mM SCFA mixes in low glucose DMEM static conditions only as these were considered to be the most physiologically relevant.

Each data set has been analysed by Two-way ANOVA with post-hoc tukey and all significant differences are presented in each figure. The text of the results in each section focuses on the patterns of significant differences that emerge from the data. In each case the results will focus examine the nature of regulation of the specific RR based on data comparisons of WT versus mutant versus complemented strain and overexpression strain for both flagella expression and motility.

4.2.1 FliC regulation by ArcAB TCS

Figure 10 evaluates the role of *arcA* in regulating FliC expression in response to SCFA mixes in DMEM under varying oxygen conditions. Results show that the deletion of *arcA* downregulates FliC expression under 30mM SCFA treatment compared to WT regardless of oxygen conditions. Complementation of *arcA* back into the mutant background restores the WT phenotype regardless of oxygen conditions. This indicates that the ArcAB TCS is modulating expression of FliC and flagella positively when the bacteria encounter small intestinal mixes of SCFA (Figure 10A-B).

Overexpression of *acrA* generated a similar phenotype to the WT and complemented mutant under 30mM SCFA mixes in DMEM high O_2 (Figure 10B). Interestingly, it generated even more pronounced FliC expression in 30mM SCFA mixes in DMEM low O_2 (Figure 10A). Suggesting that the overexpression of *arcA* is particularly sensitive to this set of conditions.

Flagella expression in LB showed similar trends compared to WT and the mutant strains (Figure S1). There was a loss of the SCFA-induced WT phenotype in Δ*arcA* under low oxygen conditions and microanaerobic conditions that was not seen in higher oxygen conditions (Figure S1).

Motility results were consistent with flagella expression results with upregulation under SCFA-induced WT phenotype being lost in Δ*arcA* (Figure 10C).

These results suggest that the ArcAB TCS is positively modulating flagella expression in response to small intestinal SCFA mixes regardless of oxygen conditions.

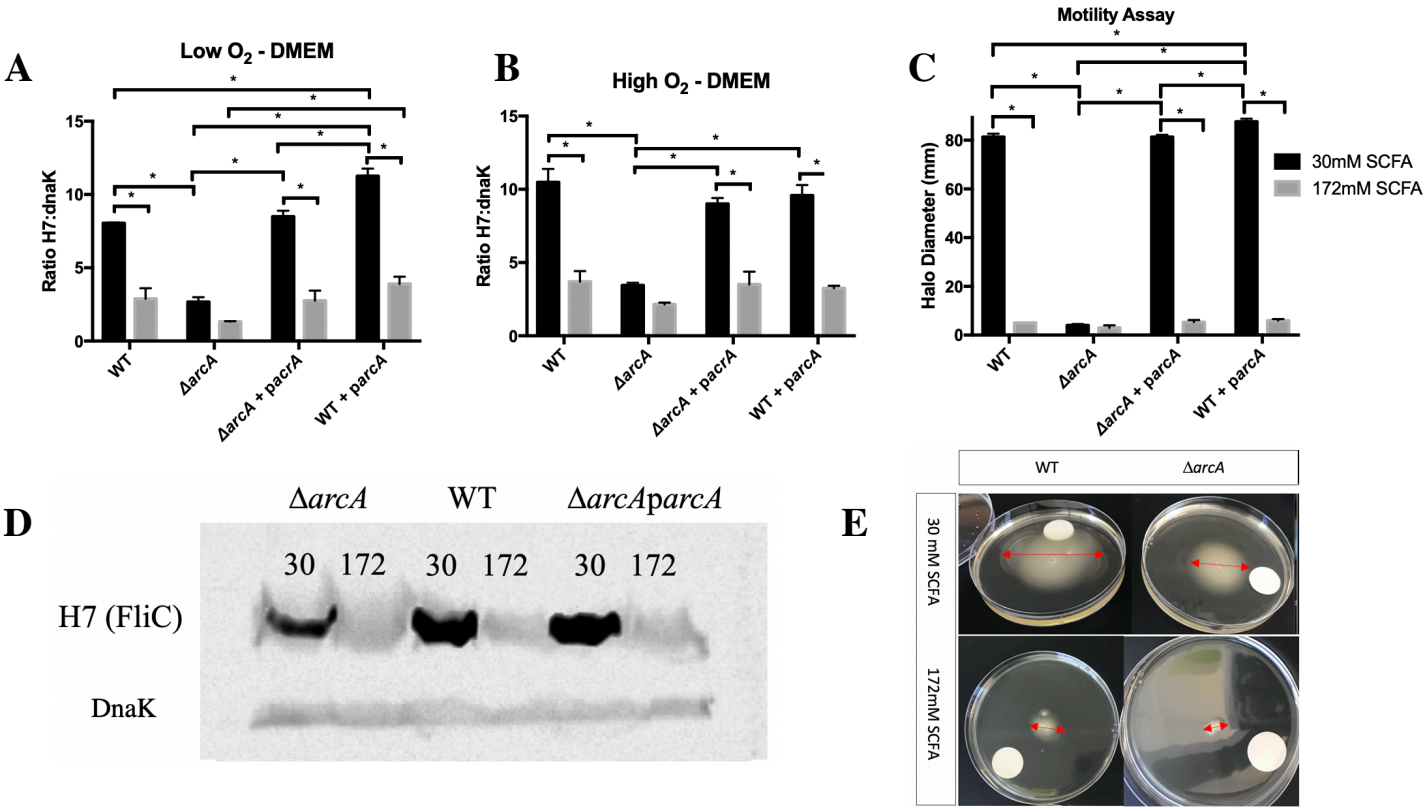

Figure 10A-E: *arcA* regulates FliC expression in response to differential oxygen conditions and SCFA levels representative of the small intestine

(A-B) EHEC 86-24, isogenic Δ*arcA*, Δ*arcA*+pWSK129:*arcA* and EHEC + pBAD:*arcA* were grown in minimal media for 6 hours at 37°C in either shaking (High O_2),or static + 5% CO_2 (low O_2). Subcultures were either treated with 30mM/172mM SCFA or NaCl osmolarity controls. FliC expression was assessed through immunoblot analysis using anti-H7. (C) Strains were subjected to SCFA or NaCl stress and grown to mid-log before being inoculated onto 0.25% agar containing the corresponding treatment. Plates were incubated for 12hrs and halo diameters were measured. (D) Representative western blot images cut to show anti-FliC (FliC 67 kDA) and anti-DnaK(DnaK 69 kDA) under 30mM and 172mM SCFA mixes. (E) Representative motility assay images under 30mM and 172mM SCFA mixes. Error bars indicate the mean ± the standard error of mean, *p< 0.05

4.2.2 FliC regulation by RcsBC TCS

Figure 11 evaluates the role of *rcsB* in regulating FliC expression in response to SCFA mixes in DMEM under varying oxygen conditions. Results show that the deletion of *rcsB* downregulates FliC expression under 30mM SCFA treatment compared to WT in low oxygen conditions. Complementation of *rcsB* back into the mutant background restores the WT phenotype regardless of oxygen conditions. This indicates that the *rcsB* behaves as a positive modulator of flagella expression specifically under small intestinal SCFA mixes and low oxygen conditions (Figure 11A-B).

However, when *rcsB* is overexpressed it appears to be acting as a negative regulator of flagella expression relative to WT under 30mM SCFA conditions regardless of the oxygen conditions of the environment (Figure 11A-B).

The response of the overexpressing strain appears to be dependent on the inclusion of sodium bicarbonate within the environmental conditions. This is apparent by the loss of repression when the overexpression strains were cultured in DMEM without sodium bicarbonate and the relative SCFA mixes (Figure 11D-E).

Regarding growth in LB, Δ*rcsB* FliC expression did not significantly differ from WT as seen in the DMEM results. However, when cultured in microaerophilic conditions Δ*rcsB* had lower FliC expression compared to WT and the complemented strain. Under all conditions overexpressed *rcsB* exhibited negative regulatory effects when cultured with SCFA mixes (Figure S1).

Motility results for the generated strains revealed that the flagella produced in the immunoblot assays are functional, with the motility results corroborating the FliC expression results. When deleted *rcsB* seems to depict positive regulation on flagella expression. Yet, when overexpressed *rcsB* plays a negative regulatory function with a decrease in motility in both strains compared to WT (Figure 11C).

The results indicate that the RcsBC TCS is positively modulating flagella expression under low oxygen and 30mM SCFA mixes, but negatively regulates flagella expression when in the presence of SCFA mixes and sodium bicarbonate regardless of oxygen conditions.

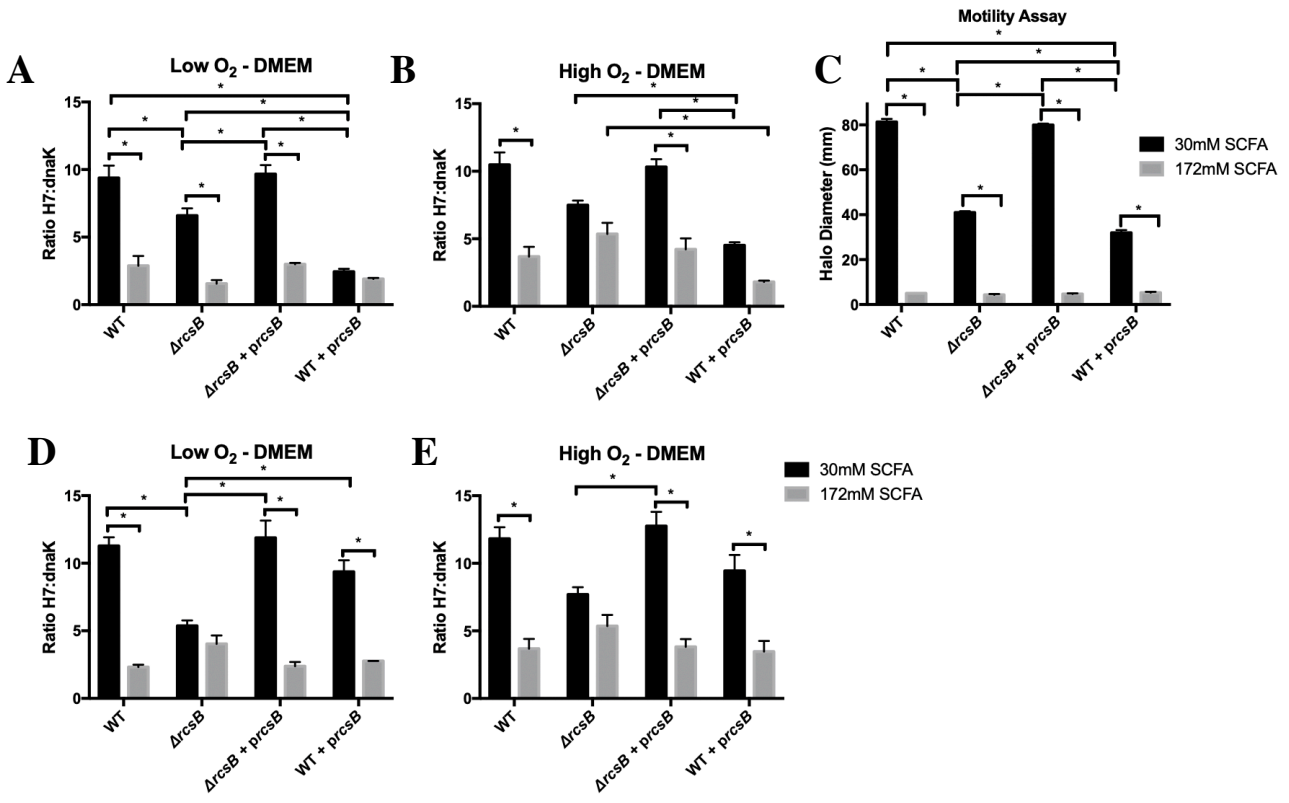

Figure 11 A-E: *rcsB* responds to SCFA levels representative of the small intestine and $NaHCO_3$

(A-B) EHEC 86-24, isogenic Δ*rcsB*, Δ*rcsB*+pWSK129:*rcsB* and EHEC + pBAD:*rcsB* were grown in DMEM for 6 hours at 37°C in either shaking (High O_2),or static + 5% CO_2 (low O_2). Subcultures were either treated with 30mM/172mM SCFA or NaCl osmolarity controls. FliC expression was assessed through immunoblot analysis using anti-H7. (C) Strains were subjected to SCFA or NaCl stress and grown to mid-log before being inoculated onto 0.25% agar containing the corresponding treatment. Plates were incubated for 12hrs and halo diameters were measured. (D-E) EHEC 86-24, isogenic Δ*rcsB*, Δ*rcsB*+pWSK129:*rcsB* and EHEC + pBAD:*rcsB* were grown in DMEM without sodium bicarbonate for 6 hours at 37°C in either shaking (High O_2),or static + 5% CO_2 (low O_2). Subcultures were either treated with 30mM/172mM SCFA or NaCl osmolarity controls. FliC expression was assessed through immunoblot analysis using anti-H7. Error bars indicate the mean ± the standard error of mean, *$p < 0.05$

4.2.3 FliC regulation by BarA/UvrY TCS

Figure 12 evaluates the role of *uvrY* in regulating FliC expression in response to SCFA mixes in DMEM under varying oxygen conditions. Results show that the deletion of *uvrY* downregulates FliC expression under 172mM SCFA treatment compared to WT in low oxygen conditions. Complementation of *uvrY* back into the mutant background restores the WT phenotype regardless of oxygen conditions (Figure 12A-B).

When overexpressed, *uvrY* downregulates flagella expression compared to WT in response to varying SCFA mixes in low oxygen. This suggests that the *uvrY* behaves as a negative regulator of flagella expression specifically under large intestinal SCFA mixes and low oxygen conditions (Figure 12A-B).

When comparing the results to the strains grown in LB, similar trends were seen to that in DMEM. There was significant upregulation in Δ*uvrY* under 172mM SCFA mixes compared to WT. In LB BarA/UvrY seems to downregulate flagella expression specifically under large intestinal SCFA mixes regardless of oxygen conditions (Figure S1).

The motility results for the *uvrY* strains revealed that the flagella phenotype results in functional flagella, with the motility results corroborating the FliC expression results. When deleted *uvrY* seems to negatively regulate flagella expression only in 172mM SCFA. When overexpressed *uvrY* downregulates flagella expression under both 30mM and 172mM SCFA mixes (Figure 12C).

The results indicate that the BarA/UvrY TCS is negatively regulating flagella expression under low oxygen and 172mM SCFA mixes regardless of media used. This data suggests that the BarA/UvrY TCS is more important in regulating flagella expression in microenvironments representative of the large intestine.

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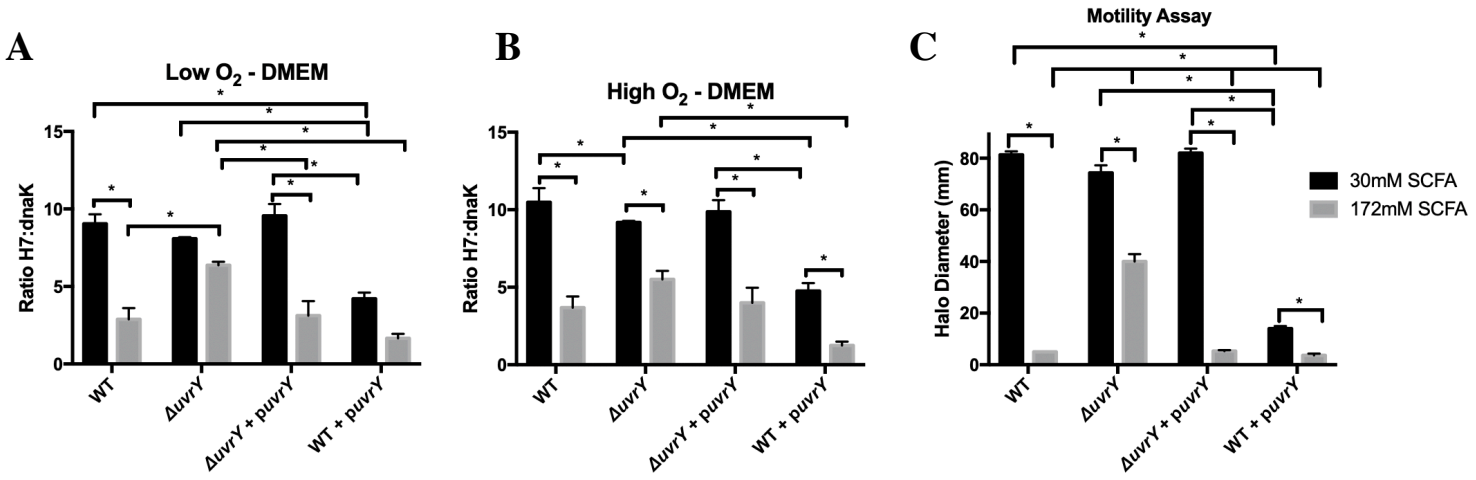

Figure 12A-C: *uvrY* responds to SCFA levels representative of the large intestine

(A-B) EHEC 86-24, isogenic Δ*uvrY*, Δ*uvrY*+pWSK129:*arcA* and EHEC + pBAD:*uvrY* were grown in minimal media for 6 hours at 37°C in either shaking (High O_2),or static + 5% CO_2 (low O_2). Subcultures were either treated with 30mM/172mM SCFA or NaCl osmolarity controls. FliC expression was assessed through immunoblot analysis using anti-H7. (C) Strains were subjected to SCFA or NaCl stress and grown to mid-log before being inoculated onto 0.25% agar containing the corresponding treatment. Plates were incubated for 12hrs and halo diameters were measured. Error bars indicate the mean ± the standard error of mean, *$p < 0.05$

5.0 Discussion

EHEC's ability to sense rapid changes within the surrounding environment relies on effective use of TCS.[86] Using these TCS, EHEC is able to tightly control expression of specific virulence factors leading to survival and passage to the site of colonization.[86] Studies of EHEC pathogenesis and survival lead to a deeper understanding of the bacteria and all of the necessary virulence factors required for survival and colonization. When *conducting in vitro* studies of bacteria less attention has been paid to the impact of specific culture conditions and how results from one study may differ from another based-on culture media alone. The standard laboratory culture media for bacterial growth is LB broth, a meticulously designed media to promote optimal growth with required growth factors, whereas, a media such as DMEM has been reported to better mimick the conditions of the human intestinal tract.[87] Previous studies that have examined EHEC LEE encoded virulence factor expression and found that when cultured in DMEM compared to LB, there were significant differences in expression of some factors.[88] Oxygen availability has also been shown to have an effect on virulence factor expression in EHEC.[36,89] The first goal of this study was the impact of oxygen levels and different media types, LB and DMEM on flagella expression. It is known that shaking bacterial cultures allows for more dissolved oxygen in the culture compared to static conditions.[90]

To explore the role of oxygen availability in different culture conditions on flagella expression, WT EHEC were cultured in small intestinal-like SCFA mixes, a previously known upregulator of flagella expression in WT EHEC [43,44,36] Results of Lackraj et al.,(2016) and Tobe et al., (2011)showed that although similar flagella expression results were found, the culturing conditions were very different between Lackraj (LB in static + 5% CO_2) and Tobe (DMEM in shaking). In the current study, we found that expression of flagella in WT EHEC was highest in LB cultures under static conditions and even higher expression in DMEM under shaking conditions (Figure 9A-B). This indicates that when EHEC is cultured in media more representative of the intestinal lumen[91] it elicits higher flagella expression than typical laboratory culture media like LB. This data also indicates that the oxygen availability of the culture conditions also plays a strong role in flagella expression and potentially other virulence factor expression. In LB media there was a relative decrease in FliC expression between shaking (high

oxygen) to static conditions with the opposite trend being found in DMEM. When the components of LB were individually supplemented into DMEM, the DMEM expression profile flipped to resemble that of complete LB media after DMEM supplementation with yeast extract. This is thought to be caused by the increase in B-vitamins and amino acids in the yeast extract available for EHEC to utilize in growth.[106] A study by Lewis et al., (2015) indicated that *E. coli* is able to utilize free amino acids in culture media as a primary nitrogen source.[92] This could also account for the overall increase in FliC expression that was found when WT EHEC was cultured in DMEM + yeast extract.

With this initial study in mind we wanted to further explore the regulation of flagella by TCS in differential host microenvironments. We chose to focus our next set of experiments on cultures in DMEM since it had been previously reported to more closely approximate the intestinal lumen.[91] Experiments conducted in LB will be provided in Appendix I. We also chose to compare shaking conditions (referred to as high oxygen conditions) and static + 5% CO_2 (referred to as low oxygen conditions) because we saw the greatest differential between these two sets of conditions in our initial WT results.

Previous work by Oshima et al. in *E. coli* K-12 showed that 7 TCSs are involved in flagella regulation.[55] K-12 strains Δ*rcsB* and Δ*uvrY* showed upregulation of flagellar gene expression under standard culture conditions (LB with shaking), suggesting that under these conditions, RcsB and UvrY function as negative regulators of flagella expression. Under the same conditions, Δ*arcA* showed decreased flagellar gene expression indicating ArcA is a positive regulator.[44] Jee In Kim (2016) then selected these TCSs in *E. coli* K-12 to evaluate their roles in regulating flagella expression in response to SCFA mixes representative of the small and large human intestine. In that study, she found that indeed, *uvrY* and *rcsB* are negative regulators but curiously, that *arcA* appeared to function as a negative regulator in contrast to the finding of Oshima et al, (2002).[44] Furthermore, Jee In demonstrated that these three TCS genes played a role in generating the SCFA-induced flagellar phenotype seen in WT K-12. It should be noted that culture conditions in this study were LB in static +5% CO_2 conditions which may explain different results from that of Oshima et al, (2002).

The study by Jee In Kim, (2016)[44] provided strong evidence of the roles of these three genes in the regulation of SCFA-flagellar phenotype in the non-pathogenic *E. coli* K-12 strain. However, we wanted to assess whether they also played a role in the SCFA-regulated flagellar expression in the pathogen, EHEC.

With regard to the TCS ArcAB, we hypothesized that there would be a significant difference in flagella expression between the Δ*arcA* isogenic mutant and wild-type EHEC when they are introduced to combinations of conditions representative of intestinal microenvironments. We found that in DMEM, *arcA* in EHEC regulates flagella expression positively in response to small intestinal SCFA mixes, a finding that is consistent with Oshima et al.[55] It should be noted that when comparing to the work of Oshima or Kim, there were differences in culture conditions. Both studies utilized LB broth and shaking as their means of culturing *E. coli* K-12 while the current study utilised DMEM in shaking and static conditions. What is also interesting is that in this study the response was more robust in higher oxygen environments than those in low oxygen environments. This finding is interesting as it is previously known that the ArcAB TCS is a global regulator under microaerophilic and anaerobic environments.[97] Yet when we look at the results in LB (supplemental data), we see that there is an increase in FliC expression in WT as oxygen levels decrease, and little to no change in the Δ*arcA* mutant, indicating that under standard laboratory culturing conditions, the ArcAB TCS is playing a role in the expression of flagella under microaerophilic conditions. In DMEM when we look at 30mM SCFA we see that disruption of *arcA* leads to significant differences from WT and the complemented strain under indicating positive regulation of flagella expression by the ArcAB TCS in small intestinal-like environments. Coupled with the fact that we do not see this differential in 172mM SCFA mixes we believe the ArcAB TCS may play a more important role in the regulation of flagella expression in the small intestine under a SCFA mix typical of that environment. When focusing on the motility phenotype of the strains, the trends follow that of the FliC protein expression results with a distinct loss of the SCFA-induced WT phenotype in the Δ*arcA*. Similarly Kato et al., (2014) showed that deletion of *arcA* and not *arcB* led to decreased FliC expression and a decrease in motility caused by the class-2 flagella gene *fliA* (a RNA polymerase sigma factor that controls expression of flagella-related genes) in LB and shaking conditons.[12] When further exploring this, Kato et al, (2007) determined that a cohort of class-2 flagella synthesis genes such

as *fliJ, fliLMN, flgA, and flgCEG* were significantly decreased in the Δ*arcA* mutant compared to WT,[81] indicating that the ArcAB TCS has positive control on flagella expression. However, no direct binding of *arcA* to any of these flagellar gene promoters has been characterized, suggesting that regulation by *arcA* of flagella expression is indirect. It is instead thought that there are specific cellular functions regulated by the ArcAB TCS, specifically *arcA* that may be necessary for the function of FlhDC. We propose that ArcAB is sensing SCFA and causing a downregulation of flagella expression potentially via the downregulation of class-2 flagella synthesis genes, although the mechanism by which this occurs has not yet been elucidated.[81] Kato et al, (2007) has proposed that ArcA may be regulating other cellular processes allowing for the cellular optimization for the expression of FlhDC.[81] This regulation does not appear to require ArcB indicating the potential for TCS 'cross-talk' where the RR may be regulated by other HKs. It should also be noted that deletion of *arcA* in *Citrobacter rodentium,* a murine surrogate, resulted in an attenuation of virulence within the mouse model and was attributed to a defect in T3SS and a lack of host epithelial cell binding.[82] This indicates that a loss of *arcA* may also be attenuating other aspects of virulence in EHEC and is another avenue for further exploration of this TCS as it responds to intestinal microenvironmental cues.

In summary, our results support the idea that *arcA* is playing an important role in positively modulating flagella expression in EHEC in response to cues representative of the small intestine including 30mM SCFA mixes as well as higher oxygen levels.

With regard to the RcsBC TCS, we hypothesized that there would be a significant difference in flagella expression between the Δ*rcsB* isogenic mutant and wild-type EHEC when they are introduced to combinations of conditions representative of intestinal microenvironments. It was found that in DMEM, disruption of *rcsB* resulted in significantly decreased flagella expression compared to WT under 30mM SCFA at all oxygen conditions and complementation restored the flagella expression phenotype. This is consistent with Kim, (2016) K-12 work and suggests that RcsBC is positively regulating flagella expression in response to a SCFA mix typical of the small intestine. What is interesting is that when overexpressed on pBAD, *rcsB* appears to be negatively regulating flagella expression in both low and high oxygen environments, indicating that when overexpressed, the RcsBC TCS has negative regulatory effects. These contrasting

results may suggest potential TCS cross-talk or underlying branched pathways that are implicated in the greater story of the RcsBC system. Tobe et al. (2005) proposed that the activation of the RcsBC system led to both positive and negative regulation of LEE genes in both LB and DMEM through the intermediate proteins GrvA and PchA, the former being a global regulator of virulence and the latter being necessary in LEE expression and biofilm formation.[83] This could be an explanation for our differing results between Δ*rcsB* and WT+p*rcsB* in both DMEM and LB media.

RcsBC has also been previously shown to sense $NaHCO_3$.[62] We found that when Δ*rcsB* is grown in media without $NaHCO_3$, there are no significant differences in flagella expression from 30mM to 172mM SCFA. When overexpressed in the absence of $NaHCO_3$, *rcsB* restores the phenotypes to WT levels. This data suggests that the RcsBC TCS when overexpressed, is sensing the metabolite $NaHCO_3$ and downregulating flagella expression. However, in the absence of $NaHCO_3$, RcsBC appears to serve as a positive regulator for flagella expression. Since the SCFA-induced WT phenotype is not lost in Δ*rcsB* in low oxygen it is thought that the RcsBC system is positively regulating SCFA-induced flagella expression in response to small intestinal SCFA levels as well as small intestinal metabolite $NaHCO_3$, but negatively regulating when overexpressed in these conditions. These results suggest that the RcsBC system is a more complex system with potentially several inputs and cross-talks. Since the RcsBC TCS has been implicated in colonic acid production in *E. coli* and capsular synthesis in other bacterial strains,[54] it is thought that as the bacterial cell surface changes in response to conditions flagella or other protruding bacterial fimbriae could be altered in response. This could also be causing the differences we see between WT, the isogenic mutant, the complemented strain and the overexpressing strain.

When grown in LB (supplemental data), the data suggest the interpretation that RcsBC system is negatively modulating flagella expression under high and low oxygen conditions but not under microanaerobic conditions. Interestingly we see a significant difference in 172mM SCFA treatment in low oxygen in overexpression strain compared to the *rcsB* mutant indicating that at low oxygen conditions, overexpression of *rcsB* may be positively modulating flagella expression in response to 30mM SCFAs. When fold changes are compared there is no loss of the WT

SCFA-induced phenotype in either the *rcsB* mutant or the overexpression strain, indicating in LB the RcsBC TCS may not be modulating expression of flagella in response to SCFAs. The results are consistent with dual role of *rcsB* as it responds to changing concentrations of bicarbonate in combination with SCFA throughout the intestinal tract.

With regard to BarA/UvrY TCS, we hypothesized that there would be a significant difference in flagella expression between the Δ*uvrY* isogenic mutant and wild-type EHEC when they are introduced to combinations of conditions representative of intestinal microenvironments. The most interesting results for this TCS are with respect to the 172mM SCFA mixes, where the data suggest that UvrY is sensing this molecular cue and negatively regulating flagella expression in response. This is consistent with previous reports that BarA/UvrY negatively regulates flagella and that it has been shown to respond to acetate.[57, 58] In our study, *uvrY* appears to respond to SCFA mixes typically found in the large intestine and downregulates flagella expression in response. These results are consistent with the idea that as the bacteria move towards the site of attachment, flagellar genes may be turned off as they are energetically costly and cause a heightened immune response by the host.[56] A different study by Patel et al. revealed a link between quorum sensing and the BarA/UvrY TCS, indicating that through *uvrY* activation there is significant expression of the regulatory gene *luxS* required for quorum sensing.[84] The study reported that deletion of *uvrY* led to the decrease of motility on 0.25% agar plates, yet the phenotype was restored upon complementation as well as plasmid expression of *luxS*. Therefore, the BarA/UvrY TCS may induce production of autoinducer-2 through luxS and that production indirectly regulates motility through an unknown TCS.[84] However, Patel et al. did not focus on the TCS's ability to sense SCFAs. Camacho et al. reported that a feedback loop exists between the BarA/UvrY TCS and the noncoding RNAs of the carbon storage regulation (Csr) system, CsrB and CsrC.[85] These small regulatory RNAs bind to the protein CsrA and prevent it from interacting with mRNA targets.[85] There has been a reported difference in CsrA expression between LB and DMEM. Therefore it is thought that since BarA and UvrY can be activated by SCFA separately the addition of this proposed CsrA feedback loop may be having expression effects which we see when we compare different nutrient availabilities.[85] In our study when *uvrY* is disrupted, we see that expression of flagella and motility is upregulated under 172mM SCFA conditions, indicating that the BarA/UvrY TCS is modulating expression of flagella by

downregulating flagella expression in environments more representative of the site of colonization in the large intestine.

Taken together, these results can be used to develop a potential model of flagella regulation by the three TCS throughout the small and large intestine in response to molecular cues including SCFA mixes, oxygen levels and bicarbonate concentrations (Figure 13). An overall model can also be developed using these results to show that the complex mixes of metabolites, oxygen levels, sodium bicarbonate levels studied in this thesis are modulating expression through the sensing of several TCSs. Based on the work in this thesis, the proposed model (Figure 14) indicates where in the intestinal tract the TCSs may be more active to enhance or repress flagella expression to aid in reaching the site of colonization. The significance of these findings provides critical insights into how EHEC modulates flagella expression and motility in response to differential host microenvironmental cues through sensing the cues with TCS. Hopefully these insights into the complex TCS can help inform strategies to prevent and treat EHEC infections leading to a decrease in the burden on the healthcare system.

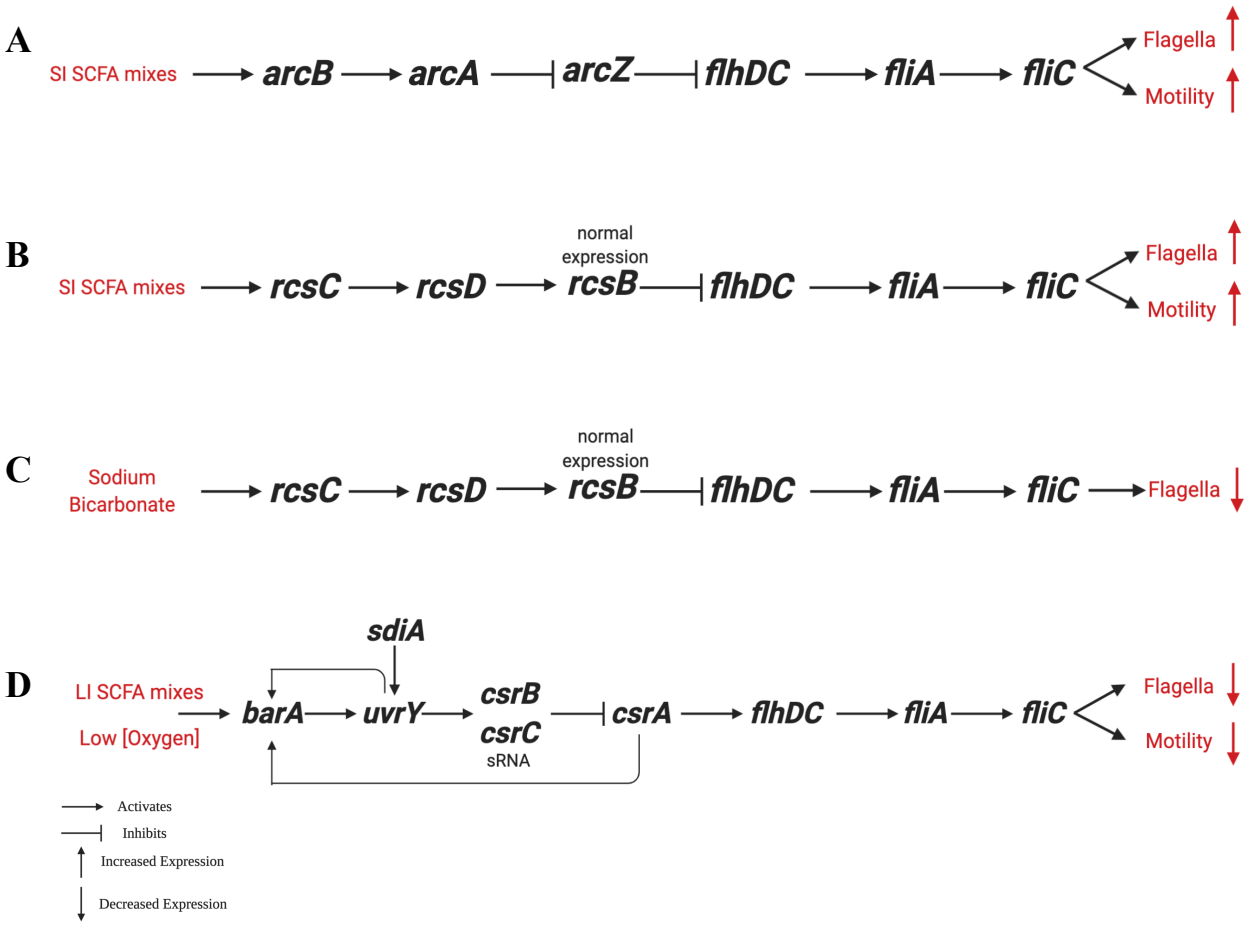

Figure 13: Proposed activation of TCSs by host microenvironmental conditions

(A) The ArcAB TCS positively modulates flagella expression in response to small intestinal (SI) SCFA mixes. (B) The RcsBC TCS positively modulates flagella expression under SI-SCFA mixes. (C) The RcsBC TCS negatively modulates flagella expression in the presence of sodium bicarbonate. (D) The BarA/UvrY TCS negatively modulates flagella expression in response to large intestinal (LI) SCFA mixes and low oxygen environments. Red indicates contributions from this study.

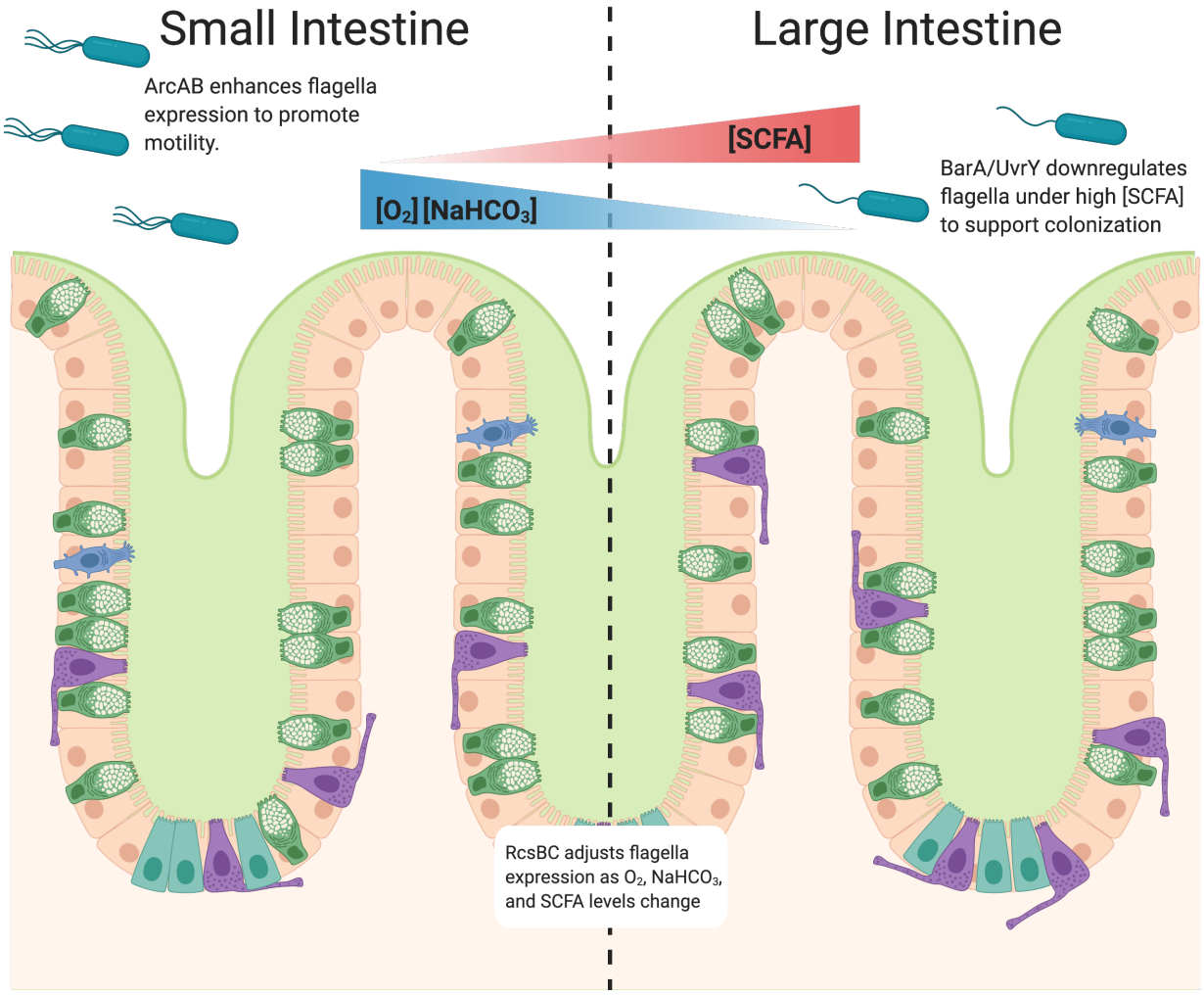

Figure 14: Proposed model of TCS modulation in different microenvironmental conditions found in the intestinal tract

EHEC 86-24 utilizes TCSs to modulate expression of flagella in response to changing concentrations of SCFAs, $NaHCO_3$, and O_2 found within specific microenvironments within the host GI tract. The ArcAB TCS seems to modulate expression of flagella in response to small intestinal conditions, BarA/UvrY seems to modulate expression of flagella in response to large intestinal conditions, and RcsBC seems to respond to conditions found in both the small and large intestine indicating a flagella 'adjustment'.

6.0 Future Work

Ways to further study TCS in EHEC in varying microenvironmental conditions would be to produce double and triple mutants of the TCS. This would be able to help us better understand the signals being sensed by these TCS. Right now, we know how they respond to SCFA mixes, oxygen, and media but there could be redundancies in EHECs flagella regulation that we are not seeing because the other TCS studied are still operating normally in our single response regulator mutants. An issue could arise based on the regulatory systems of each TCS, as some directly affect the master regulator, FlhDC while others play a role on Class 2 and Class 3 genes. A way to combat this would be to determine where the RR of each TCS binds on the flagellar operons and construct double and triple mutants with TCS that have similar binding sites. Another mutation to look at would be to create mutants of both the HK and the RR of the TCS to completely remove function of the selected TCS. Coupling this strategy with the double and triple mutant strategy, it would be especially beneficial when studying the RcsBC TCS as we have shown that it has more complex regulation in response to the studied microenvironmental conditions. However, an issue may arise in the double and triple mutants where the dominant phenotype (downregulatory) may overshadow any small changes in these TCS. A more comprehensive approach would to be incorporating all 32 TCS within one study to better understand any cross talks based on the microenvironments being tested.

Our study focused on how EHEC flagella expression was modulated by the SCFAs produced by the commensal gut microbiota. An interesting way to further study this would be to look into the typical commensals that produce SCFA and how EHEC responds in co-cultures. This would be beneficial because not only would you be studying the metabolites produced by the commensals, but you would also be looking at the commensal-pathogen interactions. A study done by Cameron et al.,(2018) showed that the commensal *Bacteroides* processes EHECs T3SS and enhance translocation into the host cell.[93] So it would be very interesting to know if expression of flagella is also mediated by commensal bacteria and not just their major metabolites. But if we do just focus on the metabolites, it would be beneficial to test ranges of SCFA mixes and

individual SCFAs on the constructed mutants to better understand what is driving the phenotypic changes as was done in the Kim (2016) study in *E. coli* K-12.[44]

Another way to further this study would be to use a method similar to the 'RoboGut'[94] and study the commensal-pathogen response in an environment that is more representative of the host GI-tract. If we could re-create the conditions of the host GI-tract, we could potentially get more representative results of what is happening during an EHEC infection and how the specific TCS are modulating expression of virulence factors, specifically flagella.

Appendix I

1.0 Supplementary Results

1.1 In LB, *arcA* responds to differential oxygen conditions and SCFA levels representative of the small intestine

In every LB condition tested WT EHEC continues to show the 'WT SCFA phenotype' with higher FliC expression in 30mM SCFA compared to 172 mM SCFA. Under 30mM SCFA in shaking (High O_2) conditions, we see that FliC expression in Δ*arcA* is significantly decreased compared to both WT and the pBAD overexpression strain. By contrast, there is no significant difference in FliC expression in the 172mM SCFA treatments.

Under static + 5% CO_2 (low O_2) conditions with 30mM SCFA we see that again FliC expression in Δ*arcA* significantly differs from both WT and the pBAD over expression strain. There is no significant difference in FliC expression between 172mM SCFA treated WT and Δ*arcA* in low O_2 conditions suggesting that when in nutrient rich environments, the ArcAB TCS is sensing low levels of SCFA to positively modulate flagella expression.

Interestingly when we decrease the oxygen levels to that of a microanaerobic environment using mineral oil overlay on a static culture, we see that the SCFA-induced WT phenotype is lost in Δ*arcA* and restored in pBAD + *arcA*. We also see a significant increase in FliC expression under 172mM SCFA in Δ*arcA* compared to WT and pBAD + *arcA* indicating that the ArcAB TCS is necessary for the SCFA-induced WT phenotype.

1.2 In LB, *rcsB* responds to SCFA levels representative of the small intestine

Under 30mM SCFA, for both oxygen conditions, FliC expression of Δ*rcsB* is unchanged compared to WT but is significantly increased compared to pBAD + *rcsB*, suggesting that *rcsB* negatively regulates flagella expression under these conditions. By contrast, there is no significant difference in FliC expression in the 172mM SCFA treatments.
There is no significant difference between 172mM SCFA treated WT and Δ*rcsB* in low O_2 conditions but a significant difference between Δ*rcsB* and pBAD + *rcsB*. These results indicate that in nutrient rich environments, overexpression of *rcsB* negatively regulates FliC expression in response to 30mM SCFA regardless of oxygen and most dramatically in response to 172mM SCFA combined with low oxygen – which is most relevant for the large intestinal lumen.

1.3 In LB, *uvrY* responds to SCFA levels representative of the small and large intestine

The data from experiments using LB essentially corroborate the data from minimal media. They reveal that overexpression of uvrY leads to a downregulation of flagella compared to the *uvrY* mutant regardless of SCFA or oxygen levels, indicating that *uvrY* is negatively regulating flagella expression under all of these conditions.

More importantly, these LB experiments are consistent with the minimal media results in that the disruption of the *uvrY* leads to a significant increase in FliC expression under 172mM SCFA regardless of oxygen levels, a phenotype which is dramatically reversed in the overexpression strain under the same conditions.

Therefore, the minimal media and LB data confirm that *uvrY* is likely responding to the 172mM SCFA regardless of oxygen levels (the response is preserved for shaking, static and static with mineral oil overlay) and is responsible for downregulating flagella in response to this cue which is relevant for the large intestine

The results from the response to 30mM SCFA is less consistent – in the case of LB, we see a significant decrease in FliC expression for the *uvrY* mutant relative to WT only for low oxygen conditions including the mineral oil overlay. The relevance of this finding is unclear.

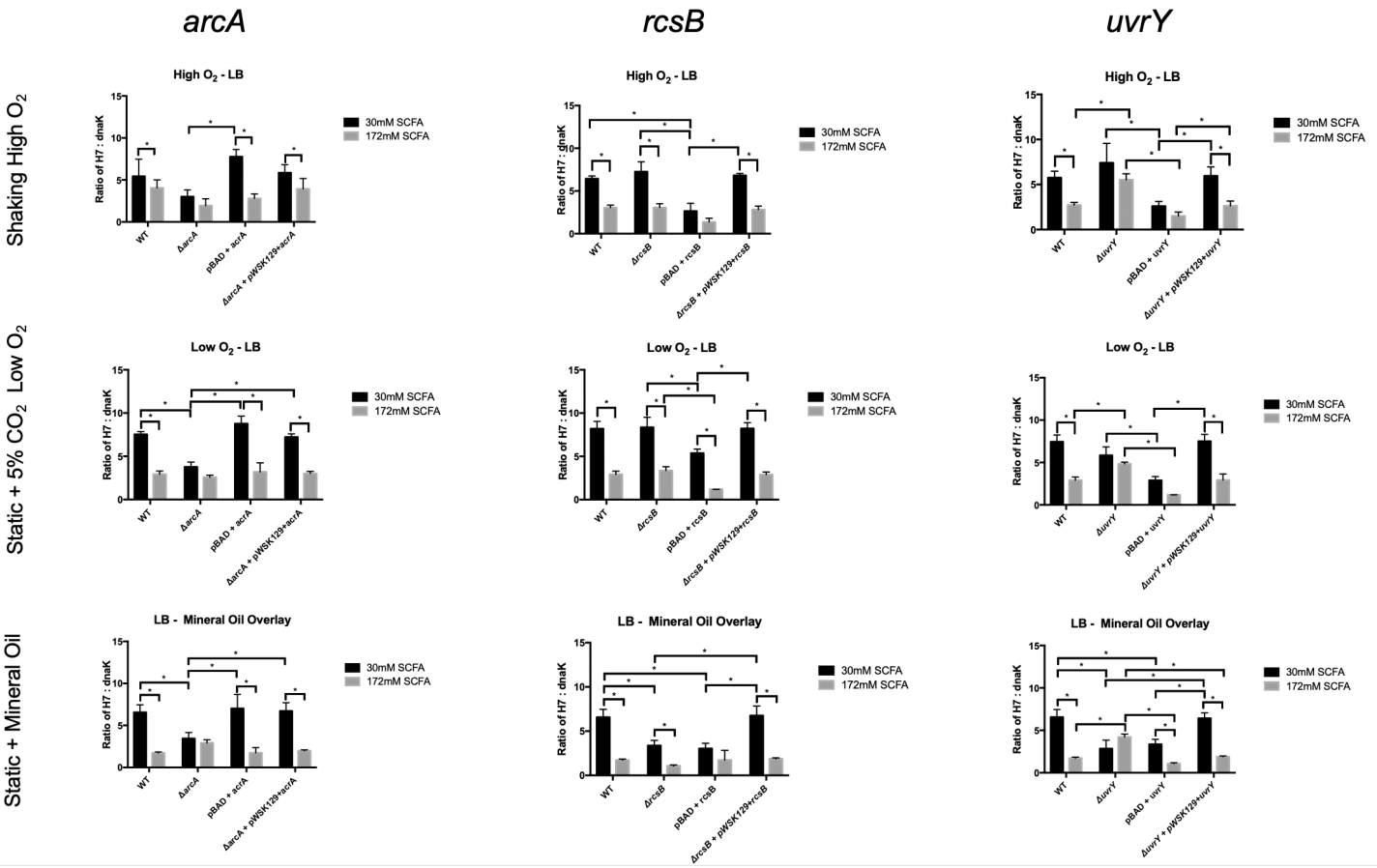

Figure S1: Flagellin expression in WT O157:H7:86-24, isogenic mutants, complemented, and overexpressing strains cultured with SCFA+ LB in high and low oxygen conditions and in microaerophilic conditions

EHEC 86-24, isogenic mutants, complemented strains and overexpressing strains were grown in LB for 6 hours at 37°C in either shaking (High O_2), static + 5% CO_2 (low O_2), or static + 5% CO_2 with a mineral oil overlay. Subcultures were either treated with 30mM/172mM SCFA or NaCl osmolarity controls. FliC expression was assessed through immunoblot analysis using anti-H7. (Error bars indicate the mean ± the standard error of mean, *p< 0.05

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