# INVESTIGATION OF THE REGULATION OF EXPRESSION OF *E. COLI* COMMON PILUS SUBUNIT, ECPA, OF ENTEROHAEMORRHAGIC *E. COLI* O157:H7 UNDER ACID STRESS

by

**Hannah Tollman**
Bachelor of Science, Winona State University, 2009

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**INVESTIGATION OF THE REGULATION OF EXPRESSION OF *E. COLI* COMMON PILUS SUBUNIT, ECPA, OF ENTEROHAEMORRHAGIC *E. COLI* O157:H7 UNDER ACID STRESS**

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## Abstract

*Escherichia coli* (EHEC) O157:H7 is a pathogenic bacterial species that is most commonly linked to severe diarrhea, but is also the leading cause of the potentially fatal hemolytic-uremic syndrome (HUS). In order to establish infection in the colon, EHEC must endure different stresses encountered in the gastrointestinal (GI) tract, such as acid stress in the stomach, bile salt stress in the small intestine, and short-chain fatty acid (SCFA) stress in the colon. These bacteria are likely able to use GI stresses as indicators of their location, impacting gene expression of adhesion, motility, and virulence factors. The *E. coli* Common Pilus (ECP) has been shown to be an important factor for EHEC adhesion to epithelial cells, which is increased after either acid or SCFA stress. It has also been demonstrated via microarray that genes of this operon are upregulated after acid stress. The aim of this study is to determine how expression of the main subunit of this structure, EcpA, is regulated upon exposure of EHEC O157:H7 to acid or SCFA-stress. Both transcriptional and translational regulation are hypothesized to be involved. Isogenic mutants have been constructed that lacked key regulators suspected to be important for each system. Two approaches are used to determine if the predicted regulatory systems are playing a role in response to stress: observing EcpA protein expression analysis through Western blotting with anti-EcpA antibodies, and examining differences in *ecp* operon promoter activity in regulatory mutants. In this study Western blots reconfirmed H-NS as a negative regulator of the *ecp* operon. However, beta-galactosidase assays show that H-NS does not modulate *ecpA* expression in direct response to acute acid stress. This suggests an alternate regulatory response in EHEC O157:H7 to acute acid stress resulting in the upregulation of *ecpA* expression previously observed with microarray analysis.

**Acknowledgements**

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## 1.1 General Introduction

*Escherichia coli* (*E. coli*) is a species of rod shaped, gram-negative bacteria that most frequently colonize the guts of the host organism[7,45]. There are many strains of *E. coli* found in many different host organisms. Most strains of *E. coli* are harmless and, in fact, share a symbiotic relationship with their host as commensal members of a diverse microflora[45]. There are others that have evolved the potential to cause serious illness upon infection, depending on the species of the host organism[7, 45]. The enterohaemorrhagic (EHEC) pathotype is one of the *E. coli* subsets known to be pathogenic to humans[45].

*E. coli* O157:H7 is arguably the most well known serotype within the EHEC pathotype[45,78]. EHEC O157:H7 was first identified in 1982 after being implicated as the cause of several outbreaks of food poisoning in humans, some of them fatal[7,11,13,45]. Those food poisoning incidents were traced back to the consumption of beef products that had not been cooked thoroughly, leaving the bacteria viable[7,11]. Anywhere from 1-50% of cattle are colonized with EHEC O157:H7 at any given time, and those cattle shed the bacteria into their environment[7,11,45]. Processing of cattle for meat production allows for bacteria, such as EHEC, on the exterior surface of the cattle to come into contact with the interior tissues humans consume as meat; with many large-scale meat-processing plants currently in operation, there are ample opportunities for these accidentally contaminated products to make it to market[11,45]. EHEC O157:H7 outbreaks have also been traced back to dairy products, as well as contaminated produce[7,11,45]. The largest outbreak of EHEC O157:H7 infection, with 7966 confirmed cases, occurred in 1996 in Japan and was traced back to contaminated radish sprouts[7,45]. Studies have found that this type of contamination of produce can be linked back to exposure to feces of EHEC colonized cattle[7,11,45,].

## 1.2 EHEC Infection and Pathogenic Mechanisms

Human infection with EHEC O157:H7 is associated with many different symptoms that range in severity and can present in varied combinations. The most common symptoms and developments stemming from EHEC O157:H7 infection include diarrhea, hemorrhagic colitis (HC), and hemolytic uremic syndrome (HUS) [7,11,13,45,48,61,66,75]. The majority of infections will present as a bout of diarrhea without blood, and are resolved without medical intervention. More severe infections will proceed to HC, characterized by abdominal cramping, vomiting, and bloody diarrhea. Infected individuals who progress to HC have a much higher risk of developing HUS, a condition which can result in renal failure, and in some cases death[7,11,13,45,61].

EHEC O157:H7 infection includes two important and relatively unique events as compared to other enteric pathogens and *E. coli* serotypes: intimate attachment to host cells and the subsequent development of attaching and effacing (A/E) lesions, and the production of Shiga-like toxins (Stx1 and Stx2)[7,45,48,66,75]. In EHEC, intimate attachment to host cells is associated with a pathogenicity island, known as the locus of enterocyte effacement (LEE), which is composed of LEE operons 1-5. LEE operons 1-3 encode a type III secretion system (TTSS). This TTSS acts as a molecular syringe that EHEC uses to inject effector molecules into target host cells[45,66,75]. LEE4 encodes many of the effector molecules that are injected into host cells[48]. LEE5 encodes both the translocated intimin receptor (Tir) and it's specific bacterial adhesin, intimin[48]. Tir is a protein, expressed in the bacteria, that integrates itself into the host cell membrane. Tir then serves as a receptor that is specifically recognized by an intimin, present on the surface of the bacterial cell, leading to intimate adhesion of the bacteria to its host[17,22,23,45,48,66]. Along with this intimate binding, the effector molecules are also able to "hijack" cell-signaling pathways of the host cell, leading to a rearrangement of actin filaments in the host cell under the bacteria such that the host cell surface alters to form a pedestal under the bacteria[17,22,23,48,52,66]. These structures are commonly referred to as attaching and effacement lesions as they are characterized by their smoothing of the normally microvilli-coated enterocyte surface[45,75].

This colonization of columnar epithelial cells in the colon is the cause of host cell damage and can lead to the development of HC[7,45,48,].While this colonization is in itself damaging, it also the precursor event by which infected individuals develop one of the more severe conditions associated with EHE; it is the release of Shiga-like toxins by colonized EHEC O157:H7 that promotes progression to HUS[45]. EHEC O157:H7 strains carry one or both Shiga-like toxins, Stx-1 or Stx-2[7,45]. Both variations function in the same manner as Shiga toxin produced in virulent *Shigella* strains[7]. Each individual toxin is composed of an A subunit and five identical B subunits, with the A subunit serving as the active form of the toxin[45]. The B subunits bind globotriaosylceramide (Gb3) receptors, present in high numbers in human renal tissue, resulting in uptake of the A subunit[45]. Once internalized, the A subunit inhibits the eukaryotic 60S ribosomal subunit, preventing continued protein synthesis in the affected cell, resulting in cellular death[45].

## 1.3 Molecular Basis of Adhesion

It is important though for these bacteria to first colonize their host organism so that they can establish a sizable population capable of infection. While EHEC O157:H7 has been demonstrated to have a low infectious dose, 10-50 organisms are enough to cause infection; the number that survives to the point of colonization is even smaller[7,11,45,48]. Thus in order to produce sufficient amounts of toxin to impact the host, the bacterial count must increase to cause illness[11,45,48]. This is where fimbria, the structures that will serve as the main focus of this research, come into play. Fimbria, or pili, are hair-like structures that are present on the surface of many different bacterial species and can play an important role in receptor-specific adherence of bacteria to host-cells[9,32,46,56,75,]. Because there are pili that are capable of exchanging genetic information between bacteria, the alternate term, fimbria, is used to discuss specifically pili that serve exclusively as adhesins, whether to host cells, abiotic surfaces, or other bacteria[56].

## 1.4 *E. coli* Common Pilus (ECP)

Of particular interest for this study is an *E. coli* fimbrial structure known as the *E. coli* common pilus (ECP) or, alternatively, as Mat fimbria[43,57,58]. The highly conserved *ecp* operon, that encodes the components of ECP pili, is present in the genome of most strains of *E. coli*, however expression levels are not consistent across the species, and are highly dependent on growth or environmental conditions[4,8,24,41,43,47,57,58](Figure 1.1).

This fimbrial structure follows the chaperone/usher pathway of assembly, a pathway that has already been well established with *E. coli* Type 1 pili as a model[9,24,32,46,57]. The basic structural idea of these chaperone/usher fimbria is that they are multimeric repeats of a major pilin subunit that extend outwards from the bacterial cell surface, and which are "capped off" with a different pilin subunit that contains an adhesin domain capable of recognizing and adhering to a specific receptor on a host cell[9,24,32] (Figure 1.2).The subunits are transferred from the cytoplasm to a periplasmic chaperone protein that initiates a conformational folding of the subunit proteins before delivering the subunits to a membrane bound usher protein, allowing the subunits to exit the periplasmic space as they join the bottom of the growing fimbria[9,24,32].

The *ecp* operon is comprised of six genes: *ecpR*, *ecpA*, *ecpB*, *ecpC*, *ecpD*, and *ecpE*[24,57]. EcpA is the major pilin subunit for ECP and is also referred to and known as *matB* or *yagZ*[24,27,43,57,58]. This study preferentially refers to *ecpA*. For the other five genes in the *ecp* operon, four constitute the chaperone/usher proteins, and their roles are more specifically defined below (Figure 1.1). The last gene, *ecpD*, encodes the minor adhesin subunit of ECP[24].

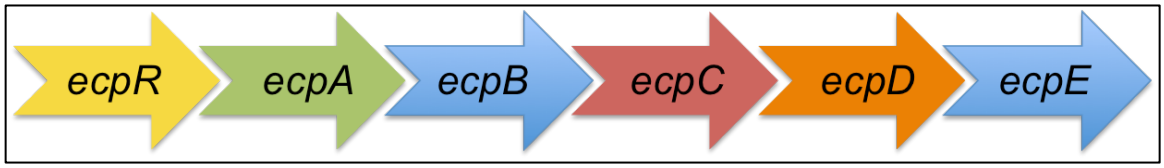



**Figure 1.1: Schematic of *ecp* operon.** *ecpR* is the gene for an operon specific promoter, *ecpA* is the gene for the major pilin subunit of the pilus, *ecpB* is the gene for one of two chaperones, *ecpC* is the gene for the usher, *ecpD* is the gene for the adhesin tip subunit, and *ecpE* is the gene for the second chaperone. Image adapted from 57.

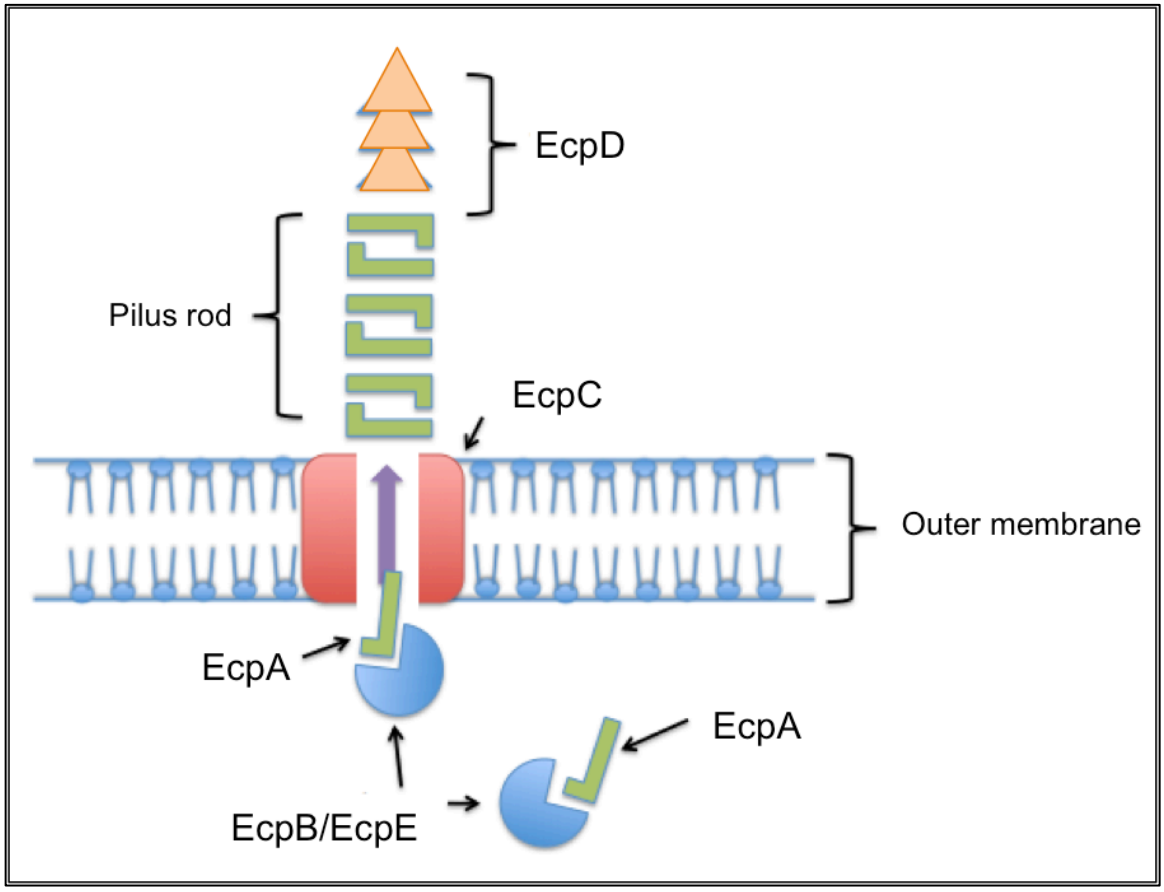



**Figure 1.2: Schematic of chaperone/usher pathway pili assembly.** Monomers of the major subunit undergo a conformational fold to allow for the formation of a multimer, which accounts for the majority of the structure. A specialized adhesin tip allows for receptor recognition and aids in adhesion to host cells.

## 1.5 Role for ECP in adhesion

Previous reports have provided strong, multi-faceted evidence that ECP is an important adhesion factor for *E. coli*[4,24,27,42,58,64]. Adhesion assays and microscopy have demonstrated that isogenic EHEC mutants lacking *ecpA* show a significant decrease in adhesion to cultured HEp-2 and HeLa cells (Figures 1.3, 1.4), as well as to neighboring cells in microconlonies[24,27,58]. Similarly, in newborn meningitis-associated *E. coli* (NMEC, clinical isolate IHE 3034) ECP was found to promote adhesion to abiotic surfaces, plastic or glass, through biofilm formation[42]. ECP was shown to be important for both early biofilm development, and as an important component of strong mature biofilms both on abiotic surfaces, and also within host organisms[24,42]. As mentioned previously, the conditions under which strains of *E. coli* express EcpA, leading to formation of ECP, varies greatly; the two examples of ECP-mediated adhesion mentioned were observed under very different growth conditions[24,27,4,58].

It has also been reported for NMEC strains the EcpR acts not only as a positive regulator for the *ecp* operon, it also negatively regulates flagellar master operon *flhDC*[41]. In this role, EcpR is modulating the shift between planktonic and sessile lifestyles[41]. This is in agreement with ECP's role as an important colonization factor in *E. coli*, downregulation of flagella is a logical response to an increase in expression of structures important for adhesion[41]. Similarly positive regulation of another important *E. coli* colonization factor, curli, has been demonstrated to negatively regulate flagellar expression[41].

These observations collectively support important roles for ECP in adhesion to host cells, other bacterial cells, as well as abiotic surfaces[4,24,27,41,42,58,64]. The adhesion capabilities conferred by ECP on pathogenic *E. coli* serotypes to both host cells and other bacterial cells is a major advantage in infection, establishing a larger population helps to ensure survival and increase severity of the infection[7,11,45,48]. The consensus from researchers in this field is that regardless of serotype and pathogenicity, expression of this highly conserved structure allows for bacterial persistence both within and outside of a host organism, and increases the likelihood of survival and transmission[4,24,27,42,58,64].

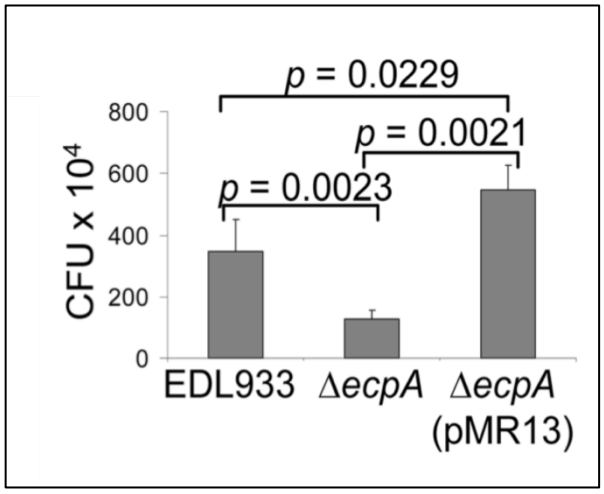



**Figure 1.3: ECP is an important factor for EHEC adhesion to epithelial cells *in vitro*.** An isogenic mutant strain lacking *ecpA* shows a significant decrease in adhesion to HEp-2 or HeLa cells when compared to the wild-type parental strain (EDL933). In the complimented isogenic mutant strain over-expressing *ecpA*, the adhesion phenotype is exaggerated (57).

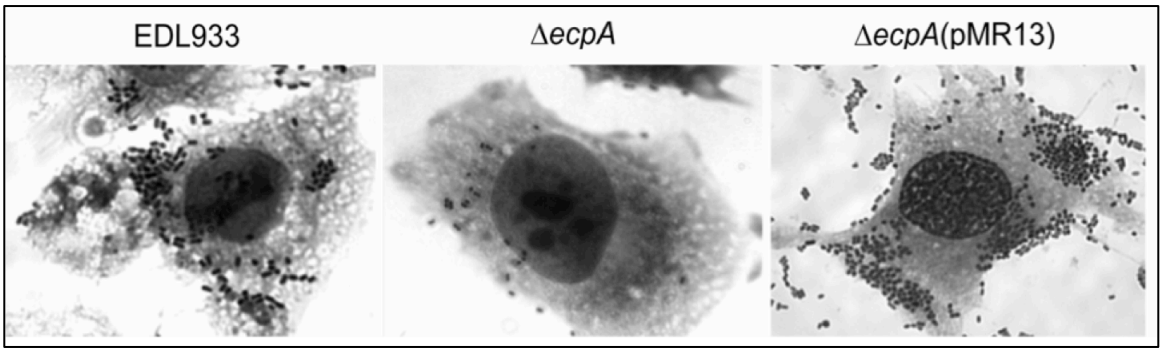



**Figure 1.4: Adhesion of EHEC is decreased significantly in the absence of EcpA.** These Giesma stained images demonstrate that an isogenic mutant lacking *ecpA* is significantly less adherent to HEp-2 or HeLa cells in comparison to the parental strain (57).

## 1.6 Acid and Short-Chain Fatty Acid Stress

The human gastrointestinal tract is a source of many bacterial environmental stresses. Among these are acute acid exposure in the stomach and exposure to short-chain fatty acids (SCFA) in the large intestine[1,3,5,15,20,26,31,50,67]. *E. coli* colonize columnar epithelial cells in the human large intestine and thus in order to reach their preferred environmental niche, all *E. coli*, both commensal and pathogenic, must survive both acid and SCFA exposure, both of which serve as sources of proton stress[3,11,19,20,45,50,63,71,].

The major damaging effect of acid or proton stresses is the alteration of the internal pH of the bacterial cell[6,19,20,26]. All bacteria have an optimum range of pH for activity of their intracellular components and processes, and to ensure a certain level of normal function they must maintain a cytoplasmic pH within that optimum range[6,19,20,26]. Protons from HCl present in the stomach are able to diffuse across the bacterial cell membrane[5,6,19]. SCFAs, on the other hand, are weak acids and diffuse across the bacterial cell membrane without an immediate impact on H+ concentration[60]. However, once inside the cell's more alkaline cytoplasm, short-chain fatty acids dissociate into a lone hydrogen ion and an organic anion, effectively decreasing the internal pH[5]. *E. coli* have systems which counteract this effect, and they will be discussed later in more detail, but it is important to note that active re-alkalization of a bacterial cell requires energy in the form for adenosine triphosphate (ATP) and reliance on these systems could potentially deplete the cell's energy; a lack of available ATP for normal cell processes is in and of itself a negative effect[54,60].

Beyond the acidification of the bacterial cytoplasm, the passive diffusion of short-chain fatty acids is damaging because it allows for the accumulation of organic anions within the cell, resulting in osmotic stress[5,60]. The build-up of these short-chain fatty acid derived organic anions, considered non-compatible solutes as they do effect cellular functions, leads to enlargement via increased turgor pressure within the cell as it works towards osmotic stability[5].

## 1.7 Resistance to proton stress in *E. coli*

*E. coli* have in place several acid resistance (AR) systems to combat proton stress. Which system the bacteria use to oppose proton stress is dependent on other environmental conditions[3,6,19,20,26,57,59,77,]. The first system, acid resistance system 1 (AR 1) is known as oxidative or glucose repressed acid resistance[3]. This is a form of acid resistance that appears to originate not specifically from acid or proton stress, but occurs rather as a general protective stress response in stationary phase bacteria[3,29]. Interestingly, and as the name would suggest, upon addition of glucose to the environment, stationary cells lose this acid stress resistance[3]. AR 1 is linked to $\sigma^S$, an alternate sigma factor that has been shown to be responsible for a major portion of the *Salmonella* acid tolerance system[3,29]. The exact mechanisms by which AR 1 combats proton stress are unclear.

Acid resistance system 2 (AR 2) and 3 (AR 3) work via similar mechanisms, which can be described in combination, however it should be noted that they do differ in their levels of efficacy; AR 2 is *E. coli*'s most effective acid stress response[20]. These systems work to combat proton stress by actively working to increase cytoplasmic pH levels[20]. Both AR2 and AR3 are comprised of an amino acid-specific decarboxylase and a cognate antiporter. AR 2 is a glutamate dependent system while AR 3 is dependent on arginine[20,77]. This means that in order for the system to be active, the requisite amino acid must be present in the extracellular environment[20]. In brief, the decarboxylase removes the α-carboxy group of its amino acid substrate, this process results in consumption of a proton from the cytoplasm[20,77]. The altered amino acid, glutamate/γ-aminobutyric acid for AR 2 and agmatine for AR 3, are then transported out of the cell via the antiporter and exchanged for a new molecule of the original amino acid substrate (Figure 1.5)[20]. In addition to raising pH levels in the cytoplasm, the processing of amino acids to remove the α-carboxy group and then releasing them back into the extracellular environment also help to increase extracellular pH levels[3].

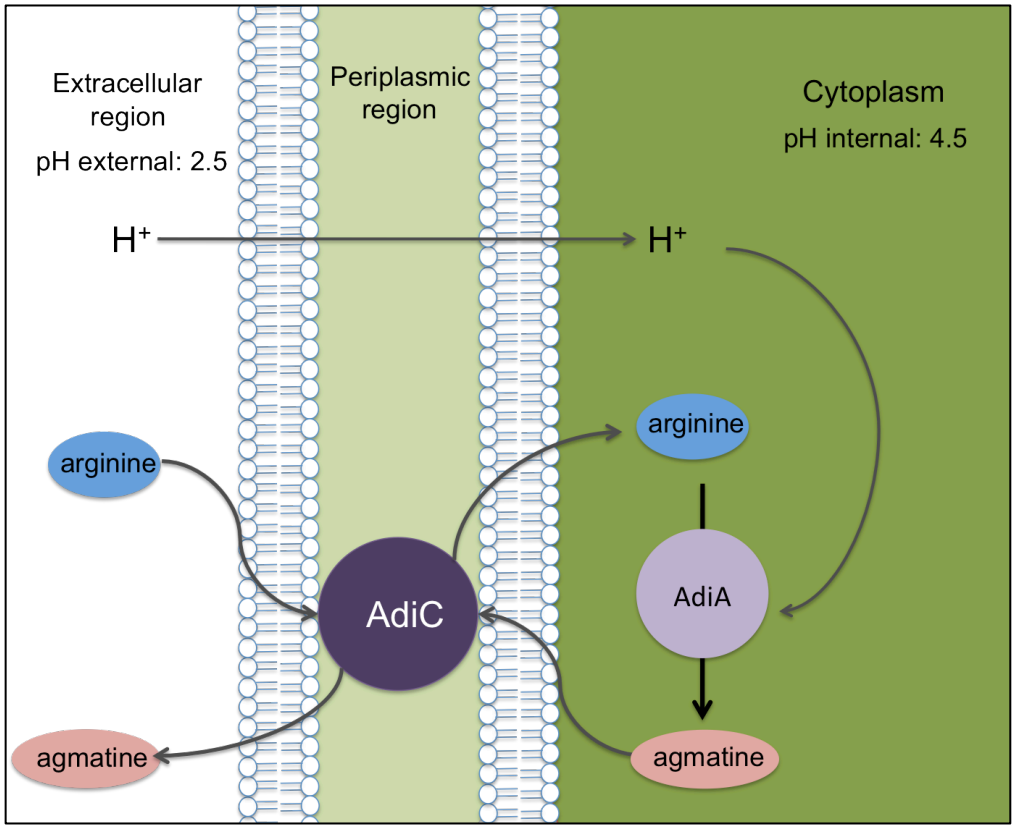



**Figure 1.5: Schematic of Acid Resistance System 3 (AR3).** In AR3, periplasmic antiporter AdiC internalizes extracellular arginine, and subsequently an arginine specific decarboxlase, AdiA, removes the α-carboxy group from arginine. This process results in conversion of arginine to agmatine as well as the consumption of a cytoplasmic proton, leading to an increase in cytoplasmic pH. Adapted from 20.

## 1.8 Impact of acid stress on ECP expression

As was previously mentioned, during gastric passage bacteria such as EHEC are exposed to acutely acidic conditions in the human stomach. It has been reported that the pH of the human stomach can reach as levels high as pH 6 and as low as pH 2[20]. DNA microarray analysis revealed a significant increase in expression of *ecpA* in EHEC after a 1-hour acid adaptation at pH 5, followed by a 30-minute acute acid stress at pH 3[31]. A similar increase in *ecpA* expression was seen in EHEC that were not adapted to acid stress at pH 5 (Figure 1.6)[31]. Both of these conditions are physiologically relevant and cover a broad range of potential stomach conditions to be potentially encountered by EHEC[20,31].

The microarray results were supported by subsequent adhesion assays that showed an increase in ECP-mediated adhesion. Acid adapted, acid stressed wild-type EHEC demonstrated an increase in levels of adhesion when compared to unstressed controls[27]. When an isogenic mutant lacking *ecpA* was analyzed for levels of adhesion under the same acid stress and control conditions, both showed a significant decrease in comparison to the parental strain. Upon complementation of *ecpA* on a plasmid, the acid-induced adhesion phenotype was partially restored (Figure 1.7)[27].

| | Relative-fold change | |
|---|---|---|
| Gene | UA30 | AA30 |
| *ecpA*; major fimbrial subunit of *E. coli* common pilus | 5.6* | 5.0* |
| *hns*; DNA-binding protein, global translational regulator | - | -1.5** |

**Figure 1.6: Microarray analysis of EHEC gene expression after acid stress.** The gene encoding the major pilin subunit of ECP, *ecpA*, was upregulated at least 5-fold under both unadapted acid stress conditions and adapted acid stress conditions. (AA30: samples were adapted to acid stress at pH 5 for 1 hour, followed by a 30 minute acute acid stress at pH 3.0. UA30 samples were not acid adapted, but were acute acid stressed at pH 3.0 for 30 minutes) (31).

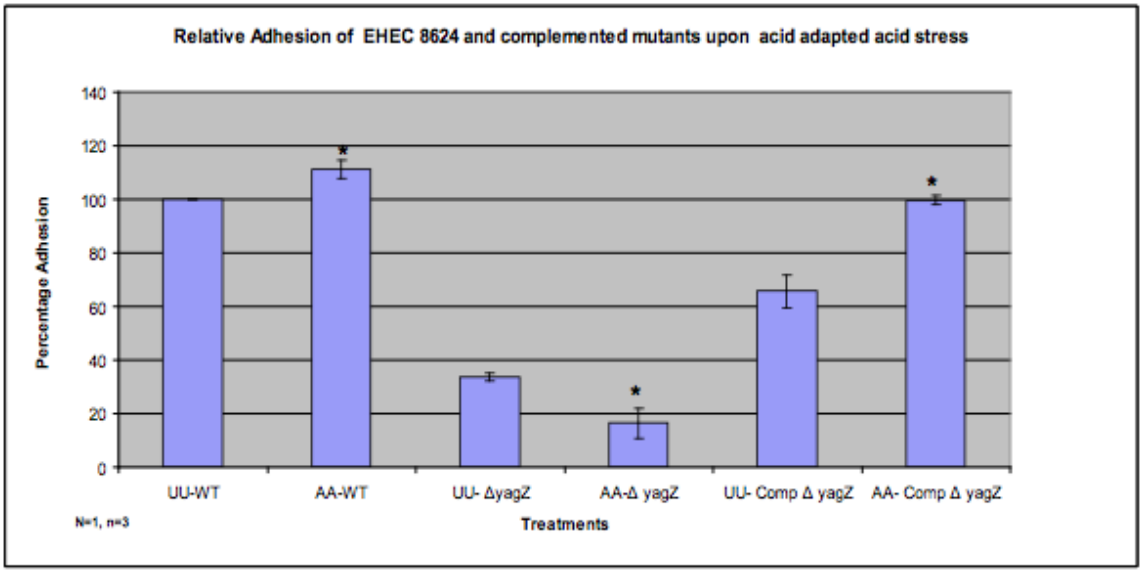



**Figure 1.7: Exposure to acid stress increases EHEC ECP-mediated adhesion to epithelial cells.** In wild-type EHEC, there is a significant increase in adhesion in acid stressed samples compared to unstressed control samples. That phenotype is lost in an isogenic mutant lacking *ecpA* (*yagZ*), but is restored upon complementation. (UU-WT: unadapted, unstressed wild-type EHEC, AA-WT: acid adapted, acid stressed 30 mins. wild-type, UU-Δ*yagZ*: unadapted, unstressed isogenic *ecpA* mutant, AA-WT: acid adapted, acid stressed 30 mins. isogenic *ecpA* mutant, UU-CompΔ*yagZ*: unadapted, unstressed complimented isogenic *ecpA* mutant, AA-CompΔ*yagZ*: acid adapted, acid stressed 30 mins. complimented isogenic *ecpA* mutant) (27)

## 1.9 Impact of SCFA stress on ECP expression

Enteric bacteria produce SCFAs as a byproduct of fermentation of soluble fiber[1,15,25,60,*80*]. The localization of SCFAs in the colon is due to the increasingly anaerobic conditions present in that portion of the gastrointestinal tract[60]. Starting in the distal ileum, SCFAs are found at a total concentration of 20-40 mM, in the proximal colon the concentration increases to 70-140 mM, and then decreases to 20-70 mM in the distal colon; human feces have been reported to contain 172mM[54,60,*80*]. The three main SCFAs present in the human colon are, in descending order, acetate, propionate, and butyrate[54,60,*80*]. The these compounds have been reported to be present in a ratio of 60:25:15 of acetate:propionate:butyrate[*80*]. SCFA stress was experimentally defined as static exposure of bacteria to a 30mM, 90mM, or 172mM mix of acetate, propionate, and butyrate for 2 hours in a 5% $CO_2$ atmosphere[27,67]. Adhesion of 90mM SCFA stressed wild-type EHEC to human epithelial cells was significantly higher than the unstressed

control samples[27,67]. As was also seen with the acid stress adhesion assays, the isogenic *ecpA* mutant strain showed a significant decrease in adhesion in comparison to the parental strain[27]. Again the phenotype was partially restored upon plasmid complementation of *ecpA* in the isogenic mutant strain (Figure 1.8)[27].

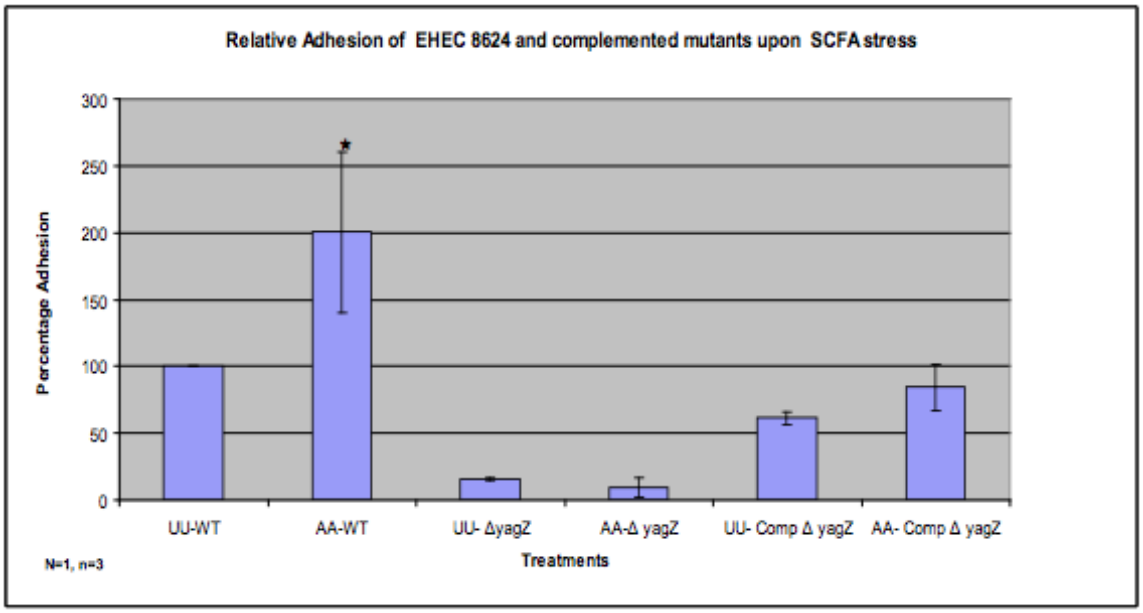



**Figure 1.8: Exposure to 90mM SCFA stress increases EHEC ECP-mediated adhesion to epithelial cells.** In wild-type EHEC, there is a significant increase in adhesion in SCFA stressed samples compared to unstressed control samples. That phenotype is lost in an isogenic mutant lacking *ecpA* (*yagZ*), but is restored upon complementation. (UU-WT: unstressed wild-type EHEC, AA-WT: 90mM SCFA mix stressed wild-type, UU-Δ*yagZ*: unstressed isogenic *ecpA* mutant, AA-WT: 90mM SCFA mix stressed isogenic *ecpA* mutant, UU-CompΔ*yagZ*: unstressed complimented isogenic *ecpA* mutant, AA-CompΔ*yagZ*: 90mM SCFA mix stressed complimented isogenic *ecpA* mutant) (27)

## 1.10 Transcriptional regulation of the *ecp* operon

Transcription of the genes of the ECP fimbrial operon (*ecp* operon) has been shown to be regulated by three proteins: the histone-like nucleoid structuring protein (H-NS), integration host factor (IHF), and the previously mentioned intra-operon regulator EcpR[43,47].

H-NS is a DNA-binding protein that has been implicated in regulating expression of upwards of 60 *E. coli* proteins, many of them are environmentally regulated or involved in stress tolerance. H-NS is most often known to negatively regulate target genes[2,14,18,36,47,53,62,69,73,74,76,79,*81*]. Results also indicate a role of H-NS in repressing the transcription of the *ecp* operon[43,47]. Additionally, the previously mentioned DNA microarray showed a slight decrease in mRNA levels for *hns* upon AA30 acid stress that correlates with an increase in transcription of *ecpA*[31].

IHF is also a DNA binding protein that is not specifically a regulator of the *ecp* operon[65]. IHF does not act directly as a positive regulator of the *ecp* operon; rather it acts to oppose the negative regulatory effects of H-NS[43,47]. The two proteins have binding sites within a small stretch of DNA in the promoter region of the *ecp* operon, and because both IHF and H-NS act through winding or bending of DNA to allow or prevent transcription of the target gene, their oppositional roles in close proximity are logical as a method to control transcription of the *ecp* operon[43,47,36,65,69].

The last member of the *ecp* operon transcriptional regulatory story is EcpR. EcpR is the first gene in the *ecp* operon, and like other first genes in an operon that encodes a chaperon/usher assembled fimbrial structure, EcpR acts as an intra-operon positive regulator, enhancing expression of the other genes in the operon through binding of promoter regions upstream of the *ecp* operon[9,33,43,47].

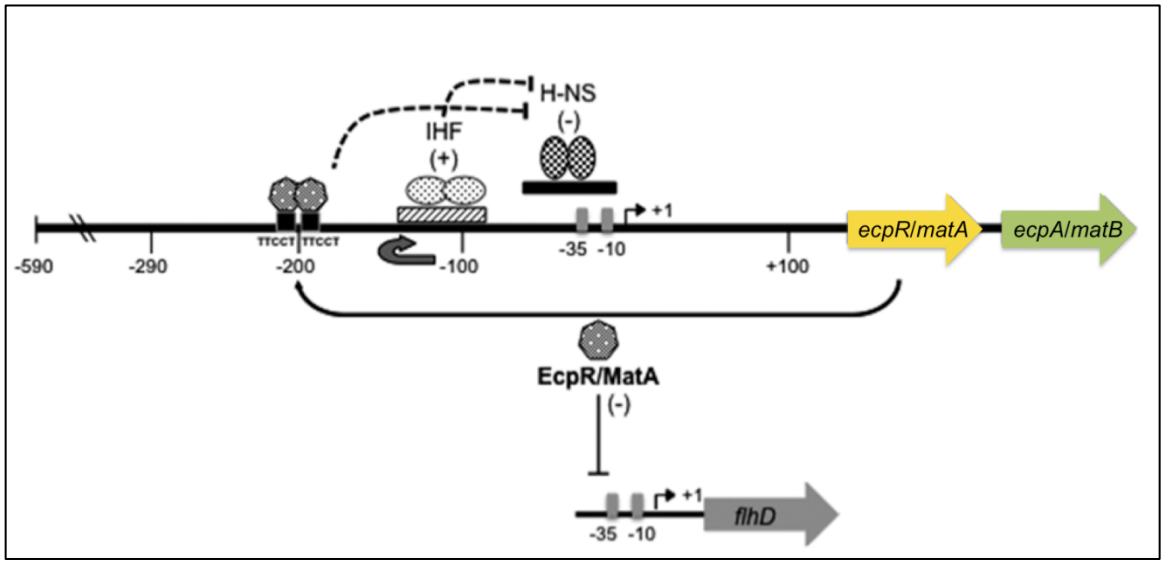



**Figure 1.9: Schematic of *ecp* operon transcriptional regulation.** Global regulator H-NS has been shown to negatively regulate this operon, while the operon specific regulator EcpR is a positive regulator. The negative regulation imposed on the operon is opposed in the presence of IHF, allowing for expression. Image modified from 46.

## 1.11 Translational regulation of the *ecp* operon

Once *ecpA* has been transcribed into mRNA, there is another regulatory system that can effect the expression of *ecpA* on a protein level. A translational regulatory system for the *ecpA* has been pieced together from previously known components of other regulatory mechanisms present in *E. coli*. The two-component transduction system BarA/UvrY has been shown to be activated, through stimulation of BarA and subsequent activation of DNA-binding protein UvrY, by exposure to aliphatic carboxylic acids such as methyl acetate (6mM) and other SCFAs[12,55]. Activation of BarA/UvrY leads to an increase in expression of two small non-coding RNAs, CsrB and CsrC[12,35,51,68,70,72].

This is the point at which the SCFA regulatory response in EHEC intersects with a global regulatory system suspected to regulate *ecpA* translation. CsrA is an RNA binding protein that acts to regulate translation of target transcripts by sequestering and degrading mRNA[34,35,68,70]. CsrB and CsrC inhibit CsrA binding of target transcripts because the small non-coding RNAs will occupy RNA binding sites on CsrA, blocking CsrA from binding additional RNA[34,35,68,70]. The *ecpA* transcript has been reported to be a target for CsrA sequestration and/or degradation (J. Puente, personal communication,

May 26, 2011). This leads to the hypothesis that upon an increase in CsrB/CsrC expression due to SCFA exposure, CsrA-mediated repression of *ecpA* translation would be decreased, and protein levels of EcpA would increase.

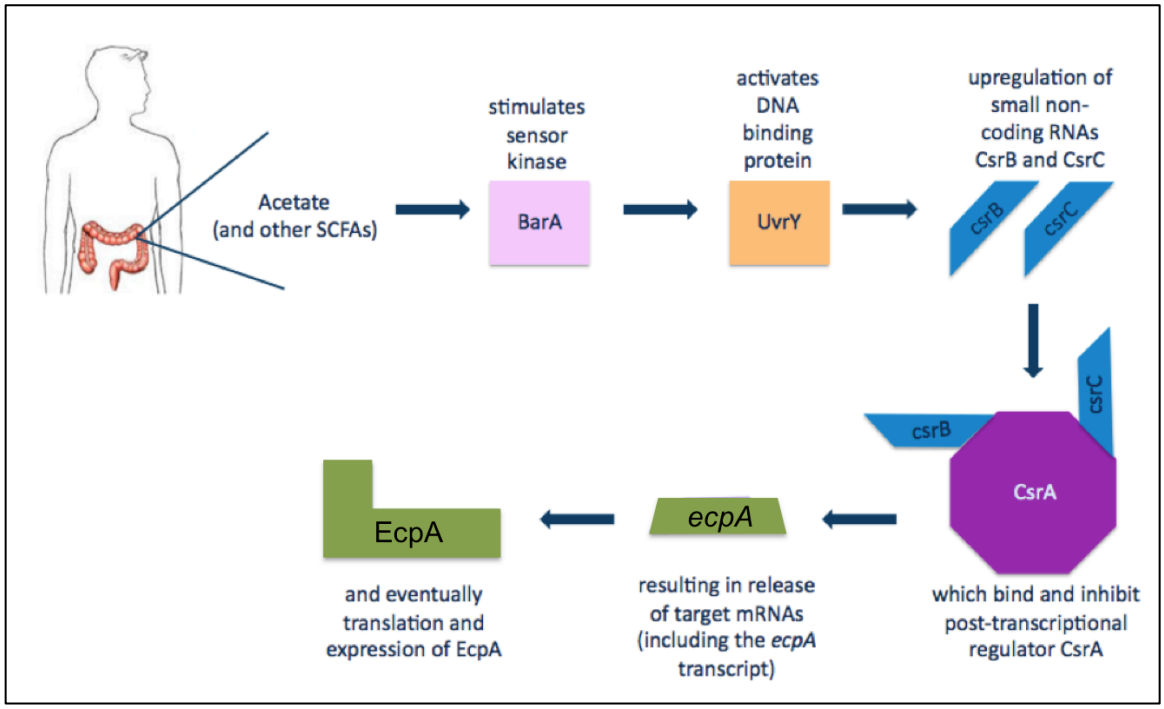



**Figure 1.10: Schematic of hypothetical *ecp* operon translational regulation.** Sensor kinase BarA is stimulated by exposure to the carboxylate group of aliphatic carboxylic acids, including SCFAs, and in turn activates its response regulator UvrY. Activation of UvrY increases expression of small non-coding RNAs CsrB and CsrC. CsrB/C are known to inhibit a global post-transcriptional regulator, CsrA. CsrA acts by sequestering the transcripts of target genes, potentially included is the mRNA for *ecpA*, and preventing them from being translated. Upregulation of CsrB/C via activation of BarA/UvrY after stimulation from SCFA exposure could lead to an increase in EcpA.

## 1.12 Rationale

The *E. coli* common pilus (ECP) has been shown to contribute to adhesion of both pathogenic and non-pathogenic *E. coli* to epithelial cells. Previous work has shown that both acid and SCFA stress enhance ECP-mediated adhesion, and that acid stress increases expression of the *ecpA* transcript for EHEC O157:H7. Understanding how these GI stresses modulate the transcriptional and translational expression of EcpA, and ultimately the *E. coli* common pilus, can contribute to our understanding of how EHEC is able to gain an adhesive advantage after exposure to acid or SCFA stress.

## 1.13 Hypothesis

Microarray analysis has shown an increase in expression of *ecpA* in acid stressed EHEC O157:H7, and adhesion assays have shown an increase in adhesion for EHEC O157:H7 upon acid and SCFA stress which is lost in mutant strains lacking *ecpA*, but partially restored in the *ecpA* complemented mutant. This increase in *ecpA* expression is modulated by environmental stresses by global transcriptional, as well as translational, regulators.

## 1.14 Objectives:

**I. Determine changes in expression of EcpA in wild-type EHEC O157:H7 after exposure to GI stresses.** EHEC will be exposed to acid or SCFA stress and levels of EcpA will be analyzed via Western blotting with α-EcpA antibodies. Promoter activity for the *ecp* operon will be assessed with beta-galactosidase activity assays.

**II. Construction of *hns*, *uvrY*, or *ecpA* deficient mutants of EHEC O157:H7.** Mutants deficient of each gene will be constructed via λ red recombinase mediated homologous recombination.

**III. Characterization of EHEC O157:H7 *hns* and *uvrY* mutants.** Regulatory mutants will be exposed to acid or SCFA stress and levels of EcpA will be assessed via Western blotting with α-EcpA antibodies. Promoter activity for the *ecp* operon will be assessed with beta-galactosidase activity assays in EDL933 *hns*::$Kan^R$.

# 2. Materials and Methods

## 2.1 Bacterial cultivation

Bacterial strains, detailed in table 2.1, were maintained at -80°C as glycerol stocks (15% glycerol, v/v) and at 4°C as bacterial working stocks on Luria broth (LB) agar plates (1% tryptone w/v, 0.5% yeast extract w/v, 1% sodium chloride w/v, 1.5% agar w/v) with antibiotics added as needed for mutant or plasmid carrying strains. Standard concentrations of antibiotics are shown in table 2.3. Bacterial working stocks were typically incubated overnight at 37°C, however strains carrying the temperature sensitive plasmids pKD46 or pCP20 were incubated at 30°C. Bacterial working stocks were subcultured every two weeks. Overnight liquid cultures were inoculated with a single colony from the working stock, with antibiotics added to standard concentrations when needed, and grown overnight at the appropriate temperature for the strain in a shaking incubator (200 rpm) for an average of 16 hours.

**Table 2.1: Plasmids and constructs used in this study**

| Name | Description | Source |
|---|---|---|
| pKD4 | Plasmid pKD4, source of kanamycin resistance cassette, with flanking FRT-sites, used to create isogenic mutants; $Amp^R$, $Kan^R$/$Neo^R$ | 16 |
| pKD46 | Plasmid pKD46, provides λ red recombinase system for homologous recombination; $Amp^R$ | 16 |
| pMC1403 | Plasmid used for cloning promoters such that they promote expression of beta-galactosidase and allow for analysis of promoter activity levels; $Amp^R$ | 10 |
| pMC1403-P*ecpR* | Promoter region of *ecpR* into XmaI/BamHI sites of the multiple cloning site of pMC1403; $Amp^R$ | This study |
| pMC1403–P*acrA* | Promoter region of *acrA* into EcoRI/BamHI sites of the multiple cloning site of pMC1403; $Amp^R$ | 38 |

## Table 2.2: Bacterial strains used in this study

| Strain | Characteristics | Source |
|---|---|---|
| EDL933 | Wild type *E. coli* O157:H7 | 44 |
| EDL933 P*ecpR* | Wild type *E. coli* O157:H7 transformed with pMC1403+P*ecpR*; $Amp^R$ | This study |
| EDL933 pMC1403 | Wild type *E. coli* O157:H7 transformed with pMC1403; $Amp^R$ | This study |
| EDL933 pKD46 | Wild type *E. coli* O157:H7 transformed with pKD46; $Amp^R$ | 82 |
| EDL933 *ecpA*::$Kan^R$ | EDL933 with $Kan^R$ chromosomal disruption of *ecpA*; $Kan^R$ | 57 |
| EDL933 *hns*::$Kan^R$ | EDL933 with $Kan^R$ chromosomal disruption of *hns*; $Kan^R$ | This study |
| EDL933 *hns*::$Kan^R$ P*ecpR* | EDL933 *hns*::$Kan^R$ transformed with pMC1403+P*ecpR*; $Amp^R$ | This study |
| EDL933 *hns*::$Kan^R$ pMC1403 | EDL933 *hns*::$Kan^R$ transformed with pMC1403, $Amp^R$ | This study |
| EDL933 *uvrY*::$Kan^R$ | EDL933 with $Kan^R$ chromosomal disruption of *uvrY*; $Kan^R$ | This study |
| 86-24 P*acrA* | Wild-type *E. coli* O157:H7 transformed with pMC1403+P*acrA*; $Amp^R$ | 38 |

## Table 2.3: Standard antibiotic concentrations used in this study

| Antibiotic | Working concentration |
|---|---|
| Ampicillin | 100 µg/mL |
| Kanamycin | 50 µg/mL |

## 2.2 Creation of isogenic mutants

EDL933 isogenic mutants were created for *ecpA*, *hns*, and *uvrY* through λ-red recombinase-mediated homologous recombination.

### 2.2.1 Plasmid purification

Plasmids used in this study, listed in Table 2.1, were all isolated with spin column minipreps (Qiagen). A 5mL overnight culture of the parental strain of interest was incubated at the appropriate temperature for the plasmid to be purified, overnight, with shaking. The following morning the miniprep was completed, as per the manufacturer's directions. This included pelleting and subsequent lysis of bacteria, separation of cellular debris from DNA, binding of plasmid DNA to a silica resin, washing samples with ethanol, and finally elution of plasmid DNA in Tris-buffer. Purified plasmid DNA was stored at -20°C.

### 2.2.2 Primer design and PCR amplification of kanamycin resistance cassette

Primers were designed, using molecular biology computer programs SerialCloner and ApE, to amplify the kanamycin resistance cassette from pKD4, with ~50 bp extensions carrying homology to regions either upstream or downstream of the target gene. The built in regions of homology will allow for the subsequent PCR product to integrate into the chromosome, essentially inserting the kanamycin resistance cassette in place of the target gene. A BLAST search was performed to confirm that both the upstream and downstream homology regions used in each primer set were unique within the genome of EDL933. This was done to ensure that homologous recombination would occur only the region of the chromosome surrounding the target gene.

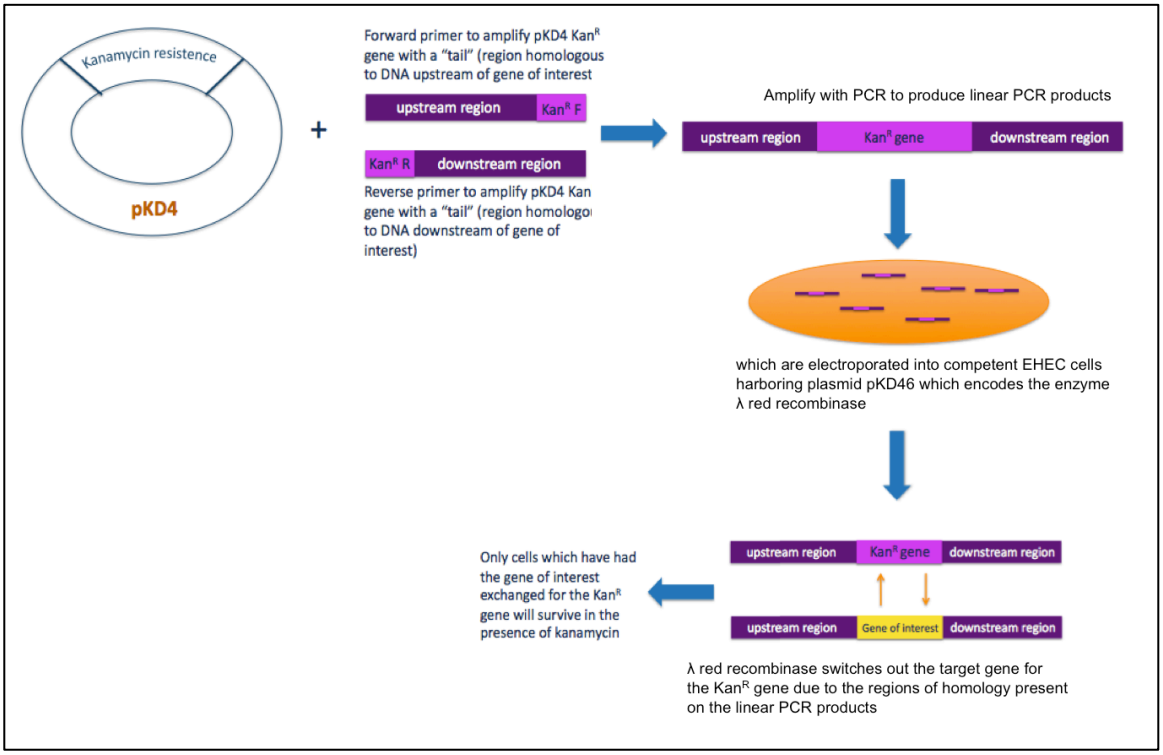



**Figure 2.1: Overview of λ red recombinase-mediated homologous recombination.** The kanamycin resistence ($Kan^R$) gene of pKD4 is amplified with PCR using primers designed to integrate regions, upstream and downstream of the $Kan^R$ gene, homologous to upstream and downstream regions of the target gene. These linear PCR products are used to transform EHEC carrying pKD46, encoding λ red recombinase, which mediates the integration of the $Kan^R$ gene into the bacterial chromosome, eliminating the target gene.

All primer sequences are listed in table 2.4. These primers were used in a 200uL PCR reaction as follows; 20uL 5X Phusion HF buffer, 1.6uL 10mM dNTP mix, 4uL 10uM forward primer, 4uL 10uM reverse primer, 4uL pKD4 miniprep, 49.6uL sterile PCR grade water, 0.8uL Phusion DNA polymerase. The reaction mixture was divided into four 50uL aliquots in flat-cap PCR tubes. All PCR reactions were run on a BioRad thermocycler. The cycling conditions were as follows; 30 second initial denaturation at 98°C, 35 cycles of a 10 second denaturation at 98°C then a 30 second annealing at primer specific temperatures (see table 2.4 for details) and then a 30 second extension at 72°C, a 10 minute final extension at 72°C, and a 4°C hold until samples were collected. Results were visualized on a 1% agarose gel. Positive results were then subjected to gel extraction purification and sample concentration (Qiagen).

### 2.2.3 Preparation of electrocompetent EHEC

In order to disrupt the target gene through integration of a kanamycin resistance cassette with λ-red recombinase mediated homologous recombination, linear PCR products generated in section 2.2.2 must be electroporated into EDL933 already carrying the λ-red recombinase encoding plasmid pKD46. An overnight culture of EDL933 (pKD46) was grown overnight at 30°C in 5mL LB broth with 50 ug/mL ampicillin (Amp). The following morning the overnight culture was diluted 1:50 in 50ug/mL Amp, 100mM L-arabinose LB broth and incubated at 30°C with shaking until the $OD_{600}$ reaches ~0.4. The subculture was placed in pre-chilled 50mL centrifuge tubes, kept on ice for 10 minutes, and then the bacteria pelleted at 2061 x g (3100 rpm) at 4°C for 10 minutes. The supernatant was removed, and the pellet resuspended in 1/10 volume of the original subculture volume of ice-cold sterile distilled water. Cells were kept on ice for 10 minutes and re-spun as described previously. This step was repeated a second time. The bacterial pellet was then resuspended in 1/20 volume of the original subculture volume of ice-cold sterile 10% glycerol water and re-spun as previously described. This step was also repeated a second time. After the second glycerol wash, the pellet was resuspended in 1/100 volume of the original subculture volume, divided into 100uL aliquots in pre-chilled microcentrifuge tubes, and kept on ice until needed for electroporation.

### 2.2.4 Electroporation

Between 1 and 5uL of concentrated and purified PCR reaction was used to transform 100uL of electrocompetent EDL933 (pKD46) in 1mm gap electroporation cuvettes using an electroporation unit (BioRad) with the following settings; 2.5kV, 25μF, 200Ω. Electroporated bacteria were immediately added to 900uL of 30°C Super Optimum Broth with Catabolite repression (SOC) media (0.5% w/v yeast extract, 2% w/v tryptone, 10mM NaCl, 2.5mM KCl, 10mM $MgCl_2$, 10mM $MgSO_4$, 20mM glucose) and allowed to recover for 3 hours at 30°C with shaking. After recovery, bacteria were spread onto LB-kanamycin plates and incubated overnight at 37°C.

### 2.2.5 Colony PCR screening

Following transformation, all colonies obtained were re-streaked onto new LB-kanamycin plates and screened, through colony PCR using primers designed to amplify a region outside of the target gene, for successful gene disruption and the integration of a kanamycin resistance cassette. The 25uL colony PCR reaction mix was as follows: 2.5uL Taq buffer with $Mg^{2+}$ (Genscript), 0.5uL 10mM dNTP mix (Genscript), 0.5uL 50uM forward primer (ACGT), 0.5uL 50uM reverse primer (ACGT), 20uL sterile PCR grade water (Genscript), 0.25uL Taq DNA polymerase (Genscript). 25uL aliquots were each inoculated with a single candidate colony. The cycling conditions were as follows; 10 minute initial denaturation at 94°C, 35 cycles of a 30 second denaturation at 94°C then a 45 second annealing at primer specific temperatures, see table 2.3 for details and then a 60 second extension at 72°C, a 7 minute final extension at 72°C, and a 4°C hold until samples were collected. Results were visualized on a 1% agarose gel. Positive results were confirmed with sequence analysis.

## Table 2.4: Primers used in this study

| Primer name | Sequence (5'-3') | A. Temp. | Product/Target | Source |
|---|---|---|---|---|
| *ecpA*/kanR F | GTTCTGGCAATAGCTCTGGTAACG GTGTTTACCGGGTGTAGGCTGGAG CTGCTTCC | 61°C | Kanamycin resistance cassette from pKD4 with *ecpA* upstream and downstream homology regions | 57 |
| *ecpA*/kanR R | TTAACTGGTCCAGGTCGCGTCGAA CTGTACGCTAACCATATGAATATCC TCCTTAG | | | |
| *ecpA* check F | AACAGCAATATTAGGGGCGTG | 51°C | Region of EDL933 chromosome outside of *ecpA* | 57 |
| *ecpA* check R | GGATAACAGCAGAGCGAGAAG | | | |
| *hns*/kanR F | TCTATTATTACCTCAACAAACCACC CCAATATAAGTTTGAGATTACTAC AGTGTAGGCTGGAGCTGCTTC | 50°C | Kanamycin resistance cassette from pKD4 with *hns* upstream and downstream homology regions | This study |
| *hns*/kanR R | AAAAAATCCCGCCGATGGCGGGAT TTTAAGCAAGTGCAATCTACAAAA GAATGGGAATTAGCCATGGTCC | | | |
| *hns* check F | TCCACGAAACGGCGTTGAGCAA | 56°C | Region of EDL933 chromosome outside of *hns* | This study |
| *hns* check R | CCTTACATTCCTGGCTATTGCACA | | | |
| *uvrY*/kanR F | TAACTATCAGTAGCGTTATCCCTAT TTCTGGAGATATTCCTGTGTAGGCT GGAGCTGCTTC | 50°C | Kanamycin resistance cassette from pKD4 with *uvrY* upstream and downstream homology regions | This study |
| *uvrY*/kanR R | TTACGGTTTTTAAAAACGCTTTTGC GTCAAACTGATCACATGGGAATTA GCCATGGTCC | | | |
| *uvrY* check F | CCGACATAGATAACCGTACCA | 50°C | Region of EDL933 chromosome outside of *uvrY* | This study |
| *uvrY* check R | CGTGACCATAACTGTGGACA | | | |
| P*ecpR* XmaI F | GAGAGACCCGGGTCCATTACACAA CACATTAAGAC | 69°C | EDL933 *ecpR* promoter region with 5'XmaI recognition site and downstream BamHI recognition site | This study |
| P*ecpR* BamHI R | GAGAGAGGATCCATGTCACTACTT TCCAAACCTGTA | | | |
| pMC1403 F | TGCCACCTGACGTCTAAGAA | 48°C | Multiple cloning region of pMC1403 | 38 |
| pMC1403 R | GTTTTCCCAGTCACGACGTT | | | |

* "A. Temp." refers to annealing temperature of a primer set
** "check" is in reference to these primers use as a means to versify successful integration of the kanamycin resistance cassette

## 2.3 Construction of beta-galactosidase promoter fusion construct

### 2.3.1 Primer design and PCR amplification of ECP promoter region

In order to observe *ecpR* promoter activity under different environmental conditions, the *ecpR* promoter region was placed upstream of a promoter-less gene encoding beta-galactosidase. Activity of the promoter region may then be assessed through the levels of beta-galactosidase activity in a sample. Primers were designed to amplify the promoter region for the ECP operon that upon amplification would contain an upstream XmaI recognition sequence and a downstream BamHI recognition sequence (see table 2.4 for details). These restriction enzymes were selected because of their presence in the multiple cloning site in front of the promoter-less *lacZ* gene of pMC1403. The primers were also designed to ensure that upon ligation of this region into pMC1403, the first three codons of *ecpR* would be in the same reading frame as the aforementioned *lacZ* gene. The promoter region was amplified with a PCR reaction with the same components and cycling conditions as described in section 2.2.1. After completion of PCR, the samples were subjected to spin column PCR cleanup (GE Healthcare).

### 2.3.2 Restriction digestion and vector dephosphorylation

Both the cleaned promoter region and an aliquot of a miniprep of pMC1403 underwent simultaneous digestion with both XmaI (New England Biolabs) and BamHI (New England Biolabs), each in a 50uL restriction reaction composed as follows: 5uL 10X NEBuffer 3 (New England Biolabs), 0.5uL 100X BSA (New England Biolabs), 1uL XmaI, 1uL BamHI, 3uL DNA (~1ug), and sterile distilled water to achieve a final volume of 50uL. This mixture was incubated at 37°C for 1 hour. The now digested pMC1403 was subjected to dephosphorylation with Antarctic phosphatase (New England Biolabs) with the addition of 5uL 10X Antarctic phosphatase buffer and 1uL Antarctic phosphatase and incubated at 37°C for 20 minutes. Both samples were then subjected to spin column enzymatic reaction cleanup.

### 2.3.3 Ligation

To successfully assemble the beta-galactosidase promoter construct through ligation of the *ecpR* promoter region into the multiple cloning site of pMC1403, A 20uL ligation mixture was prepared as follows: 4uL 5X ligase reaction buffer, 3uL digested and dephosphorylated pMC1403, 11uL digested ECP promoter region, 1U in 1uL $T_4$ DNA ligase, 1uL sterile distilled water. This mixture was incubated at room temperature for 1 hour, and then diluted with 80uL of sterile distilled water.

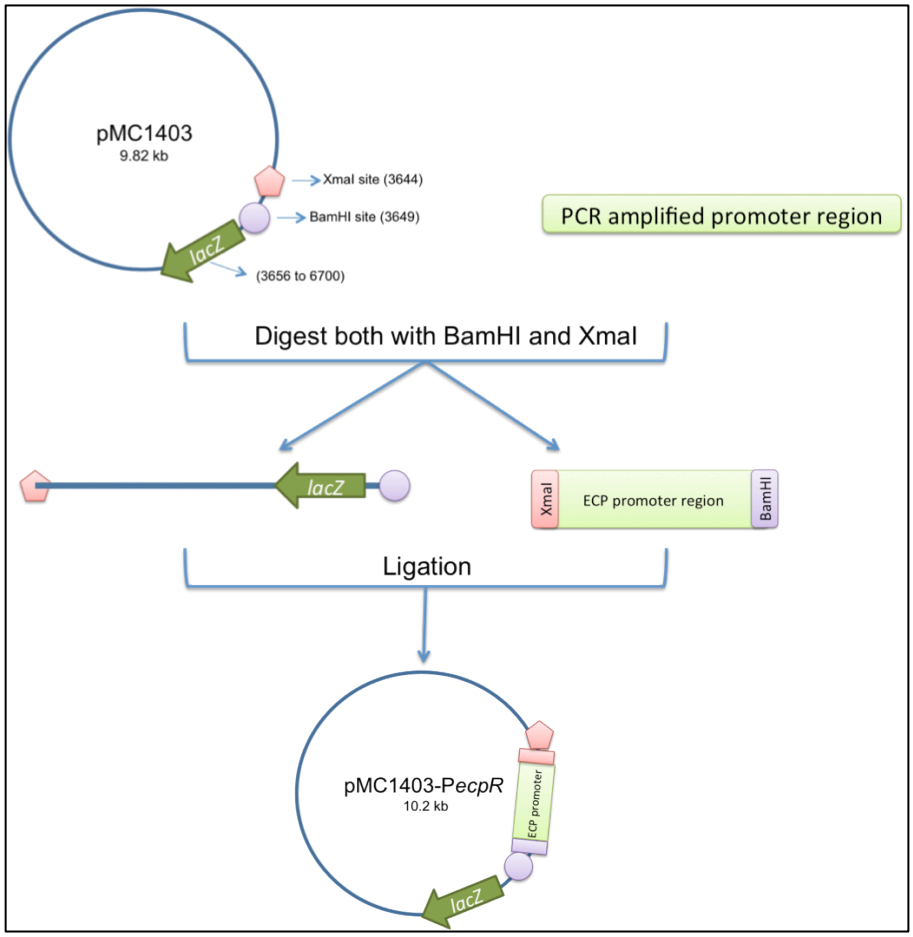



**Figure 2.2: Overview of creation of pMC1403-P*ecp*R.** Summary image of sections 2.3.1-2.3.3. Parental plasmid, pMC1403, and PCR amplified promoter region for *ecp* operon are both digested with BamHI and XmaI, the sticky ends of pMC1403 were dephosphorylated with Antarctic phosphatase, and subsequently the promoter region and plasmid were ligated such that the *ecp* operon promoter region was inserted in front of promoter-less *lacZ* of pMC1403.

### 2.3.4 Transformation of TOP10 chemically competent *E. coli* and isolation of pMC1403-P*ecpR*

The ligation reaction from section 2.3.3 was used to transform chemically competent *E. coli* so that potentially successful ligation products could be identified based on conference of ampicillin resistance. 100uL of TOP10 chemically competent *E. coli* was thawed on ice for 10 minutes. 20uL of dilute ligation reaction was added to the cells, which were kept on ice for an additional 20 minutes. The cells were then incubated at 37°C for 5 minutes, and then placed back on ice for 1 minute. 500uL of SOC media was added to the cells, followed by 1 hour recovery period at 37°C with shaking. 100uL of the transformed cells were plated onto LB-Amp. Resulting colonies were subjected to colony PCR, as described in section 2.2.5, with primers pMC1403 F and pMC1403 R. Positive results were confirmed with sequence analysis. Electrocompetent EDL933 and EDL933Δ*hns*::*kan*$^r$ were both transformed with the final construct.

## 2.4 Acid stress treatment, AA30

Overnight cultures were grown in 10mL LB broth, with antibiotics when needed, at 37°C with shaking. 1.5mL of the overnight culture was subcultured into 8.5mL of high glucose (4.5 g/L) Dubellco's Minimal Media (DMEM) supplemented with 25mM MOPS, adjusted to pH 7.4 from 7.8 with concentrated hydrochloric acid (HCl). For each strain, two such subcultures were made. The subcultures were incubated at 37°C with shaking for approximately 3 hours, or until the $OD_{600}$ was 0.4-0.6. The subcultures were then centrifuged at 2630 x g (3500 rpm) at 4°C for 10 minutes. The supernatants were discarded. For each strain, one bacterial pellet was resuspended in 10mL of control DMEM, and the other in 10mL of acid adaptation DMEM. "Control DMEM" was buffered with 25mM MOPS and adjusted to pH 7.4 from 7.8 with concentrated HCl. "Acid adaptation DMEM" was buffered with 25mM MES and adjusted to pH 5.0 from 7.8 with concentrated HCl. These acid adaptation subcultures were incubated for 1 hour at 37°C with 5%$CO_2$. These acid adaptation and control subcultures were re-spun as described before, and the supernatants were again discarded. The bacterial pellets previously resuspended in control DMEM were again resuspended in 10mL of control

DMEM. The bacterial pellets previously resuspended in acid adaptation were resuspended in 10mL of acid stress DMEM. Acid stress DMEM was unbuffered and adjusted to pH 3.0 from 7.8 with concentrated HCl. These acid stress and control subcultures were incubated for 30 minutes at 37°C with 5%$CO_2$. These acid stress and control subcultures were re-spun as described before, and the supernatants were again discarded. All bacterial pellets were resuspended in 10mL sterile 1X PBS, and re-spun as described before. The supernatant was discarded, and this PBS wash step was repeated a second time. The resulting bacterial pellets were then either freshly prepared for SDS-PAGE and Western blot analysis, stored at -80°C prior to SDS-PAGE sample preparation, or analyzed for promoter activity in beta-galactosidase assays.

## 2.5 Short-chain fatty acid (SCFA) stress treatment

Initial cultures of each strain were started in 5mL of LB broth, with antibiotics added as needed, incubated for 5-6 hours at 37°C with shaking. After 5-6 hours, the $OD_{600}$ value was taken for each strain and based on those values the amount of culture needed to obtain a 10mL subculture with an initial $OD_{600}$ value of 0.01 was calculated. This volume was added to two centrifuge tubes for each strain and centrifuged at 2630 x g (3500 rpm) at 18°C for 10 minutes. The supernatant was discarded. One sample for each strain served as the control sample and was resuspended in 10mL of LB broth, with antibiotics added as needed. The other sample for each strain served as the SCFA stressed sample and was resuspended in 9.5mL of LB broth and 0.5mL of 20X 90mM SCFA mix, with antibiotics added as needed. The 20X 90mM SCFA solution was made up in miliQ-filtered water. At a 1X concentration in either LB or DMEM, the 90mM SCFA mix included 60.5mM sodium acetate, 24.5mM sodium propionate, and 3mM sodium butyrate. All samples were incubated overnight at 37°C with shaking. The following morning, all samples were diluted. For the control samples previously incubated in only LB broth, 1mL of the overnight subculture was added to 7mL of DMEM buffered with 100mM pH 6.7 MOPS, with antibiotics added as needed. For the SCFA stressed samples previously incubated with 90mM SCFA LB broth, 1mL of the overnight subculture was added to 7mL of 90mM SCFA DMEM buffered with 100mM pH 6.7 MOPS. All samples were then incubated at 37°C with 5% $CO_2$ for 2 hours. After this incubation, all samples

were re-spun as described before. The supernatant was discarded, and the bacterial pellets all resuspended in 8mL sterile 1X PBS and re-spun as described before. This PBS wash step was repeated a second time. The resulting bacterial pellets were then either freshly prepared for SDS-PAGE and Western blot analysis, stored at -80°C prior to SDS-PAGE sample preparation, or analyzed for promoter activity in beta-galactosidase assays.

## 2.6 Beta-galactosidase promoter activity assays

After either acid stress, strains carrying either pMC1403 or pMC1403-P*ecpR* were assayed for levels of beta-galactosidase activity[49]. The final $OD_{600}$ values were taken for each sample, and 20uL of acid or SCFA stressed sample was added to 80uL of permeabilization solution (100mM $Na_2HPO_4$, 20mM KCl, 2mM $MgSO_4$, 0.8mg/mL hexadecyltrimethylammonium bromide (CTAB), 0.4mg/mL sodium deoxycholate, 5.4µL/mL beta-mercaptoethanol) in a 96-well flat bottom plate[49,83]. Samples were then incubated at 30°C for 30 minutes. 25uL of each sample was transferred to a new well on the 96-well plate, and 150uL of substrate solution (60mM $Na_2HPO_4$, 40mM $NaH_2PO_4$, 1mg/mL o-nitrophenyl-beta-D-Galactoside (ONPG), 2.7µL/mL beta-mercaptoethanol) was added to each sample to start the enzymatic reaction[49,83]. The absorbance of each sample was recorded at 414 nm at 10-minute intervals, for a total of 2 hours. Miller units were calculated as follows[83]:

$$\text{Miller Units} = 1000 \times \frac{\text{Absorbance}_{414}}{OD_{600} \times \text{reaction volume (mL)} \times \text{reaction time (minutes)}}$$

## 2.7 Western blotting

### 2.7.1 Sample preparation

Specialized SDS-PAGE sample preparation was needed in order to visualize EcpA monomers with western blotting. Exact volumes varied between acid and SCFA stressed samples, but the following volumes are given based on 10mL of original sample. Bacterial pellets obtained, either fresh or previously stored at -80°C, were resuspended in 180uL autoclaved distilled water adjusted with concentrated HCl to pH 1.5. The resuspended sample was transferred to a microcentrifuge tube and placed in a heating block at 95°C for 5 minutes. 20uL 20% w/v SDS was added for a final concentration of 2% SDS, and the samples were incubated at 95°C for 10 minutes. At this point, a total of 15uL was removed to determine the total protein concentration of each sample through a standard BCA assay. BSA standards used in these assays also contained a final SDS concentration of 2%. 49uL of 5X SDS-free sample buffer (10mM beta-mercaptoethanol, 20 % v/v glycerol, 0.2M Tris-HCl of pH 6.8 and 0.05 % w/v bromophenol blue) was added to samples, resulting in yellow or orange samples. Samples were then neutralized with 8uL 1N NaOH, confirmed with the sample color changing to a dark navy blue. Samples were again incubated at 95°C for 5 minutes, then stored at -20°C until needed.

### 2.7.2 SDS-PAGE and transfer

Samples were separated through SDS-PAGE on gels with an 6% polyacrylamide stacking gel (2.6mL distilled water, 1mL 30% acrylamide/bisacrylamide mix, 2mL 1.5M Tris-HCl pH 8.8, 40uL 20% SDS, 50uL 10% APS, 5uL TEMED) and a 15% polyacrylamide resolving gel (1.8mL distilled water, 4mL 30% acrylamide/bisacrylamide mix, 1.25mL 0.5M Tris-HCl pH 6.8, 25uL 20% SDS, 80uL 10% ammonium persulfate (APS), 8uL TEMED). Gels were run in a BioRad apparatus at a constant voltage of 150V for 90 minutes in 1X SDS-PAGE running buffer (25mM Tris-HCl pH 8.3, 200mM glycine, 0.1% SDS). Molecular weight standards were run along with samples, throughout this study several commercial molecular weight standards were used: PiNK Plus Pre-stained Protein Ladder (FroggaBio), Precision Plus Protein Western Blot Standard (BioRad), and Prescision Plus Protein All Blue Standards (BioRad). The

separated proteins were then transferred to a polyvinylidene fluoride membrane overnight at 4°C at a constant voltage of 30V in 1X transfer buffer (25mM Tris-HCl pH 8.3, 200mM glycine, 20% v/v methanol). After transfer, the gel was stained for 30 minutes in Biosafe Coomassie stain, and destained for 1 hour in distilled water, to ensure a successful transfer.

### 2.7.3 Western blotting

After transfer, membranes were incubated in a blocking solution (1X PBST with 5% w/v non-fat powdered milk) for 1 hour at room temperature with gentle shaking. The membranes were then washed twice for 5 minutes each wash in 1X PBST at room temperature with gentle shaking. Polyclonal α-EcpA, kindly provided by Dr. Jorge Girón from the University of Florida, was applied to washed membranes at a dilution of 1:3000 in 1X PBST and incubated with membranes overnight at 4°C with gentle shaking. The membranes were then washed again as described before, twice for 5 minutes each and then three times for 10 minutes each. Lanes containing a sample of Precision Plus Protein Western Blot Standard (BioRad), made up of Strep tagged recombinant proteins, were separated from the rest of the blot and incubated with horseradish peroxidase conjugated StrepTactin diluted 1:5000 in PBST and incubated for 1 hour at room temperature.Horseradish peroxidase (HRP) conjugated goat-anti-rabbit was diluted 1:10,000 in 1X PBST and incubated with the remaining membrane for 1 hour at room temperature with gentle shaking. The blots were again washed five times as described before. An enhanced chemiluminscence (ECL) solution was applied to the blot for a total exposure time of 5 minutes and the results were visualized on either x-ray film or a gel doc with chemiluminescent capabilities.

## 2.8 Statistical analysis

All figures are presented as the mean ± standard deviation. One-way ANOVA, performed with Prism 6, was used to determine significance, with P value $\leq 0.05$ considered significant.

# 3. Results

## 3.1: Construction of EDL933 isogenic mutants

In this study, two regulatory proteins, H-NS and UvrY, were suspected of playing a role in regulating expression of EcpA under acid or SCFA stress. Isogenic mutants lacking these genes were created in order to potentially understand how EcpA is regulated under acid or SCFA stress; more specifically to determine if the previously observed increases in EcpA expression were modulated by those regulators. Additionally it was also necessary to create an isogenic mutant lacking *ecpA* to serve as a negative control.

Homologous recombination was the method used to produce the isogenic mutants; specifically λ-red recombinase mediated homologous recombination[16]. First, EDL933 was transformed with pKD46, a temperature sensitive plasmid that encodes λ-red recombinase. Next, primers were designed which would amplify the kanamycin resistance cassette present on pKD4, but which also contained regions homologous to the region of EDL933 genomic DNA just upstream and downstream of the target gene. High-fidelity PCR yielded linear portions of DNA that were then used to transform EDL933 (pKD46) via electroporation. Following a recovery period, samples were plated on LB-Kan plates and candidate colonies were screened via colony PCR for successful integration of the kanamycin cassette, indicating replacement of the target gene (Figures 3.1, 3.2, and 3.3). Colonies that appeared to contain the kanamycin resistance cassette were submitted for sequence analysis to further confirm the replacement of the target gene (Figures 3.4, 3.5, and 3.6).

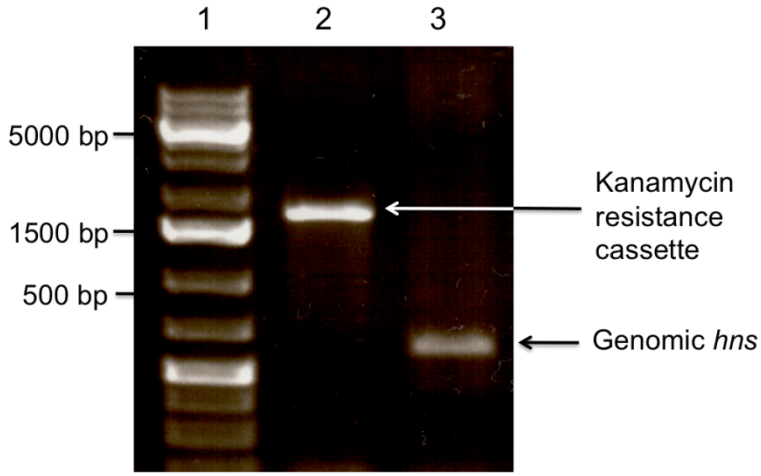



**Figure 3.1: PCR confirmation of kanamycin cassette in EDL933 *hns*::Kan$^R$.**
The lane contents were as follows: 1 – molecular weight standard, 2 - EDL933 *hns*::Kan$^R$, 3 – EDL933. The primers used to generate these products (*hns* check F and *hns* check R) were specific to a region outside of genomic *hns* in EDL933. The band in lane 2 (~1500 bp) is significantly larger than the faint band in lane 3 (~500 bp) due to the integration of the kanamycin resistance cassette from pKD4 (950 bp) in place of genomic *hns* (414 bp).

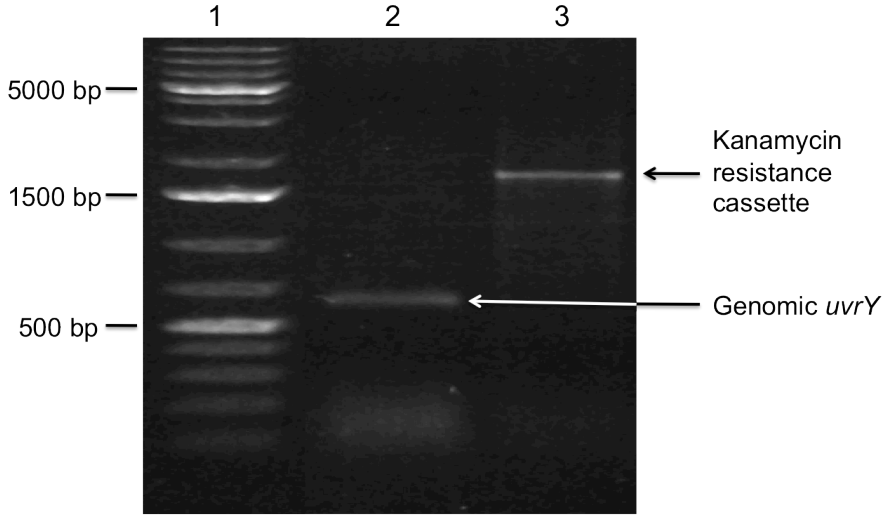



**Figure 3.2: PCR confirmation of kanamycin cassette in EDL933 *uvrY*::Kan$^R$.**
The lane contents were as follows: 1 – molecular weight standard, 2 – EDL933, 3 – EDL933 *uvrY*::Kan$^R$. The primers used to generate these products (*uvrY* check F and *uvrY* check R) were specific to a region outside of genomic *uvrY* in EDL933. The band in lane 3 (~1500 bp) is significantly larger than the band in lane 2 (~600 bp) due to the integration of the kanamycin resistance cassette from pKD4 (950 bp) in place of genomic *uvrY* (657 bp).

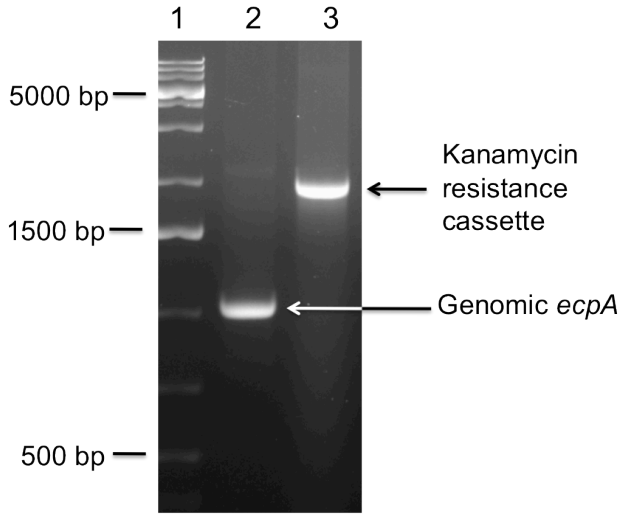



**Figure 3.3: PCR confirmation of kanamycin cassette in EDL933 *ecpA*::Kan$^{R}$.**
The lane contents were as follows: 1 – molecular weight standard, 2 – EDL933, 3 – EDL933 *ecpA*::Kan$^{R}$. The primers used to generate these products (*ecpA* check F and *ecpA* check R) were specific to a region outside of genomic *ecpA* in EDL933. The band in lane 3 (~1800 bp) is significantly larger than the band in lane 2 (~1000 bp) due to the integration of the kanamycin resistance cassette from pKD4 (950 bp) in place of genomic *ecpA* (588 bp).

```
Alignment of Sequence_1:  [portion from KanR check F [Backup 2011-06-30 12_05_46_12:05
PM]] with  Sequence_2: [genomic + KanR insert]

Similarity : 885/890 (99.44 %)

Seq_1  1     ------------TCTtATtAATTGtCTT-AACCGGACAATAAAAAATCCCGCCGATGGCG  47
                         |   | ||||||||| ||||||||||||||||||||||||||||||
Seq_2  1736  TTATAGCTTATTCTTATTAAATTGTCTTAAACCGGACAATAAAAAATCCCGCCGATGGCG  1677

Seq_1  48    GGATTTTAAgcaagtgcaAtCTACAAAAGAATGGGAATTAGCCATGGTCCATATGAaTaT  107
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  1676  GGATTTTAAGCAAGTGCAATCTACAAAAGAatgggaattagccatggtccatatgaatat  1617

Seq_1  108   CCTCCTTAGTTCCTATTCCGAAGTTCCTATTCTCTAGAAAGTATAGGAACTTCAGAGCGC  167
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  1616  cctccttagttcctattccgaagttcctattctctagaaagtataggaacttcagagcgc  1557

Seq_1  168   TTTTGAAGCTGGGGTGGGCGAAGAACTCCAGCATGAGATCCCCGCGCTGGAGGATCATCC  227
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  1556  ttttgaagctggggtgggcgaagaactccagcatgagatccccgcgctggaggatcatcc  1497

Seq_1  228   AGCCGGCGTCCCGGAAAACGATTCCGAAGCCCAACCTTTCATAGAAGGCGGCGGTGGAAT  287
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  1496  agccggcgtcccggaaaacgattccgaagcccaacctttcatagaaggcggcggtggaat  1437

Seq_1  288   CGAAATCTCGTGATGGCAGGTTGGGCGTCGCTTGGTCGGTCATTTCGAACCCCAGAGTCC  347
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  1436  cgaaatctcgtgatggcaggttgggcgtcgcttggtcggtcatttcgaaccccagagtcc  1377

Seq_1  348   CGCTCAgAAGAACTCGTCAAGAAGGCGATAGAAGGCGATGCGCTGCGAATCGGGAGCGGC  407
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  1376  cgctcagaagaactcgtcaagaaggcgatagaaggcgatgcgctgcgaatcgggagcggc  1317

Seq_1  408   GATACCGTAAAGCACGAGGAAGCGGTCAGCCCATTCGCCGCCAAGCTCTTCAGCAATATC  467
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  1316  gataccgtaaagcacgaggaagcggtcagcccattcgccgccaagctcttcagcaatatc  1257

Seq_1  468   ACGGGTAGCCAACGCTATGTCCTGATAGCGGTCCGCCACACCCAGCCGGCCACAGTCGAT  527
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  1256  acgggtagccaacgctatgtcctgatagcggtccgccacacccagccggccacagtcgat  1197

Seq_1  528   GAATCCAGAAAAGCGGCCATTTTCCACCATGATATTCGGCAAGCAGGCATCGCCATGGGT  587
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  1196  gaatccagaaaagcggccattttccaccatgatattcggcaagcaggcatcgccatgggt  1137

Seq_1  588   CACGACGAGATCCTCGCCGTCGGGCATGCGCGCCTTGAGCCTGGCGAACAGTTCGGCTGG  647
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  1136  cacgacgagatcctcgccgtcgggcatgcgcgccttgagcctggcgaacagttcggctgg  1077

Seq_1  648   CGCGAGCCCCTGATGCTCTTCGTCCAGATCATCCTGATCGACAAGACCGGCTTCCATCCG  707
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  1076  cgcgagccctgatgctcttcgtccagatcatcctgatcgacaagaccggcttccatccg  1017

Seq_1  708   AGTACGTGCTCGCTCGATGCGATGTTTCGCTTGGTGGTCGAATGGGCAGGTAGCCGGATC  767
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  1016  agtacgtgctcgctcgatgcgatgtttcgcttggtggtcgaatgggcaggtagccggatc  957

Seq_1  768   AAGCGTATGCAGCCGCCGCATTGCATCAGCCATGATGGATACTTTCTCGGCAGGAGCAAG  827
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  956   aagcgtatgcagccgccgcattgcatcagccatgatggatactttctcggcaggagcaag  897

Seq_1  828   GtGAGATGACAGGAGATCCTGCCCCGGCACTTCGCCCaATAGCAGCCAGTCCCTTCCCGC  887
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  896   gtgagatgacaggagatcctgccccggcacttcgcccaatagcagccagtcccttcccgc  837

Seq_1  888   TTC-------------------------------------------------------  890
             |||
Seq_2  836   ttcagtgacaacgtcgagcacagctgcgcaaggaacgcccgtcgtggccagccacgatag  777
```

**Figure 3.4: Sequence alignment result for EDL933 *hns*::Kan$^{R}$ .**
Seq_1 is representative of the sequence obtained from the candidate strain, while Seq_2 is representative of the desired EDL933 genomic sequence after successful replacement of *hns* with a kanamycin resistance cassette. The results, obtained with SerialCloner 2-6, confirmed the replacement of EDL933 genomic *hns* with the kanamycin resistance cassette of pKD4.

```
Alignment of Sequence_1:  [uvrY2 - F.xdna] with  Sequence_2: [uvrY mutant insertion -
final]

Similarity : 839/839 (100.00 %)

Seq_1  839   ----------------------------------------------ATTGGGCGAAGTGC  826
                                                           ||||||||||||||
Seq_2  1861  cagctgtgctcgacgttgtcactgaagcgggaagggactggctgctattgggcgaagtgc  1920

Seq_1  825   CGGGGCAGGATCTCCTGTCATCTCACCTTGCTCCTGCCGAGAAAGTATCCATCATGGCTG  766
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  1921  cggggcaggatctcctgtcatctcaccttgctcctgccgagaaagtatccatcatggctg  1980

Seq_1  765   ATGCAATGCGGCGGCTGCATACGCTTGATCCGGCTACCTGCCCATTCGACCACCAAGCGA  706
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  1981  atgcaatgcggcggctgcatacgcttgatccggctacctgcccattcgaccaccaagcga  2040

Seq_1  705   AACATCGCATCGAGCGAGCACGTACTCGGATGGAAGCCGGTCTTGTCGATCAGGATGATC  646
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  2041  aacatcgcatcgagcgagcacgtactcggatggaagccggtcttgtcgatcaggatgatc  2100

Seq_1  645   TGGACGAAGAGCATCAGGGGCTCGCGCCAGCCGAACTGTTCGCCAGGCTCAAGGCGCGCA  586
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  2101  tggacgaagagcatcaggggctcgcgccagccgaactgttcgccaggctcaaggcgcgca  2160

Seq_1  585   TGCCCGACGGCGAGGATCTCGTCGTGACCCATGGCGATGCCTGCTTGCCGAATATCATGG  526
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  2161  tgcccgacggcgaggatctcgtcgtgacccatggcgatgcctgcttgccgaatatcatgg  2220

Seq_1  525   TGGAAAATGGCCGCTTTTCTGGATTCATCGACTGTGGCCGGCTGGGTGTGGCGGACCGCT  466
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  2221  tggaaaatggccgcttttctggattcatcgactgtggccggctgggtgtggcggaccgct  2280

Seq_1  465   ATCAGGACATAGCGTTGGCTACCCGTGATATTGCTGAAGAGCTTGGCGGCGAATGGGCTG  406
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  2281  atcaggacatagcgttggctacccgtgatattgctgaagagcttggcggcgaatgggctg  2340

Seq_1  405   ACCGCTTCCTCGTGCTTTACGGTATCGCCGCTCCCGATTCGCAGCGCATCGCCTTCTATC  346
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  2341  accgcttcctcgtgctttacggtatcgccgctcccgattcgcagcgcatcgccttctatc  2400

Seq_1  345   GCCTTCTTGACGAGTTCTTCTGAGCGGGACTCTGGGGTTCGAAATGACCGACCAAGCGAC  286
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  2401  gccttcttgacgagttcttctgagcgggactctggggttcgaaatgaccgaccaagcgac  2460

Seq_1  285   GCCCAACCTGCCATCACGAGATTTCGATTCCACCGCCGCCTTCTATGAAAGGTTGGGCTT  226
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  2461  gcccaacctgccatcacgagatttcgattccaccgccgccttctatgaaaggttgggctt  2520

Seq_1  225   CGGAATCGTTTTCCGGGACGCCGGCTGGATGATCCTCCAGCGCGGGGATCTCATGCTGGA  166
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  2521  cggaatcgttttccgggacgccggctggatgatcctccagcgcggggatctcatgctgga  2580

Seq_1  165   GTTCTTCGCCCACCCCAGCTTCAAAAGCGCTCTGAAGTTCCTATACTTTCTAGAGAATAG  106
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  2581  gttcttcgcccaccccagcttcaaaagcgctctgaagttcctatactttctagagaatag  2640

Seq_1  105   GAACTTCGGAATAGGAACTAAGGAGGATATTCATATGGACCAtGGcTAATTCCCATGTGA  46
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Seq_2  2641  gaacttcggaataggaactaaggaggatattcatatggaccatggctaattcccatGTGA  2700

Seq_1  45    TCAGTTTGACGCAAAAGCGTTTTTAAAAACCGTAA-------------------------  11
             ||||||||||||||||||||||||||||||||||
Seq_2  2701  TCAGTTTGACGCAAAAGCGTTTTTAAAAACCGTAAGTGATCAGTTTGACGCAAAAGCGTT  2760
```

**Figure 3.5: Sequence alignment result for EDL933 *uvrY*::Kan$^{R}$ .**
Seq_1 is representative of the sequence obtained from the candidate strain, while Seq_2 is representative of the desired EDL933 genomic sequence after successful replacement of *uvrY* with a kanamycin resistance cassette. The results, obtained with SerialCloner 2-6, confirmed the replacement of EDL933 genomic *uvrY* with the kanamycin resistance cassette of pKD4.

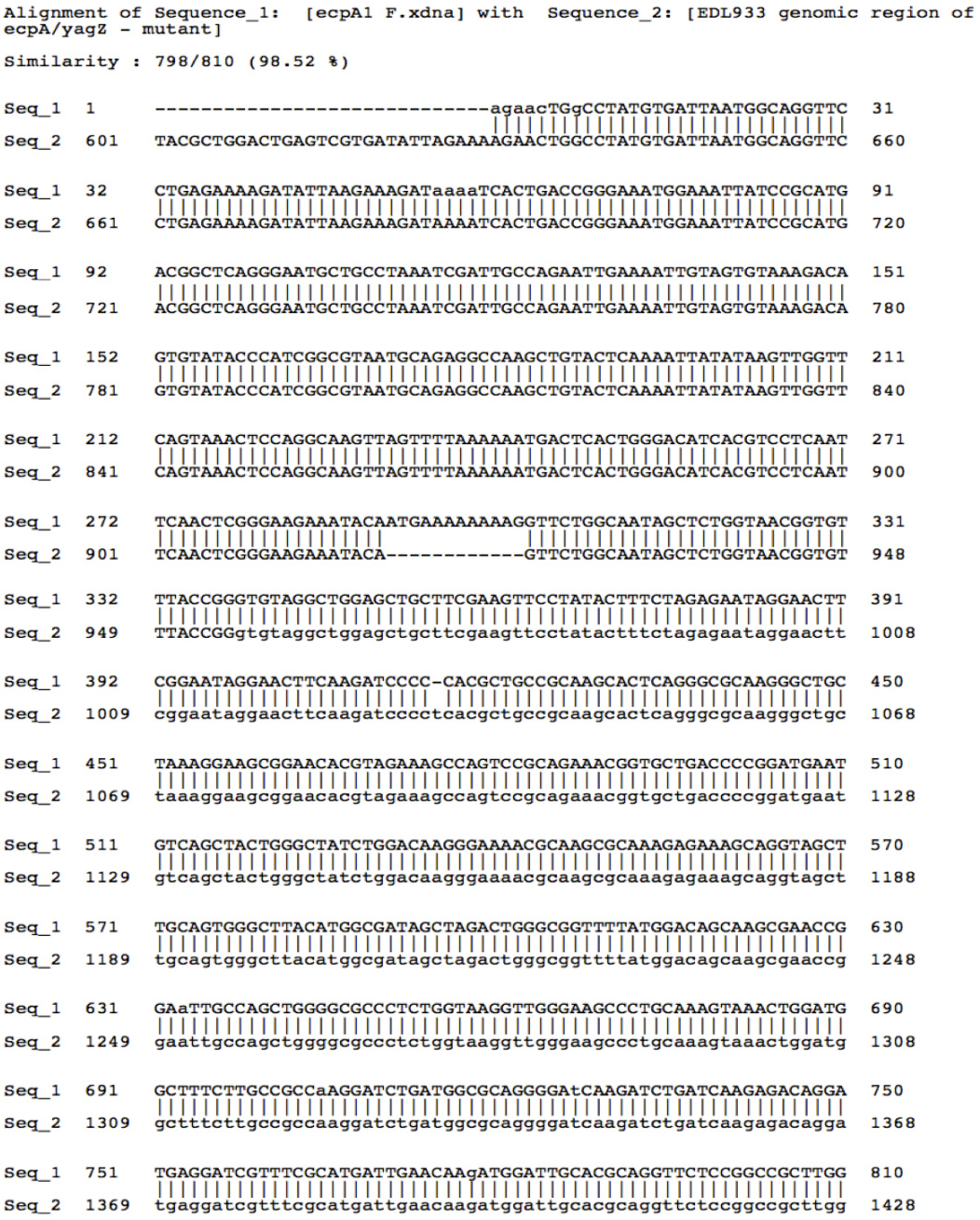



**Figure 3.6: Sequence alignment result for EDL933 *ecpA*::Kan$^{R}$ .**
Seq_1 is representative of the sequence obtained from the candidate strain, while Seq_2 is representative of the desired EDL933 genomic sequence after successful replacement of *ecpA* with a kanamycin resistance cassette. The results, obtained with SerialCloner 2-6, confirmed the replacement of EDL933 genomic *ecpA* with the kanamycin resistance cassette of pKD4.

## 3.2: EcpA Expression in *Escherichia coli* O157:H7 EDL933 and isogenic mutants

In order to confirm previously reported results of *ecpA* upregulation after acid stress, several varied attempts were made to observe expression of EcpA through Western blotting. It was reported by another group that simply boiling bacterial lysates in Laemmli buffer was insufficient to separate ECP into EcpA monomers, so for the most part, whole cell lysates were prepared by boiling pelleted EHEC in acidic water, pH 1.5, before the addition of Laemmli buffer[47,58]. The lysates were neutralized with 1N NaOH[47,58].

The results observed were in agreement with those which have been previously reported, and can be summarized as follows: EcpA expression was only observed during growth in minimal media (DMEM) and never in LB, regardless of temperature or atmosphere, and EcpA expression was most evident in samples grown statically at 26°C without $CO_2$ for at least 11 hours. It should be noted that the image is indicative of only two experiments and these results were more often not observed in replicates (Figure 3.7, 3.9). Under most conditions tested, EDL933 *hns*::Kan$^R$ demonstrated a strong band of approximately 21kDa, even when a comparable band was not detected in EDL933. However due to the presence of many non-specific bands for those samples, and the lack of an *hns*/*ecpA* double mutant, it cannot be confirmed that this band was specific to EcpA (Figure 3.8). EDL933 *uvrY*::Kan$^R$ was observed to have a profile of protein expression similar to that EDL933*ecpA*::Kan$^R$ when visualized with α-EcpA antibodies (Figure 3.9); but it should be noted that this result is only indicative of a single experiment and was not successfully replicated during this study.

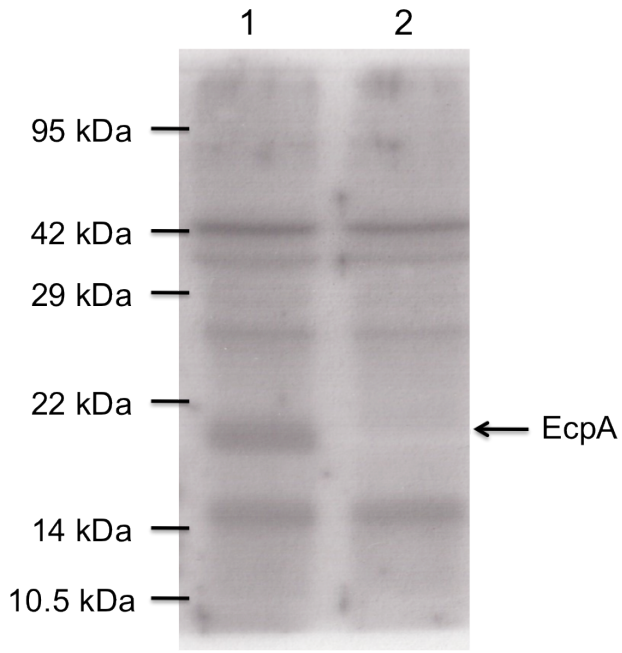



**Figure 3.7: Western blotting of EcpA in EDL933 and EDL933 *ecpA*::Kan$^R$ with α-EcpA.** Whole cell lysates of EDL933 and EDL933Δ*ecpA*::Kan$^R$ were prepared after overnight incubation at 37°C with shaking in LB followed by a static subculture in DMEM for 11 hours at 26°C. Each lane was loaded with 90μg total protein. Blots were probed with rabbit α-EcpA (1° antibody) at a dilution of 1:3000 followed by HRP-conjugated goat-anti-rabbit (2° antibody) at a dilution of 1:10,000. The lane contents were: 1 – EDL933, 2 - EDL933 *ecpA*::Kan$^R$. A 21kDa protein is observed only the in the EDL933 sample, demonstrating that EcpA can be expressed by EDL933 in these particular conditions.

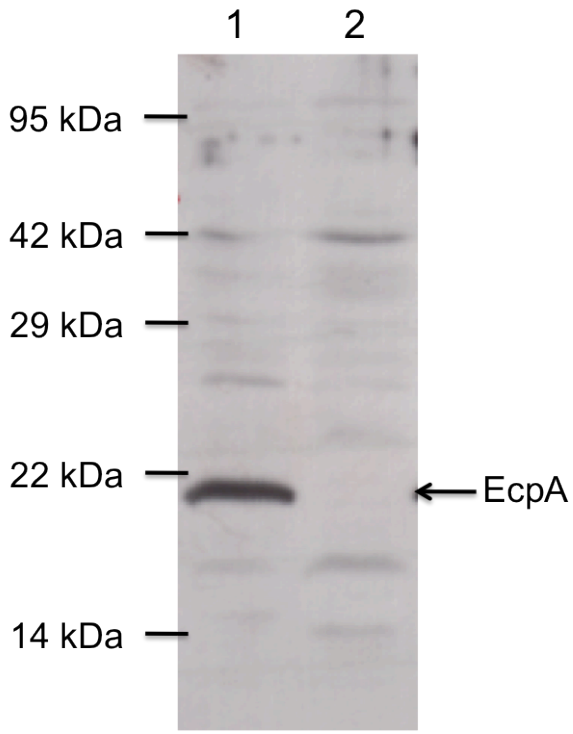



**Figure 3.8: Western blotting of EcpA in EDL933 and EDL933 *hns*::Kan$^R$ with α-EcpA.** Whole cell lysates of EDL933 and EDL933Δ*hns*::Kan$^R$ were prepared after overnight incubation at 37°C with shaking in LB followed by a static subculture in DMEM for 5 hours at 37°C with 5% $CO_2$. Each lane was loaded with 90μg total protein. Blots were probed with rabbit α-EcpA (1° antibody) at a dilution of 1:3000 followed by HRP-conjugated goat-anti-rabbit (2° antibody) at a dilution of 1:10,000. The lane contents were: 1 - EDL933 *hns*::Kan$^R$, 2 – EDL933. A strong 21kDa protein is observed in the EDL933 *hns*::Kan$^R$ sample but not in the EDL933 sample, potentially demonstrating that conditions that do not lead to EcpA expression in EDL933 might still allow a strain lacking H-NS to express EcpA.

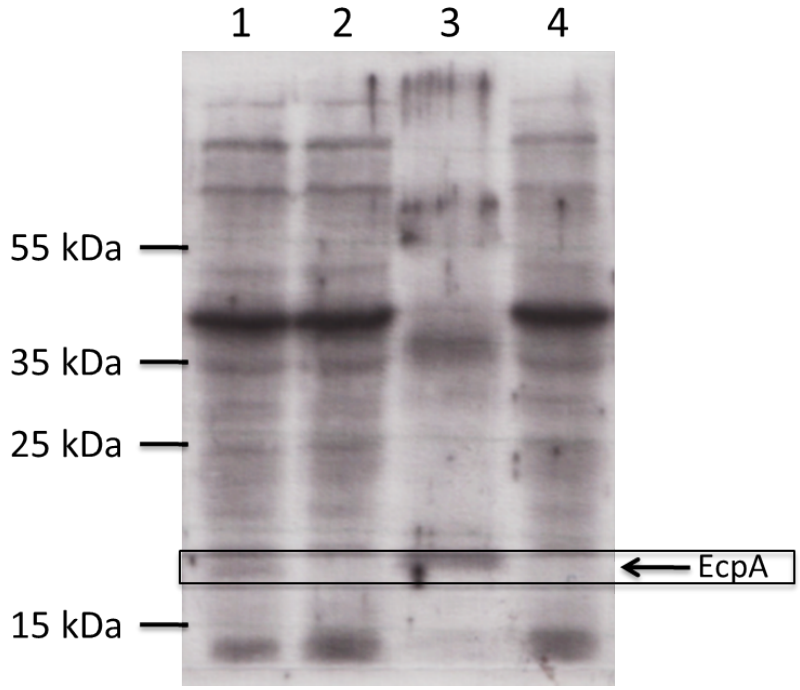



**Figure 3.9: Western blotting of EcpA in EDL933 and isogenic mutants with α-EcpA.** Whole cell lysates of EDL933 and EDL933Δ*hns*::Kan$^R$ were prepared after overnight incubation at 37°C with shaking in LB followed by a static subculture in DMEM for 11 hours at 26°C. Each lane was loaded with 90µg total protein. Blots were probed with rabbit α-EcpA (1° antibody) at a dilution of 1:3000 followed by HRP-conjugated goat-anti-rabbit (2° antibody) at a dilution of 1:10,000. The lane contents were: 1 – EDL933, 2 - EDL933 *ecpA*::Kan$^R$, 3 – EDL933 *hns*::Kan$^R$, 4 – EDL933 *uvrY*::KanR. A strong 21kDa protein is again observed in the EDL933 *hns*::Kan$^R$ sample however this time there is also a faint band, also 21kDa, in the EDL933 that is not present in either the EDL933 *ecpA*::Kan$^R$ sample or the EDL933 *uvrY*::Kan$^R$ sample. This same result was observed in one of four replicates of this experiment.

## 3.3: Construction of *ecpR* promoter fusion construct pMC1403-P*ecpR*, and subsequent transformation of EDL933 and EDL933 *hns*::Kan$^{R}$

A previous study has previously demonstrated the location of a promoter region for *ecpR*, and did so by integrating this promoter region upstream of a promoter-less chloramphenicol acetyltransferase (CAT) gene present, and performing routine CAT assays to observe promoter activity[47]. In this study, the majority of that promoter region was integrated upstream of a promoter-less gene encoding the enzyme beta-galactosidase (*lacZ*) on pMC1403. Construction of this promoter reporter construct makes it possible to observe changes in *ecpR* promoter activity under different environmental conditions, including acid or SCFA stress, as well as in isogenic regulatory mutants.

Primers were designed to amplify the promoter region from EDL933 genomic DNA, and were also designed to include recognition sites for BamHI and XmaI. Both the resulting PCR products and pMC1403 were digested with both BamHI and XmaI, followed by ligation and transformation of DH5α and eventual plating of the transformation reactions on LB-Amp. Candidate colonies were screened via colony PCR for integration of the promoter region into pMC1403. Colonies that yielded positive results with PCR underwent mini-prep plasmid isolation, and subsequent sequence analysis to confirm successful in-frame integration of the promoter region (Figure 3.10). The final plasmid, now referred to as pMC1403-P*ecpR*, was then used to transform both EDL933 and EDL933 *hns*::Kan$^{R}$ (Figures 3.11 and 3.12).

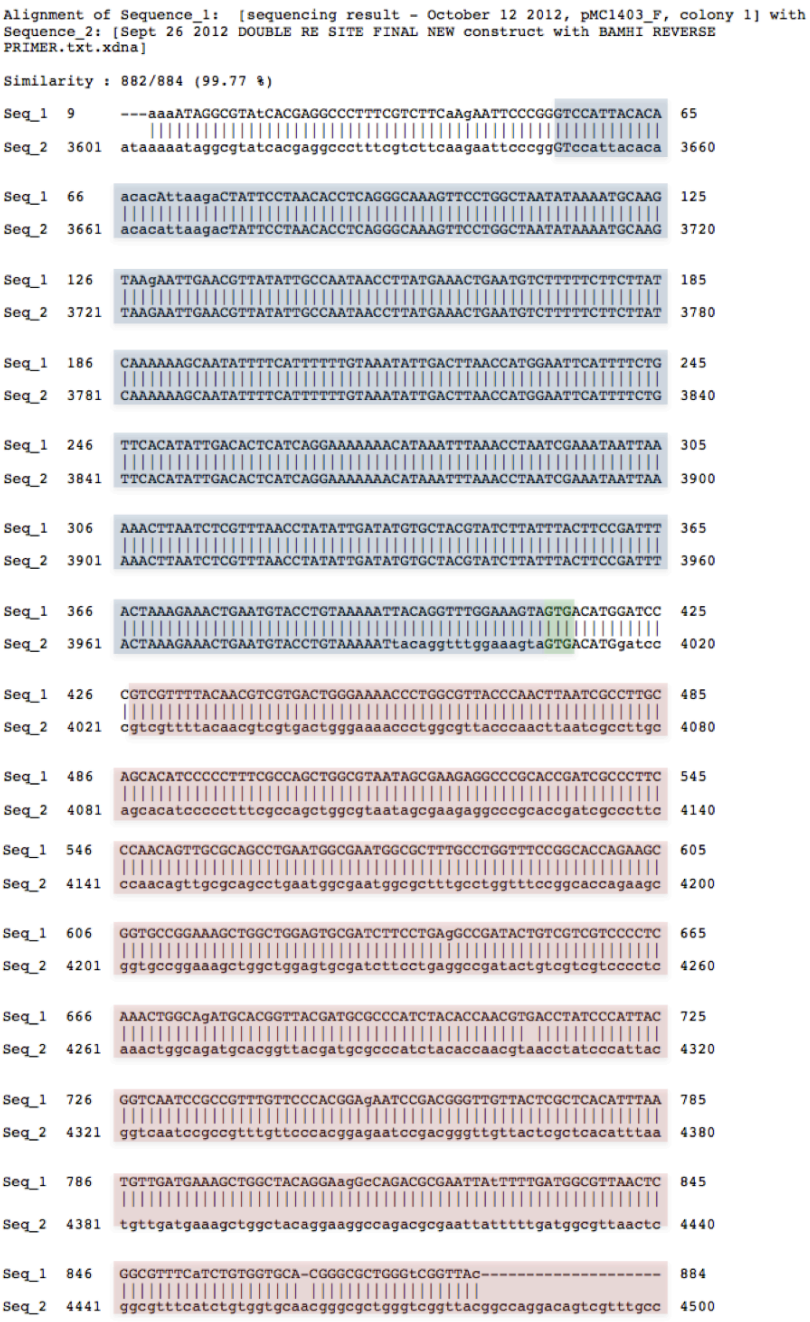



**Figure 3.10: Sequence alignment result for pMC1403-P*ecpR*.**
Seq_1 is representative of the sequence obtained from the candidate plasmid, while Seq_2 is representative of the desired promoter construct sequence after successful integration of the *ecpR* promoter region into the BamHI and XmaI sites of pMC1403. Results, obtained with SerialCloner 2-6, confirmed the in-frame integration of the *ecpR* promoter region in front of the promoter-less *lacZ* present on pMC1403. Highlighted in blue is the inserted promoter region, in green in the *ecpR* start codon, and in red is *lacZ*.

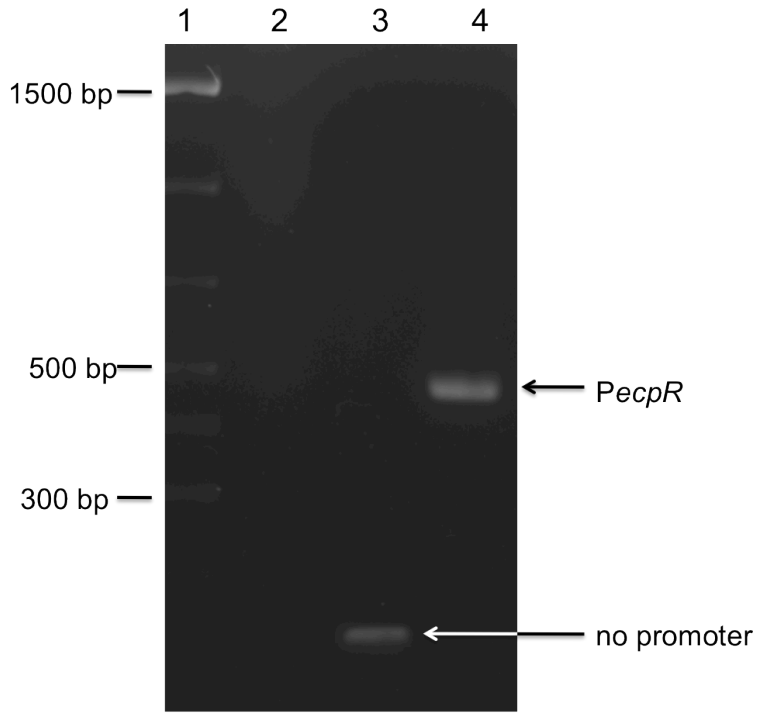



**Figure 3.11: Colony PCR confirmation of transformation of EDL933 with pMC1403 and pMC1403-P*ecpR*.** The lane contents were as follows: 1 – molecular weight standard, 2 – EDL933, 3 – EDL933(pMC1403), 4 – EDL933(pMC1403-P*ecpR*). The primers used to generate these products (pMC1403 F and pMC1403 R) were specific to regions just outside of the multiple cloning site of pMC1403. The band in lane 4 (~500 bp) is significantly larger than the faint band in lane 3 (~130 bp) due to the insertion of the *ecpR* promoter region into the BamHI and XmaI sites of pMC1403. The lack of a band in lane 2 confirms that either pMC1403 or pMC1403-P*ecpR* served as template DNA for the PCR reaction, rather than EDL933 genomic DNA.

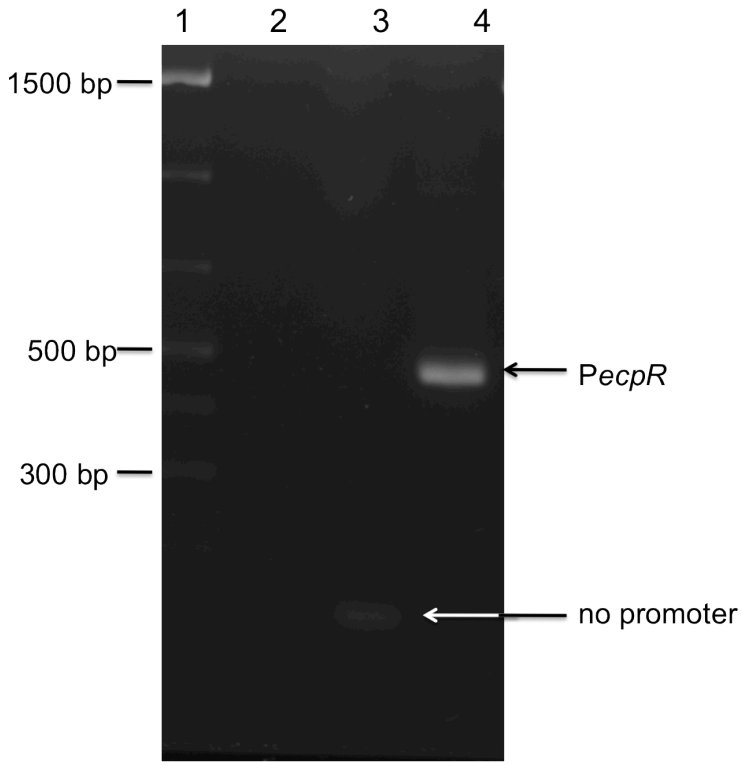



**Figure 3.12: PCR confirmation of transformation of EDL933 *hns*::Kan$^R$ with pMC1403 and pMC1403-P*ecpR*.** The lane contents were as follows: 1 – molecular weight standard, 2 – EDL933 *hns*::Kan$^R$, 3 – EDL933 *hns*::Kan$^R$ (pMC1403), 4 – EDL933 *hns*::Kan$^R$ (pMC1403-P*ecpR*). The primers used to generate these products (pMC1403 F and pMC1403 R) were specific to regions just outside of the multiple cloning site of pMC1403. The band in lane 4 (~500 bp) is significantly larger than the faint band in lane 3 (~130 bp) due to the insertion of the *ecpR* promoter region into the BamHI and XmaI sites of pMC1403. The lack of a band in lane 2 confirms that either pMC1403 or pMC1403-P*ecpR* served as template DNA for the PCR reaction, rather than EDL933Δ*hns*::Kan$^R$ genomic DNA.

## 3.4: Promoter activity of P*ecpR* under acid stress

To establish evidence of promoter activity with strains transfected with pMC1403-P*ecpR*, strains of EDL933 carrying either the empty vector (pMC1403) or the promoter construct (pMC1403-P*ecpR*) were grown in conditions previously reported to allow P*ecpR* promoter activity and tested for promoter activity. Samples were grown overnight in LB at 37°C with shaking, followed by a 6 hour subculture in DMEM also at 37°C with shaking. These samples were analyzed for *ecpR* promoter activity via beta-galactosidase activity assays (Figure 3.13).

To determine the impact of acid stress on *ecpR* promoter activity, EDL933 (pMC1403) and EDL933 (pMC1403-P*ecpR*) underwent acid stress (AA30). Unstressed (UU30) controls of each were also processed. These samples were then analyzed for *ecpR* promoter activity via beta-galactosidase activity assays (Figure 3.14, 3.16).

Additionally, to establish whether H-NS plays a role in modulating EcpA expression in response to acid stress, EDL933 *hns*::Kan$^R$ (pMC1403) and EDL933 *hns*::Kan$^R$ (pMC1403-P*ecpR*) were also exposed to AA30 and UU30 conditions and assayed for promoter activity with beta-galactosidase activity assays (Figure 3.15, 3.16).

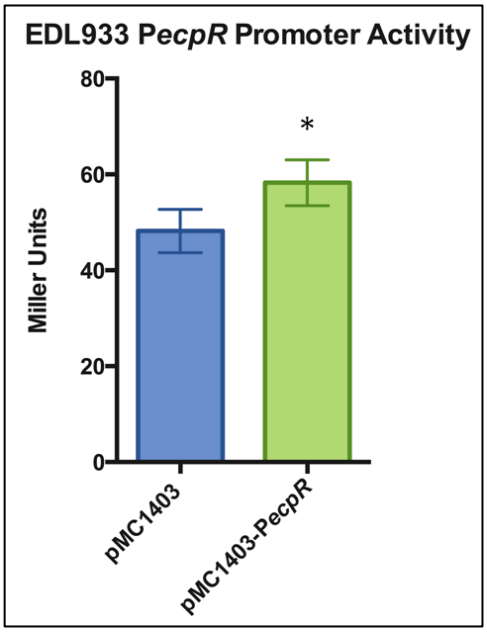



**Figure 3.13: Confirmation of pMC1403-P*ecpR* activity.** Promoter activity in EDL933 (pMC1403) and EDL933 (pMC1403-P*ecpR*) was assessed under unstressed conditions previously reported to demonstrate P*ecpR* activity, samples were cultured overnight in LB with shaking at 37°C, then subcultured for 6 hours in DMEM with shaking at 37°C. This was done in order to confirm that the promoter construct is functioning as expected. Significant differences ($P \leq 0.05$) were observed between the carrying the empty vector (pMC1403) and the strain carrying the promoter construct (pMC1403-P*ecpR*). This 1.8 fold increase in beta-galactosidase activity is representative of two independent experiments.

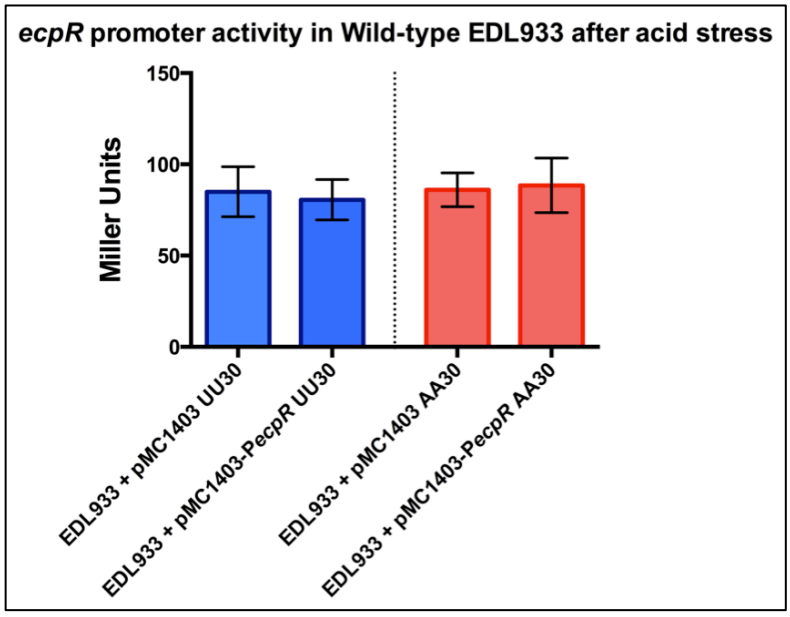



**Figure 3.14: *ecpR* promoter activity in EDL933 after acid stress.** Promoter activity in EDL933 (pMC1403) and EDL933 (pMC1403-P*ecpR*) was assessed for both UU30 (unadapted, unstressed 30 mins) samples and AA30 (acid adapted, acid stressed 30 mins) samples. No significant differences ($P > 0.05$) were observed between strains carrying the empty vector (pMC1403) and strains carrying the promoter construct (pMC1403-P*ecpR*). (N=3, n=9)

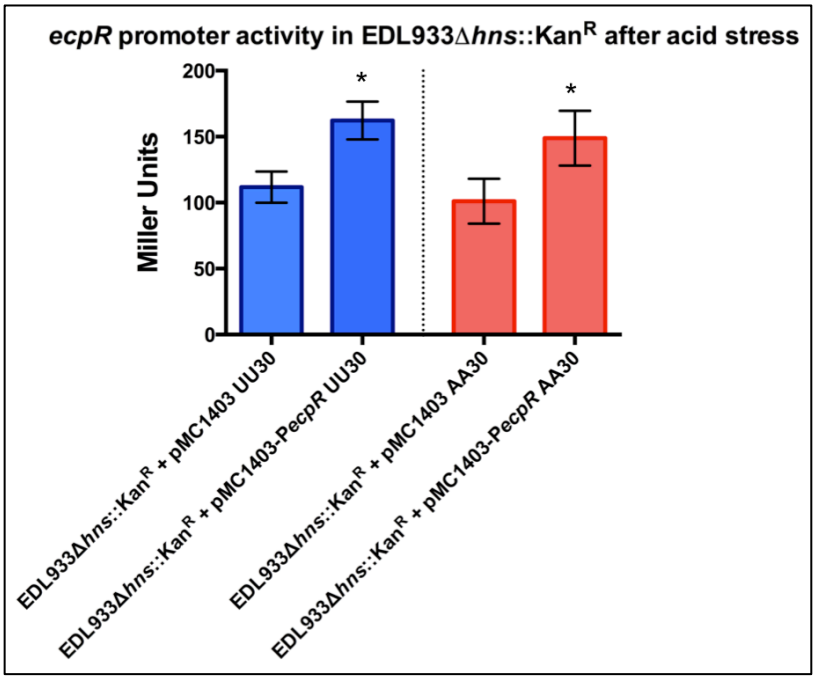



**Figure 3.15: *ecpR* promoter activity in EDL933 *hns*::Kan$^R$ after acid stress.**
Promoter activity in EDL933 *hns*::Kan$^R$ (pMC1403) and EDL933 *hns*::Kan$^R$ (pMC1403-P*ecpR*) was assessed for both UU30 (unadapted, unstressed 30 mins) samples and AA30 (acid adapted, acid stressed 30 mins) samples. Significant differences (*P ≤ 0.05) were observed between strains carrying the empty vector (pMC1403) and strains carrying the promoter construct (pMC1403-P*ecpR*). (N=3, n=9)

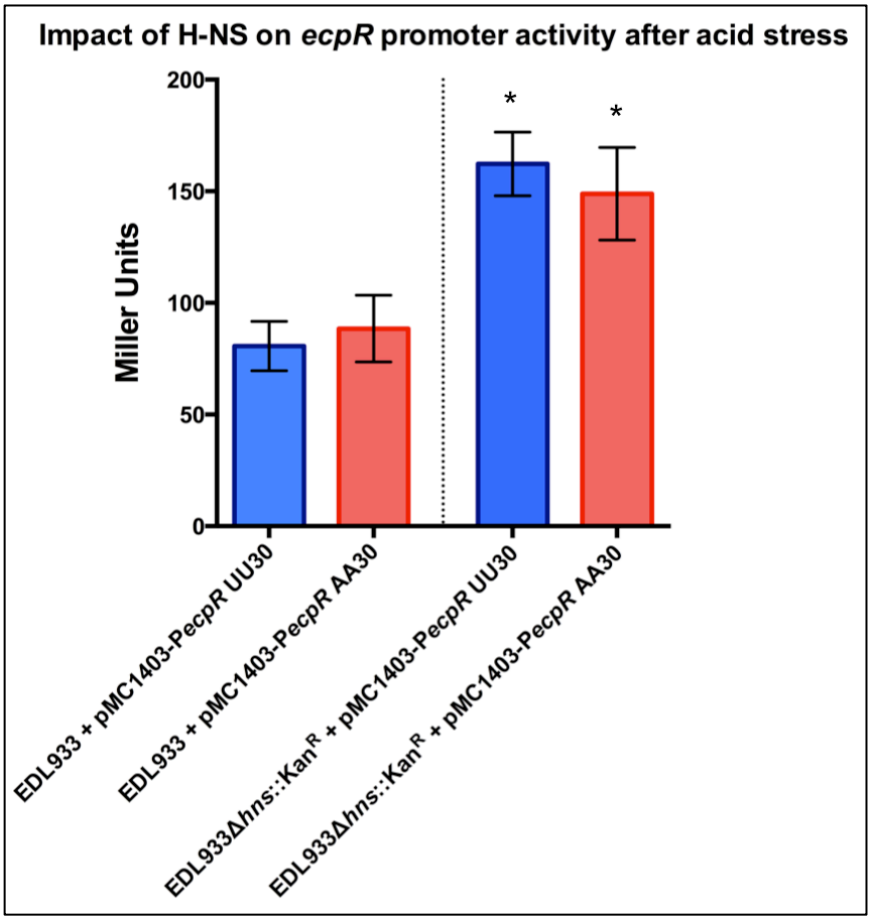



**Figure 3.16: Effect of H-NS on *ecpR* promoter activity in EDL933 acid stress.** Promoter activity in EDL933 (pMC1403-P*ecpR*) and EDL933 *hns*::Kan$^R$ (pMC1403-P*ecpR*) was assessed for both UU30 (unadapted, unstressed 30 mins) samples and AA30 (acid adapted, acid stressed 30 mins) samples. Significant differences ($P \leq 0.05$) were observed between EDL933 and EDL933 *hns*::Kan$^R$ samples for both UU30 and AA30 samples. No significant differences (*$P > 0.05$) were observed between UU30 treated samples and AA30 samples.
(N=3, n=9)

# 4. Discussion

The human body has in place many lines of defense against enteric pathogens. Among these are acute acid exposure in the stomach, and SCFA exposure in the large intestine. Enterohaemorrhagic *E. coli* O157:H7 is an enteric pathogen, with major health implications for humans, that has adapted to survive those defenses. In fact, rather than succumb to those gastrointestinal stresses, this pathogen has evolved to be able to use the stresses as signals of location within a host, allowing for successful colonization and infection. In a DNA microarray analysis of gene expression in O157:H7 it was shown that many genes related to virulence and adhesion were upregulated after acid stress[31]. Among the many genes to be affected was *ecpA*, the major subunit of the *E. coli* common pilus. This structure was also demonstrated to be an important adhesion factor for EHEC; a mutant strain lacking *ecpA* showed a significant decrease in adhesion to epithelial cells[27,57]. This phenotype, a decrease in adhesion, was also observed in EHEC that had been exposed to either acid or SCFA stress conditions[27].

While previous studies have suggested a link between acid or SCFA stress and expression levels of EcpA, the exact mechanisms of regulation for EcpA under those conditions is not fully understood. Two other research groups have determined that the ECP operon is under negative regulation by H-NS, a global regulator; and that it was under positive regulation by EcpR, encoded in the first gene of the operon[43,47]. Additionally, it was suggested through a personal communication that the mRNA for *ecpA* is a target for a global post-transcriptional regulator, CsrA. CsrA is known to be inhibited through activation of upstream pathways by exposure to SCFAs[12,]. This led to the formation of the hypothesis that EcpA expression is modulated on both a transcriptional and translational level by different regulatory mechanisms. Under acid stress, it was hypothesized that decreases in H-NS would allow for an increase in *ecpR* promoter activity, and in turn, transcription of *ecpA*. Under SCFA stress, it was hypothesized that inhibition of CsrA would allow for an increase in translation of *ecpA* mRNAs. Both scenarios would provide an explanation for the previous results which indicated an upregulation of *ecpA* mRNA after acid stress, and an increase in ECP-

mediated adhesion, potentially due to an increase in levels of ECP and thus EcpA, after either acid or SCFA stress.

Isogenic mutants of EDL933 lacking either *hns* or *uvrY* were constructed so that regulatory mechanisms for changes in *ecpA* expression could be elucidated. Both H-NS and UvrY are already known to regulate expression of other genes, and in fact, it was previously demonstrated that H-NS regulates the *ecp* operon, however neither has been directly implicated in regulation of *ecpA* in response to acid or SCFA stress. λ-red recombinase-mediated homologous recombination was employed to switch out the chromosomal target gene for a kanamycin resistance cassette. In addition, a mutant lacking the study's gene of interest, *ecpA*, was constructed as a negative control when analyzing protein expression levels. Primers were designed to amplify a kanamycin resistance cassette, and included regions of homology just outside of the target gene. This allowed EHEC already expressing λ-red recombinase via pKD46 to undergo homologous recombination. Replacement of target genes was confirmed with both colony PCR (Figure 3.1, 3.2, 3.3) and sequence alignment (Figure 3.4, 3.5, 3.6). The confirmed mutants were used to characterize *ecpA* expression and regulation in both Western blots and beta-galactosidase activity assays.

Western blots using α-EcpA antibodies demonstrated that ECP expression is under very specific environmental control (Figure 3.7, 3.8, 3.9), a result that is consistent when reviewing other bodies of research involving Western blot detection of EcpA (Table 4.1). In this study, no expression of EcpA was detected in samples of EDL933 grown in LB, nor in samples grown at 37°C without $CO_2$ supplementation. Exposure to acid stress (AA30 conditions) or SCFA stress (90mM mix) did not appear to impact EcpA expression, despite previously reported microarray or adhesion assay results that would suggest otherwise.

## Table 4.1: Impact of strain and culture conditions on EcpA expression by Western blot

| | Overnight conditions | | | | | |
|---|---|---|---|---|---|---|
| **Strain** | Temp. | Media | Shaking? | Environment | **Band Intensity** | **Reference** |
| EHEC O157:H7 EDL933 | 37°C | DMEM | yes | $O_2$ | + | Rendón et al., 2007 (58) |
| EHEC O157:H7 EDL933 | 37°C | DMEM | no | 5% $CO_2$ | ++ | |
| EHEC O157:H7 EDL933 | 37°C | DMEM | no | anaerobic | + | |
| EHEC O157:H7 EDL933 | 37°C | LB | yes | $O_2$ | 0 | |
| EHEC O157:H7 EDL933 | 37°C | LB | no | 5% $CO_2$ | 0 | |
| EHEC O157:H7 EDL933 | 37°C | LB | no | anaerobic | 0 | |
| EHEC O157:H7 EDL933 | 20°C | DMEM | yes | $O_2$ | ++ | |
| EHEC O157:H7 EDL933 | 20°C | DMEM | no | 5% $CO_2$ | +++ | |
| EHEC O157:H7 EDL933 | 20°C | DMEM | no | anaerobic | ++ | |
| EHEC O157:H7 EDL933 | 20°C | LB | yes | $O_2$ | 0 | |
| EHEC O157:H7 EDL933 | 20°C | LB | no | 5% $CO_2$ | 0 | |
| EHEC O157:H7 EDL933 | 20°C | LB | no | anaerobic | 0 | |
| EHEC O157:H7 EDL933 | 26°C | DMEM | no | $O_2$ | + | |
| EHEC O157:H7 86-24 | 26°C | DMEM | no | $O_2$ | + | |

**Table 4.1: Impact of strain and culture conditions on EcpA expression by Western blot (continued)**

| Strain | Growth conditions | | | | | Band Intensity | Reference |
|---|---|---|---|---|---|---|---|
| | Temp. | Media | Shaking? | Environment | Growth Phase | | |
| NMEC O18ac:K1:H7 IHE 3034 | 37°C | DMEM | yes | $O_2$ | Mid-logarithmic | 0 | Lehti et al., 2013 (43) |
| NMEC O18ac:K1:H7 IHE 3034 | 37°C | DMEM | yes | 5% $CO_2$ | Mid-logarithmic | 0 | |
| NMEC O18ac:K1:H7 IHE 3034 | 37°C | LB, pH 7.1 | yes | $O_2$ | Mid-logarithmic | + | |
| NMEC O18ac:K1:H7 IHE 3034 | 37°C | LB, pH 5.5 | yes | $O_2$ | Mid-logarithmic | + | |
| NMEC O18ac:K1:H7 IHE 3034 | 37°C | LB + 0.1M acetate, pH 7.1 | yes | $O_2$ | Mid-logarithmic | ++ | |
| NMEC O18ac:K1:H7 IHE 3034 | 37°C | DMEM | yes | $O_2$ | Stationary | 0 | |
| NMEC O18ac:K1:H7 IHE 3034 | 37°C | DMEM | yes | 5% $CO_2$ | Stationary | 0 | |
| NMEC O18ac:K1:H7 IHE 3034 | 37°C | LB, pH 7.1 | yes | $O_2$ | Stationary | 0 | |
| NMEC O18ac:K1:H7 IHE 3034 | 37°C | LB, pH 5.5 | yes | $O_2$ | Stationary | + | |
| NMEC O18ac:K1:H7 IHE 3034 | 37°C | LB + 0.1M acetate, pH 7.1 | yes | $O_2$ | Stationary | + | |
| NMEC O18ac:K1:H7 IHE 3034 | 20°C | DMEM | yes | $O_2$ | Mid-logarithmic | + | |
| NMEC O18ac:K1:H7 IHE 3034 | 20°C | LB, pH 7.1 | yes | $O_2$ | Mid-logarithmic | ++ | |
| NMEC O18ac:K1:H7 IHE 3034 | 20°C | LB, pH 5.5 | yes | $O_2$ | Mid-logarithmic | +++ | |
| NMEC O18ac:K1:H7 IHE 3034 | 20°C | LB + 0.1M acetate, pH 7.1 | yes | $O_2$ | Mid-logarithmic | ++ | |
| NMEC O18ac:K1:H7 IHE 3034 | 20°C | DMEM | yes | $O_2$ | Stationary | + | |
| NMEC O18ac:K1:H7 IHE 3034 | 20°C | LB, pH 7.1 | yes | $O_2$ | Stationary | ++ | |
| NMEC O18ac:K1:H7 IHE 3034 | 20°C | LB, pH 5.5 | yes | $O_2$ | Stationary | +++ | |
| NMEC O18ac:K1:H7 IHE 3034 | 20°C | LB + 0.1M acetate, pH 7.1 | yes | $O_2$ | Stationary | ++ | |

## Table 4.1: Impact of strain and culture conditions on EcpA expression by Western blot (continued)

| | Overnight conditions | | | | Subculture conditions | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Strain** | Temp. | Media | Shaking? | Environment | Temp. | Media | Shaking? | Environment | Time | **Band Intensity** | **Reference** |
| EHEC O157:H7 EDL933 | 37°C | LB | yes | $O_2$ | 26°C | DMEM | no | $O_2$ | NS* | + | Martínez-Santos et al., 2012 (47) |
| EHEC O157:H7 EDL933 *hns*::Kan$^R$ | 37°C | LB | yes | $O_2$ | 26°C | DMEM | no | $O_2$ | NS | +++ | |

| | Overnight conditions | | | | Subculture conditions | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Strain** | Temp. | Media | Shaking? | Environment | Temp. | Media | Shaking? | Environment | Time | **Band Intensity** | **Reference** |
| EHEC O157:H7 EDL933 | 37°C | LB | yes | $O_2$ | 26°C | DMEM | no | $O_2$ | 11 hrs | + | This study |
| EHEC O157:H7 EDL933 *uvrY*::Kan$^R$ | 37°C | LB | yes | O2 | 26°C | DMEM | no | $O_2$ | 11 hrs | 0 | |
| EHEC O157:H7 EDL933 *hns*::Kan$^R$ | 37°C | LB | yes | $O_2$ | 26°C | DMEM | no | $O_2$ | 11 hrs | +++ | |

* NS: not specified

There was preliminary evidence (Figure 3.9) that the BarA/UvrY two-component transduction system is indeed regulating expression of EcpA, potentially through inhibition of CsrA. Western blotting revealed that a sample of EDL933 *uvrY*::Kan$^R$, subcultured in DMEM statically for at 26°C for 11 hours had the same protein expression profile as EDL933 *ecpA*::Kan$^R$; both lacked a 21kDa band present in EDL933 and EDL933 *hns*::Kan$^R$, all samples were cultured in the same conditions. Previous researchers have demonstrated that BarA/UvrY is activated in *E. coli* grown in minimal media[35]. BarA/UvrY upregulates transcription of two small non-coding RNAs, CsrB and CsrC, resulting in inhibition of global post-transcriptional regulator CrsA, and allowing for translation of CsrA's target mRNAs[12,35,68]. This result, while only indicative of a single replicate, suggests that in EHEC cultured in minimal media (DMEM) and lacking UvrY, translation of *ecpA* mRNA is inhibited, preventing expression of EcpA. While exposure to SCFAs at concentrations as low as 6mM have also been shown to activate BarA/UvrY, in this study no differences in expression of EcpA were observed between EDL933 and EDL933 *uvrY*::KanR samples exposed to a 90mM SCFA mix.

On the other hand, Western blots consistently revealed a strong band at 21kDa, likely that of EcpA, in samples of EDL933 *hns*::Kan$^R$ probed with α-EcpA (Figure 3.8). This was seen in a variety of culture conditions, including AA30 and UU30 conditions, 90mM SCFA stressed and unstressed control samples, unstressed DMEM subculture at 37°C with 5% $CO_2$, and unstressed DMEM subculture at 26°C without $CO_2$. This result is consistent with previous research results indicating a role for H-NS as a negative regulator of the *ecp* operon[43,47]. Due to a lack of similar bands consistently present in EDL933 samples, it is difficult to conclude with certainty that the band observed is EcpA, but it is consistent with the absence of the band in EDL933 *ecpA*::Kan$^R$.

While H-NS is a negative regulator of *ecpA*, it does not appear to be directly regulating *ecpA* directly in response to acid stress conditions. Promoter activity assays showed an increase in EDL933 *hns*::Kan$^R$ samples under both AA30 and UU30 conditions when compared to EDL933 samples of the same conditions. However there

was no significant difference observed between AA30 and UU30 promoter activity levels in both EDL933 and EDL933 *hns*::Kan$^R$ (Figures 3.15, 3.16).

A recent publication by Lehti et al. investigated differential regulation of ECP/Mat fimbria in a broad range of *E. coli* isolates covering several phylogenetic groups[43]. They observed that while there is extensive homology in *ecp* operon upstream regulatory regions, the conditions under which promoter activity was induced varied greatly[43]. They hypothesize that while there is a conserved regulatory mechanism that acts on the *ecp* operon, involving H-NS, IHF, and EcpR, that there are likely strain-specific transcription factors, under environmental control, that dictate the expression of the *ecp* operon[43]. The lack of difference in promoter activity in acid stressed versus control EHEC indicates that H-NS likely not the regulatory protein directly responsible for increases in *ecpA* mRNA levels under AA30 conditions previously observed with the DNA microarray, and that the EHEC-specific transcription factors involved remain unknown. It should also be noted that the microarray, while performed with samples from an EHEC O157:H7 strain, was not performed with EDL933, the primary strain used in this work. The use of a closely related but different strain, 86-24, could also be the cause of conflicting results between promoter activity observed in EDL933 AA30 samples and the increase in *ecpA* expression in 86-24 AA30 samples from the microarray, due to differences in strain-specific transcription factors regulating expression of the *ecp* operon.

The EHEC *ecp* operon promoter region used in this study, P*ecpR*, was first reported by other researchers, Martinez-Santos et al[47]. In their search for an *ecp* operon transcriptional regulatory system they analyzed several regions upstream of the *ecp* operon via chloramphenicol acetyltransferase (CAT) resistance assays and found this region to contain both positive and negative regulatory elements[47]. In that study, the regions with promoter activity extended 198 bp downstream of the transcriptional start site (TSS), however during design of pMC1403-PecpR, used in this study, it was decided that altering the 3' end of the promoter region such that it only extended 120 bp downstream of the TSS, to the end of the third codon of *ecpR*, would have no impact on

promoter activity as all regulatory elements described by the previous research were still present in the region used (Figure 4.1). It was also assumed for this study that the use of a different reporter system for promoter activity, beta-galactosidase activity rather than CAT resistance, would be insignificant as both are well characterized systems for assessing promoter activity levels.

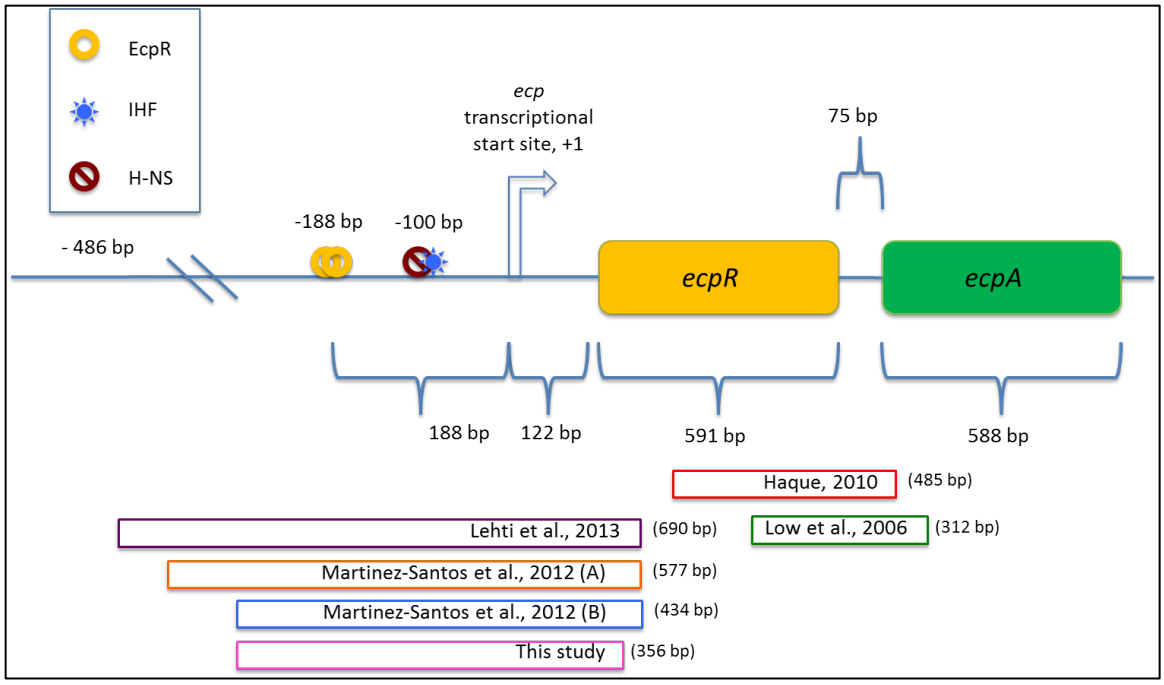



**Figure 4.1: Comparison of *ecp* operon promoter regions used for *ecp* operon transcriptional analysis.** Potential *ecp* operon promoter regions shown here were used in either CAT resistance assays or beta-galactosidase activity assays to determine *ecp* transcription levels in different commensal *E. coli*, NMEC, or EHEC strains under various environmental conditions. See Table 4.2 for more detail. Image is not drawn to scale.

It was surprising then that in beta-galactosidase assays performed on samples of EDL933 grown in conditions reported in the previous study to induce *ecp* operon promoter activity did not yield high levels of beta-galactosidase activity. Rather the values obtained were close to values obtained with the empty reporter vector while a previously characterized positive control vector, pMC1403-P*acrA*, routinely provided very high levels of activity. Beta-galactosidase assays performed with EDL933 after AA30 treatment also showed low levels of promoter activity. Lehti et al, who looked at phylogenic differences in *ecp* operon and promoter regions, have also examined

transcriptional regulation of the *ecp* operon in NMEC and K12 strains, through beta-galactosidase activity assays[43]. Their findings indicate that the *ecp* operon regulatory region, which is highly conserved when compared to EHEC strains, extends farther upstream than initially believed, but that there is also a positive regulatory element found in the intergenic region between *ecpR* and *ecpA*. It is therefore likely that the region examined in this study was not the optimal region for analyzing *ecp* operon promoter activity; additionally it is possible that there are differences in the configuration and spacing of inserted promoter regions with regard to the reporter gene in different reporter systems. These issues could account for the lack of activity observed with an already established promoter region.

## Table 4.2: Impact of strain, culture conditions, and promoter region on *ecp* operon promoter activity

| | | | Growth conditions | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Strain** | **Promoter region** | **Reporter system** | Temp. | Media | Shaking? | Environment | Growth phase | **Results** | **Negative control** | **Negative control activity** | **Reference** |
| EHEC O157:H7 ZAP193 | Low et al., 2006 | Single copy *lacZ* fusion | 37°C | LB | no | $O_2$ | Exponential | ~25 MU* | wild-type strain | not reported | Low et al., 2006 (46) |
| EHEC O157:H7 ZAP193 | Low et al., 2006 | Single copy *lacZ* fusion | 37°C | LB | no | $O_2$ | Stationary | ~20 MU | wild-type strain | not reported | |
| EHEC O157:H7 ZAP193 | Low et al., 2006 | Single copy *lacZ* fusion | 37°C | LB | no | $O_2$ | 24 hr static growth | ~20 MU | wild-type strain | not reported | |
| EHEC O157:H7 ZAP193 | Low et al., 2006 | Single copy *lacZ* fusion | 37°C | MEM | no | $O_2$ | Exponential | ~12 MU | wild-type strain | not reported | |
| EHEC O157:H7 ZAP193 | Low et al., 2006 | Single copy *lacZ* fusion | 37°C | MEM | no | $O_2$ | Stationary | ~12 MU | wild-type strain | not reported | |
| EHEC O157:H7 ZAP193 | Low et al., 2006 | Single copy *lacZ* fusion | 37°C | MEM | no | $O_2$ | 24 hr static growth | ~25 MU | wild-type strain | not reported | |
| EHEC O157:H7 ZAP193 | Low et al., 2006 | Single copy *lacZ* fusion | 37°C | M9 | no | $O_2$ | Exponential | ~12 MU | wild-type strain | not reported | |
| EHEC O157:H7 ZAP193 | Low et al., 2006 | Single copy *lacZ* fusion | 37°C | M9 | no | $O_2$ | Stationary | ~15 MU | wild-type strain | not reported | |
| EHEC O157:H7 ZAP193 | Low et al., 2006 | Single copy *lacZ* fusion | 37°C | M9 | no | $O_2$ | 24 hr static growth | ~20 MU | wild-type strain | not reported | |
| EHEC O157:H7 ZAP193 | Low et al., 2006 | Single copy *lacZ* fusion | 28°C | LB | no | $O_2$ | Exponential | ~20 MU | wild-type strain | not reported | |
| EHEC O157:H7 ZAP193 | Low et al., 2006 | Single copy *lacZ* fusion | 28°C | LB | no | $O_2$ | Stationary | ~35 MU | wild-type strain | not reported | |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| EHEC O157:H7 ZAP193 | Low et al., 2006 | Single copy *lacZ* fusion | 28°C | LB | no | $O_2$ | 24 hr static growth | ~45 MU | wild-type strain | not reported | |
| EHEC O157:H7 ZAP193 | Low et al., 2006 | Single copy *lacZ* fusion | 34°C | LB | no | $O_2$ | Exponential | ~40 MU | wild-type strain | not reported | |
| EHEC O157:H7 ZAP193 | Low et al., 2006 | Single copy *lacZ* fusion | 34°C | LB | no | $O_2$ | Stationary | ~25 MU | wild-type strain | not reported | |
| EHEC O157:H7 ZAP193 | Low et al., 2006 | Single copy *lacZ* fusion | 37°C | LB | no | $O_2$ | Exponential | ~25 MU | wild-type strain | not reported | |
| EHEC O157:H7 ZAP193 | Low et al., 2006 | Single copy *lacZ* fusion | 37°C | LB | no | $O_2$ | Stationary | ~15 MU | wild-type strain | not reported | |
| EHEC O157:H7 ZAP193 | Low et al., 2006 | Single copy lacZ fusion | 37°C | LB | no | O2 | 24 hr static growth | ~25 MU | wild-type strain | not reported | |
| EHEC O157:H7 ZAP193 | Low et al., 2006 | Single copy *lacZ* fusion | 37°C | McIlvanines Minimal Buffer, pH 7 | no | $O_2$ | NS** | ~20 MU | wild-type strain | not reported | |
| EHEC O157:H7 ZAP193 | Low et al., 2006 | Single copy *lacZ* fusion | 37°C | McIlvanines Minimal Buffer, pH 5.2 | no | $O_2$ | NS | ~10 MU | wild-type strain | not reported | |
| EHEC O157:H7 ZAP193 | Low et al., 2006 | Single copy *lacZ* fusion | 37°C | LB | no | Anaerobic | NS | ~12 MU | wild-type strain | not reported | |
| EHEC O157:H7 ZAP193 | Low et al., 2006 | Single copy *lacZ* fusion | 37°C | LB | yes | $O_2$ | NS | ~15 MU | wild-type strain | not reported | |

* MU: Miller Units
** NS: not specified

## Table 4.2: Impact of strain, culture conditions, and promoter region on *ecp* operon promoter activity (continued)

| | | | Overnight growth conditions | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Strain** | **Promoter region** | **Reporter system** | Temp. | Media | Shaking? | Environment | **Treatment** | **Results** | **Negative control** | **Negative control activity** | **Reference** |
| EHEC O157:H7 86-24 | Haque, 2010 | Promoter-less *lacZ* vector, pMC1403 | 37°C | LB | yes | $O_2$ | AA30 | 30.14 MU | pMC1403 | not reported | Haque, 2010 (27) |
| EHEC O157:H7 86-24 | Haque, 2010 | Promoter-less *lacZ* vector, pMC1403 | 37°C | LB | yes | $O_2$ | UU30 | 37.95 MU | pMC1403 | not reported | |
| EHEC O157:H7 86-24 | Haque, 2010 | Promoter-less *lacZ* vector, pMC1403 | 37°C | LB with 90mM SCFA mix | yes | $O_2$ | 90mM SCFA stressed | 7.08 MU | pMC1403 | not reported | |
| EHEC O157:H7 86-24 | Haque, 2010 | Promoter-less *lacZ* vector, pMC1403 | 37°C | LB | yes | $O_2$ | unstressed control | 23.91 MU | pMC1403 | not reported | |

| | | | Overnight conditions | | | | Subculture conditions | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Strain** | **Promoter region** | **Reporter system** | Temp. | Media | Shaking? | Environment | Temp. | Media | Shaking? | Environment | Time | **Results** | **Negative control** | **Negative control activity** | **Reference** |
| EHEC O157:H7 EDL933 | Martinez-Santos et al., 2012 (A) | Promoter-less *cat* vector, pKK232-8 | 37°C | LB | yes | $O_2$ | 37°C | DMEM | yes | $O_2$ | 6 hrs | ~225 umole/min/mg CAT specific activity | pKK232-8 | no activity observed | Martinez-Santos et al., 2012 (47) |
| EHEC O157:H7 EDL933 | Martinez-Santos et al., 2012 (B) | Promoter-less *cat* vector, pKK232-8 | 37°C | LB | yes | $O_2$ | 37°C | DMEM | yes | $O_2$ | 6 hrs | ~225 umole/min/mg CAT specific activity | pKK232-8 | no activity observed | |

## Table 4.2: Impact of strain, culture conditions, and promoter region on *ecp* operon promoter activity (continued)

| Strain | Promoter region | Reporter system | Growth conditions: Temp. | Media | Shaking? | Environment | Growth phase | Results | Negative control | Negative control activity | Reference |
|---|---|---|---|---|---|---|---|---|---|---|---|
| NMEC O18ac:K1:H7 IHE 3034 | Lehti et al., 2013 | Single copy *lacZ* fusion | 37°C | LB | yes | $O_2$ | Mid-exponential | ~3000 MU | wild-type strain | ~100 MU | Lehti et al., 2013 (43) |
| NMEC O18ac:K1:H7 IHE 3034 | Lehti et al., 2013 | Single copy lacZ fusion | 20°C | LB | yes | $O_2$ | Mid-exponential | ~8000 MU | wild-type strain | ~100 MU | |

| Strain | Promoter region | Reporter system | Overnight growth conditions: Temp. | Media | Shaking? | Environment | Treatment | Results | Negative control | Negative control activity | Reference |
|---|---|---|---|---|---|---|---|---|---|---|---|
| EHEC O157:H7 EDL933 | This study | Promoter-less *lacZ* vector, pMC1403 | 37°C | LB | yes | $O_2$ | AA30 | 88.5 MU | pMC1403 | 86.1 MU | This study |
| EHEC O157:H7 EDL933 | This study | Promoter-less *lacZ* vector, pMC1403 | 37°C | LB | yes | $O_2$ | UU30 | 80.6 MU | pMC1403 | 85.0 MU | |
| EHEC O157:H7 EDL933 *hns*::Kan$^R$ | This study | Promoter-less *lacZ* vector, pMC1403 | 37°C | LB | yes | $O_2$ | AA30 | 148.9 MU | pMC1403 | 101.1 MU | |
| EHEC O157:H7 EDL933 *hns*::Kan$^R$ | This study | Promoter-less *lacZ* vector, pMC1403 | 37°C | LB | yes | $O_2$ | UU30 | 162.3 MU | pMC1403 | 111.7 MU | |
| EHEC O157:H7 EDL933 | This study | Promoter-less lacZ vector, pMC1403 | 37°C | LB | yes | $O_2$ | 6 hr. DMEM subculture( 37°C, with shaking, $O_2$) | 58.3 MU | pMC1403 | 48.2 MU | |

The ultimate goal of this work was to elucidate the regulatory mechanisms at work in ECP-mediated adhesion under acid or SCFA stress. Several approaches were attempted, but within the scope of this study, none were successful in discovering exactly what molecular mechanisms were responsible for increases in expression of EcpA specifically under physiologically relevant exposure to acute acid stress mimicking passage through the stomach, or SCFA stress experienced during colonization of the distal colon. This could have been due to several factors; prominently among them was a lack of strongly specific α-EcpA antisera; the results observed varied greatly between experimental replicates. This led to inconclusive results with regard to EcpA production levels under acid or SCFA stress. Without western blot results that clearly indicate changes in EcpA production in the parental strain under acid or SCFA stress, it is in turn not possible to determine if the hypothesized regulators, H-NS and UvrY, are responsible for directly modulating expression of EcpA under acid or SCFA stress. Without baseline EcpA production levels from acid or SCFA stressed EDL933, no comparison was possible.

Overall, there still remains the potential of a regulatory protein in EHEC EDL933 modulating *ecpA* transcription under AA30 conditions; however it does not appear to be H-NS, a known *ecp* operon and global regulator. The link between SCFA activation of BarA/UvrY and increased translation of *ecpA* mRNA via interaction with CsrA was not confirmed, but remains a viable possibility and is summarized, in cooperation with acid stress modulation of *ecpA*, in Figure 4.1.

ECP is known to be an important factor in EHEC adhesion to abiotic surfaces, to host epithelial cells, and to other bacterial cells, depending on their environment. Because EHEC is an enteric pathogen that colonizes the colon, it has in place survival strategies to withstand environmental stresses encountered in the human gastrointestinal tract such as acute acid and SCFA exposure. It is an exciting possibility then that EHEC have not only evolved to survive those stresses, but also to use them as cues to sense their location within a host organism and modulate gene expression accordingly.

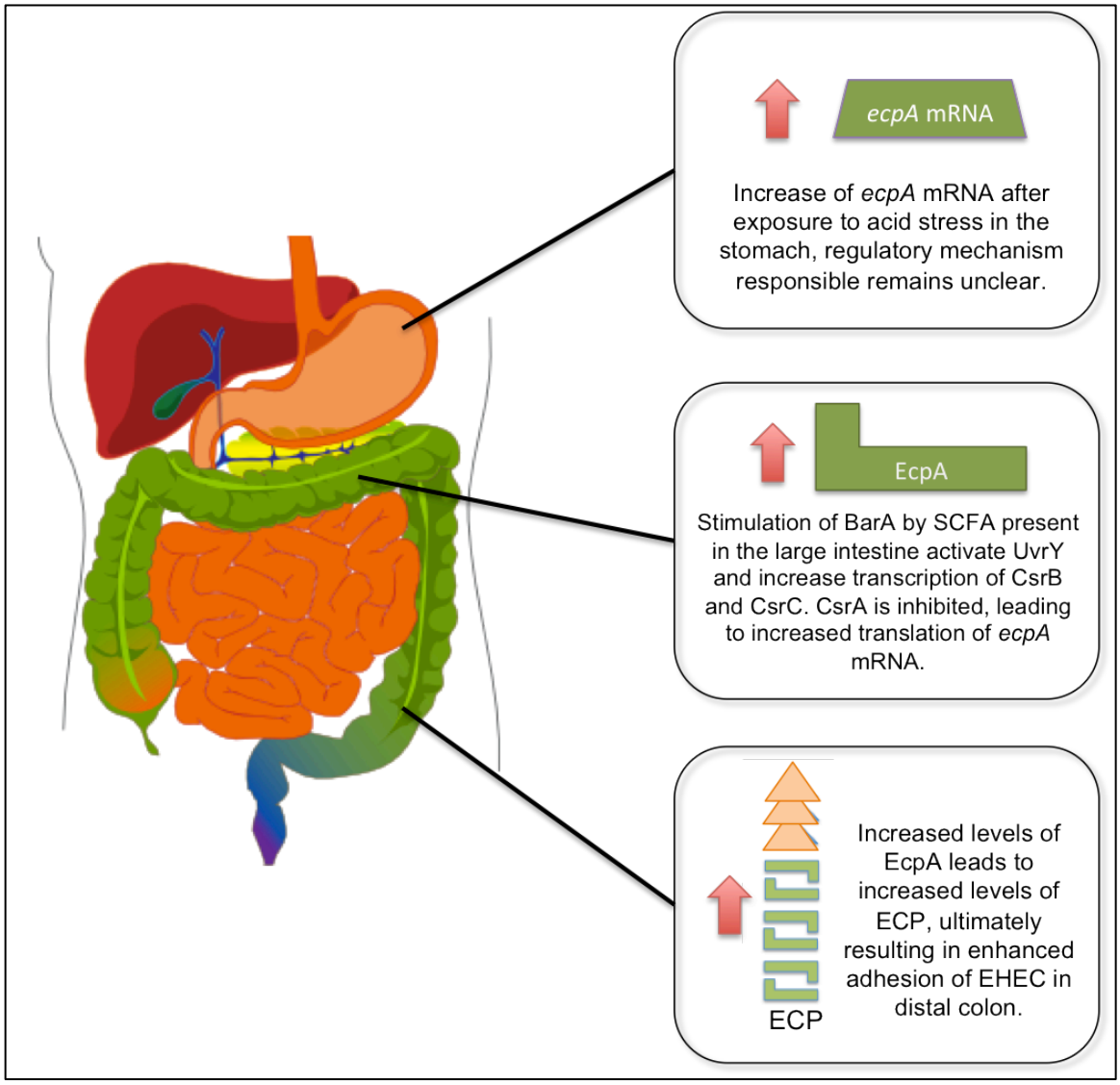



**Figure 4.2: Potential mechanisms modulating *ecpA* expression in EHEC O157:H7 under acid and SCFA stress.** Previous results have indicated that ECP-mediated adhesion to epithelial cells, as well as *ecpA* transcription is enhanced in acid stressed (AA30) EHEC. Similar increases were seen with adhesion of SCFA (90mM) stressed EHEC to epithelial cells. Currently, the exact regulatory mechanisms that modulate *ecpA* transcription during acid stress are unknown. It is hypothesized that exposure to SCFA in the large intestine results in an increase in translation of *ecpA* mRNA, leading to an increase in EcpA levels. Increased EcpA levels would provide an explanation for increased ECP-mediated adhesion in EHEC exposed to either acid or SCFA stress, as EcpA serves as the major pilin subunit of ECP. Human digestive system image obtained from http://stin-e-taxi.blogspot.ca/2011/12/15.html.

# 5. Future Work

In order to successfully observe changes in the level of EcpA expression under acid or SCFA stress, it would be advantageous to add an epitope tag such as green fluorescent protein (GFP) to *ecpA* in the genome of EDL933. Such a tag would allow for specific probing of EcpA with commercial α-GFP antibodies,

In addition to GFP tagging EcpA, a double mutant strain lacking both *hns* and *ecpA* could be created. This strain could be used to verify that the strong band present in EDL933 *hns*::Kan$^R$ samples on Western blots is in fact EcpA as is currently suspected.

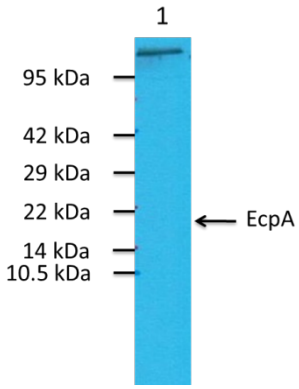
The current study was unable to demonstrate direct effects of H-NS or UvrY in regulation of EcpA under acid or SCFA stress conditions, so it would also be interesting to determine if the regulatory mutants demonstrate changes in adhesion levels to cultured epithelial cells under those stresses. Incorporation of a GFP tag could prove to be useful in this application as well; regulatory mutants could be tagged and fluorescent microscopy used to quantify adherent bacteria expressing EcpA.

It is also understood that in the course of human infection, acid stress and SCFA do not occur in isolation. The bacteria will first encounter acute acid stress in the stomach, and will eventually colonize in the colon in the presence of SCFAs. As discussed previously, there are both transcriptional and translational regulatory systems hypothesized to be involved in *ecpA*/EcpA expression; transcriptional regulation was hypothesized to be in response to acid stress while translational regulation is in responsive to SCFA stress. Exposing EHEC to these two stresses in succession could potentially result in an even higher level of adhesion or EcpA expression than either stress has previously demonstrated individually. This could be explored on an mRNA level with reverse transcriptase real-time PCR.

And lastly, ECP is a structure that is employed by commensal and pathogenic *E. coli* strains such as EHEC. The use of a common structure for adhesion is a strategy that

might allow EHEC to adhere to host cells without immediately initiating an immune response. Perhaps EHEC are able to sense the presence of commensal *E. coli* in the colon, leading to a preferential use of ECP for adhesion. The impact of co-incubation of commensal *E. coli* with EHEC, separated only by a filter, on ECP-mediated adhesion or EcpA expression levels, could provide insight on the importance of commensal bacteria in EHEC pathogenesis.

# 6. Appendix A: Supplementary Data




**Overnight Growth Conditions:** DMEM; 28°C static, no $CO_2$
**Subculture Conditions:** n/a
**Sample Preparation:** boiled in Laemelli buffer
**SDS-PAGE:** 15% PAGE; 130V, 90 minutes
**Transfer:** 100V; 60 minutes at room temperature
**Blocking:** TBST with 5% non-fat milk; 60 mins @ RT
**1° Antibody:** Batch 1 α-EcpA (1:3000 in TBST with 5% non-fat milk); overnight at 4°C
**2° Antibody:** HRP conjugated goat-anti-rabbit (1:10,000 in TBST); 60 mins @ RT

**Figure S1** Lane contents are as follows: 1 – 86-24 (25uL)

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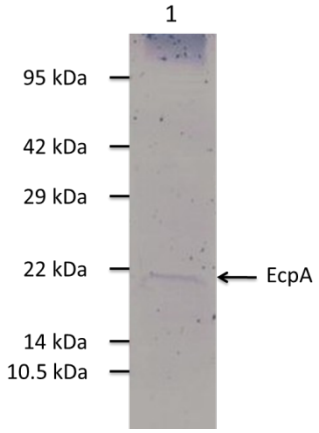


**Overnight Growth Conditions:** DMEM; 30°C static, no $CO_2$
**Subculture Conditions:** n/a
**Sample Preparation:** boiled in water pH1.5, then boiled in Laemelli buffer
**SDS-PAGE:** 15% PAGE; 130V, 90 minutes
**Transfer:** 100V; 60 minutes at room temperature
**Blocking:** TBST with 5% non-fat milk; 60 minutes at room temperature
**1° Antibody:** Batch 1 α-EcpA (1:2000 in TBST with 5% non-fat milk); 60 minutes at room temperature then overnight at 4°C
**2° Antibody:** Alkaline phosphatase conjugated goat-anti-rabbit (1:30,000 in TBST); 60 minutes at room temperature

**Figure S2** Lane contents are as follows: 1 – 86-24 (25uL)

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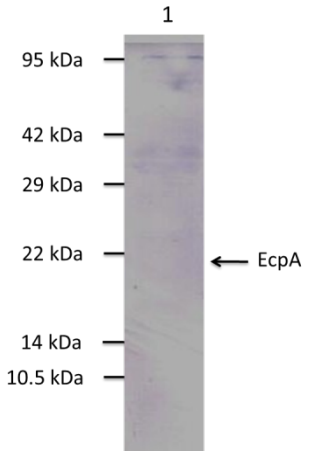


**Overnight Growth Conditions:** LB; 37°C with shaking, no $CO_2$
**Subculture Conditions:** n/a
**Sample Preparation:** boiled in water pH1.5, then boiled in Laemelli buffer
**SDS-PAGE:** 15% PAGE; 130V, 90 minutes
**Transfer:** 100V; 60 minutes at room temperature
**Blocking:** TBST with 5% non-fat milk; 60 minutes at room temperature
**1° Antibody:** Batch 1 α-EcpA (1:2000 in TBST with 5% non-fat milk); 60 minutes at room temperature then overnight at 4°C
**2° Antibody:** Alkaline phosphatase conjugated goat-anti-rabbit (1:30,000 in TBST); 60 mins @ RT

**Figure S3** Lane contents are as follows:
1 – 86-24 (6.65uL, 30ug total protein)

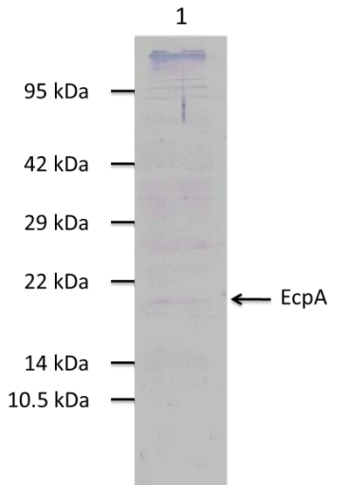


**Figure S4** Lane contents are as follows:
1 – 86-24 (20uL, 80ug total protein)

**Overnight Growth Conditions:** DMEM; 30°C static, no $CO_2$
**Subculture Conditions:** n/a
**Sample Preparation:** boiled in water pH1.5, then boiled in Laemelli buffer
**SDS-PAGE:** 15% PAGE; 130V, 90 minutes
**Transfer:** 100V; 60 minutes at room temperature
**Blocking:** TBST with 5% non-fat milk; 60 minutes at room temperature
**1° Antibody:** Batch 1 α-EcpA (1:2000 in TBST with 5% non-fat milk); 60 minutes at room temperature then overnight at 4°C
**2° Antibody:** Alkaline phosphatase conjugated goat-anti-rabbit (1:30,000 in TBST); 60 mins @ RT

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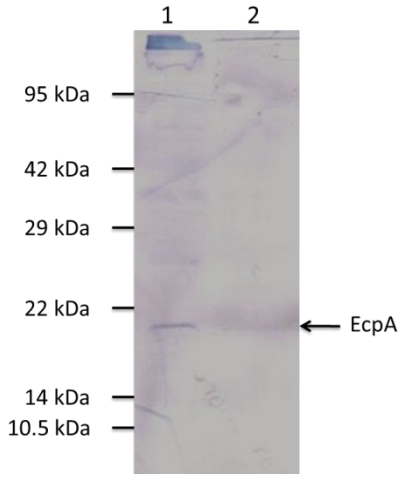
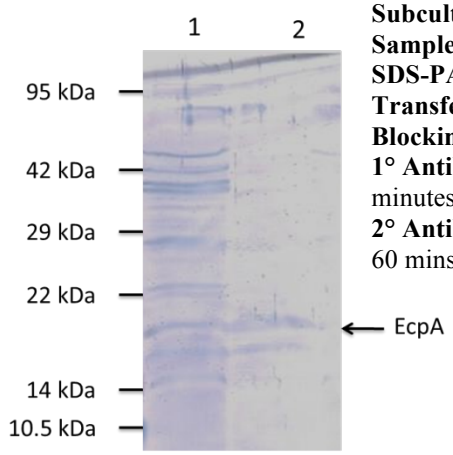



**Figure S5** Lane contents are as follows:
1 – 86-24, UU30 (13uL, 80ug total protein)
2 – 86-24, AA30 (18.5uL, 80ug total protein)

**Overnight Growth Conditions:** LB; 37°C with shaking, no $CO_2$
**Subculture Conditions:** AA30 or UU30
**Sample Preparation:** boiled in water pH1.5, then boiled in Laemelli buffer
**SDS-PAGE:** 15% PAGE; 130V, 90 minutes
**Transfer:** 110V; 60 minutes at room temperature
**Blocking:** TBST with 5% non-fat milk; overnight at 4°C
**1° Antibody:** Batch 1 α-EcpA (1:2000 in TBST with 5% non-fat milk); 60 minutes at room temperature then overnight at 4°C
**2° Antibody:** Alkaline phosphatase conjugated goat-anti-rabbit (1:30,000 in TBST); 60 mins @ RT




**Overnight Growth Conditions:** LB; 37°C with shaking, no $CO_2$
**Subculture Conditions:** AA30 or UU30
**Sample Preparation:** boiled in water pH1.5, then boiled in Laemelli buffer
**SDS-PAGE:** 15% PAGE; 150V, 90 minutes
**Transfer:** 300mA; 90 minutes at room temperature
**Blocking:** TBST with 5% non-fat milk; overnight at 4°C
**1° Antibody:** Batch 2 α-EcpA (1:500 in TBST with 5% non-fat milk); 60 minutes at room temperature then overnight at 4°C
**2° Antibody:** Alkaline phosphatase conjugated goat-anti-rabbit (1:30,000); 60 mins @ RT

**Figure S6** Lane contents are as follows:
1 – EDL933, UU30 (39.3uL, 260ug total protein)
2 – EDL933, AA30 (55uL, 260ug total protein)

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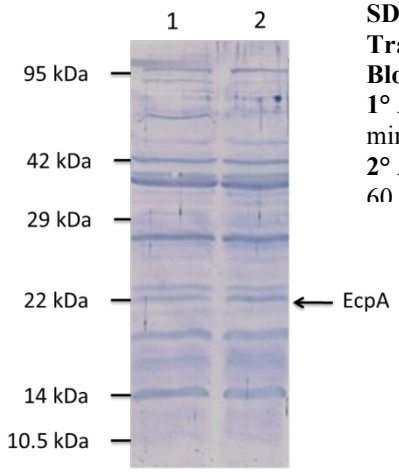


**Overnight Growth Conditions:** LB with or without 90mM SCFA; 37°C with shaking, no $CO_2$
**Subculture Conditions:** 90mM SCFA stressed or unstressed control
**Sample Preparation:** boiled in water pH1.5, then boiled in Laemelli buffer
**SDS-PAGE:** 15% PAGE; 150V, 90 minutes
**Transfer:** 300mA; 90 minutes at room temperature
**Blocking:** TBST with 5% non-fat milk; overnight at 4°C
**1° Antibody:** Batch 2 α-EcpA (1:500 in TBST with 5% non-fat milk); 60 minutes at room temperature then overnight at 4°C
**2° Antibody:** Alkaline phosphatase conjugated goat-anti-rabbit (1:30,000); 60 mins @ RT

**Figure S7** Lane contents are as follows:
1 – EDL933, unstressed control (25uL, 68ug total protein)
2 – EDL933, 90mM SCFA stressed (55uL, 68ug total protein)

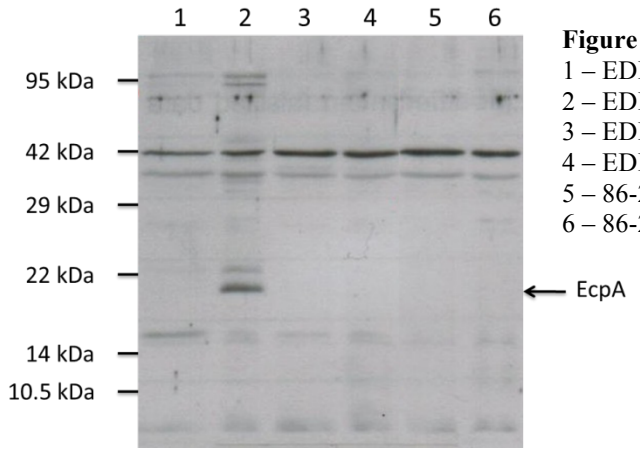


**Figure S8** Lane contents are as follows:
1 – EDL933 *hns*::Kan$^R$, AA30 (21.8uL, 50ug total protein)
2 – EDL933 *hns*::Kan$^R$, UU30 (10.2uL, 50ug total protein)
3 – EDL933, AA30 (23.3uL, 50ug total protein)
4 – EDL933, UU30 (13.6uL, 50ug total protein)
5 – 86-24 *ecpA*::Kan$^R$, AA30 (19.5uL, 50ug total protein)
6 – 86-24 *ecpA*::Kan$^R$, UU30 (8.8uL, 50ug total protein)

**Overnight Growth Conditions:** LB; 37°C with shaking, no $CO_2$
**Subculture Conditions:** AA30 or UU30
**Sample Preparation:** boiled in water pH1.5, then boiled in Laemelli buffer
**SDS-PAGE:** 15% PAGE; 130V, 90 minutes
**Transfer:** 100V; 60 minutes at room temperature
**Blocking:** TBST with 5% non-fat milk; overnight at 4°C
**1° Antibody:** Batch 2 α-EcpA (1:500 in TBST with 5% non-fat milk); 60 minutes at room temperature then overnight at 4°C
**2° Antibody:** Horseradish peroxidase conjugated goat-anti-rabbit (1:10,000); 60 mins @ RT

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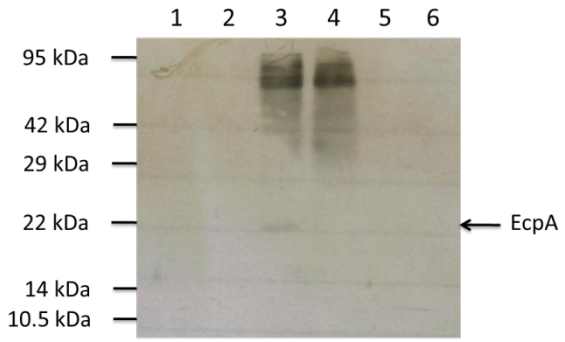


**Figure S9** Lane contents are as follows:
1 – EDL933, UU30 (55uL, 70ug total protein)
2 – EDL933, AA30 (55uL, 70ug total protein)
3 – EDL933 *hns*::Kan$^R$, UU30 (55uL, 70ug total protein)
4 – EDL933 *hns*::Kan$^R$, AA30 (55uL, 70ug total protein)
5 – EDL933 *ecpA*::Kan$^R$, UU30 (55uL, 70ug total protein)
6 – EDL933 *ecpA*::Kan$^R$, AA30 (55uL, 70ug total protein)

**Overnight Growth Conditions:** LB; 37°C with shaking, no $CO_2$
**Subculture Conditions:** AA30 or UU30
**Sample Preparation:** boiled in water pH1.5, then boiled in Laemelli buffer
**SDS-PAGE:** 15% PAGE; 130V, 90 minutes
**Transfer:** 100V; 60 minutes at room temperature
**Blocking:** TBST with 5% non-fat milk; overnight at 4°C
**1° Antibody:** Batch 2 α-EcpA (1:1000 in TBST with 5% BSA); 60 minutes at room temperature then overnight at 4°C
**2° Antibody:** Horseradish peroxidase conjugated goat-anti-rabbit (1:10,000 in TBST); 60 minutes at room temperature

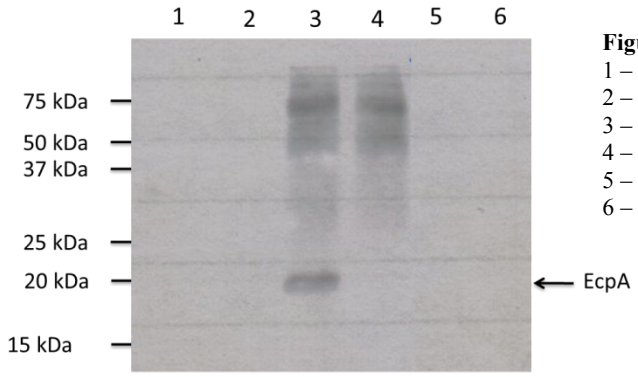


**Figure S10** Lane contents are as follows:
1 – EDL933, UU30 (55uL, 70ug total protein)
2 – EDL933, AA30 (55uL, 70ug total protein)
3 – EDL933 *hns*::Kan$^R$, UU30 (55uL, 70ug total protein)
4 – EDL933 *hns*::Kan$^R$, AA30 (55uL, 70ug total protein)
5 – EDL933 *ecpA*::Kan$^R$, UU30 (55uL, 70ug total protein)
6 – EDL933 *ecpA*::Kan$^R$, AA30 (55uL, 70ug total protein)

**Overnight Growth Conditions:** LB; 37°C with shaking, no $CO_2$
**Subculture Conditions:** AA30 or UU30
**Sample Preparation:** boiled in water pH1.5, then boiled in Laemelli buffer
**SDS-PAGE:** 15% PAGE; 130V, 90 minutes
**Transfer:** 30V; 12 hours at 4°C
**Blocking:** TBST with 5% non-fat milk; overnight at 4°C
**1° Antibody:** Batch 2 α-EcpA (1:1000 in TBST with 5% BSA); 60 minutes at room temperature then overnight at 4°C
**2° Antibody:** Horseradish peroxidase conjugated goat-anti-rabbit (1:10,000 in TBST); 60 minutes at room temperature

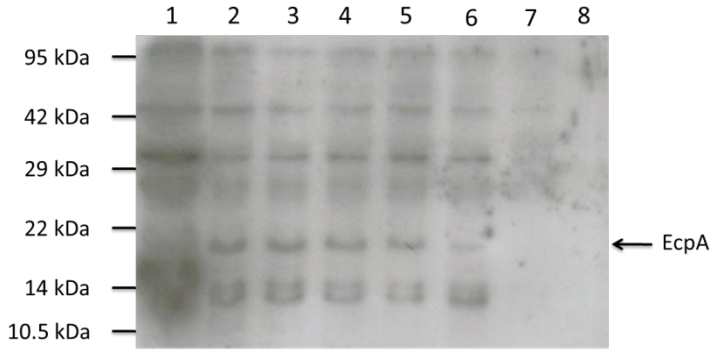


**Figure S11** Lane contents are as follows:
1 – EDL933 (58uL, 290ug total protein)
2 – EDL933 (50uL, 250ug total protein)
3 – EDL933 (42uL, 210ug total protein)
4 – EDL933 (34uL, 170ug total protein)
5 – EDL933 (26uL, 130ug total protein)
6 – EDL933 (18uL, 90ug total protein)
7 – EDL933 (10uL, 50ug total protein)
8 – EDL933 (2uL, 10ug total protein)

**Initial Growth Conditions:** LB; 37°C with shaking, no $CO_2$
**Subculture Conditions:** DMEM, 26°C static, no $CO_2$
**Sample Preparation:** boiled in water pH1.5, then boiled in Laemelli buffer
**SDS-PAGE:** 15% PAGE; 130V, 90 minutes
**Transfer:** 30V; 12 hours at 4°C
**Blocking:** TBST with 5% non-fat milk; overnight at 4°C
**1° Antibody:** Batch 2 α-EcpA (1:500 in TBST with 5% non-fat milk); 60 minutes at room temperature then overnight at 4°C
**2° Antibody:** Horseradish peroxidase conjugated goat-anti-rabbit (1:10,000 in TBST); 60 minutes at room temperature

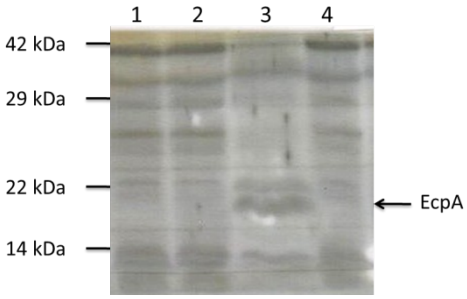


**Figure S12** Lane contents are as follows:
1 – EDL933 (16.8uL, 90ug total protein)
2 – EDL933 *ecpA*::Kan$^R$ (15.1uL, 90ug total protein)
3 – EDL933 *hns*::Kan$^R$ (75.7uL, 90ug total protein)
4 – EDL933 *uvrY*::Kan$^R$ (14.2uL, 90ug total protein)

**Overnight Growth Conditions:** LB; 37°C with shaking, no $CO_2$
**Subculture Conditions:** DMEM, 26°C static, 7 hours
**Sample Preparation:** boiled in water pH1.5, then boiled in Laemelli buffer
**SDS-PAGE:** 12.5% PAGE; 125V, 120 minutes
**Transfer:** 30V; 12 hours at 4°C
**Blocking:** TBST with 5% non-fat milk; overnight at 4°C
**1° Antibody:** Batch 2 α-EcpA (1:500 in TBST with 5% non-fat milk); 60 minutes at room temperature then overnight at 4°C
**2° Antibody:** Horseradish peroxidase conjugated goat-anti-rabbit (1:10,000 in TBST); 60 minutes at room temperature

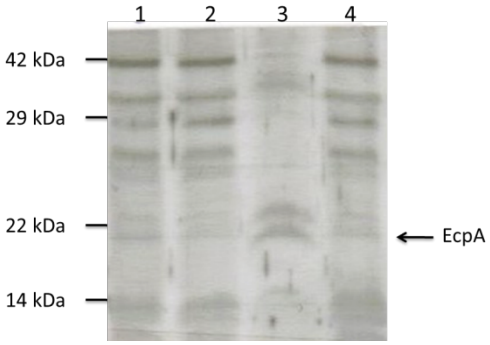


**Figure S13** Lane contents are as follows:
1 – EDL933 (15.6uL, 90ug total protein)
2 – EDL933 *ecpA*::Kan$^R$ (16uL, 90ug total protein)
3 – EDL933 *hns*::Kan$^R$ (73uL, 90ug total protein)
4 – EDL933 *uvrY*::Kan$^R$ (14.6uL, 90ug total protein)

**Overnight Growth Conditions:** LB; 37°C with shaking, no $CO_2$
**Subculture Conditions:** DMEM, 26°C static, 7 hours
**Sample Preparation:** boiled in water pH1.5, then boiled in Laemelli buffer
**SDS-PAGE:** 12.5% PAGE; 125V, 120 minutes
**Transfer:** 30V; 12 hours at 4°C
**Blocking:** TBST with 5% non-fat milk; overnight at 4°C
**1° Antibody:** Batch 2 α-EcpA (1:500 in TBST with 5% non-fat milk); 60 minutes at room temperature then overnight at 4°C
**2° Antibody:** Horseradish peroxidase conjugated goat-anti-rabbit (1:10,000 in TBST); 60 minutes at room temperature

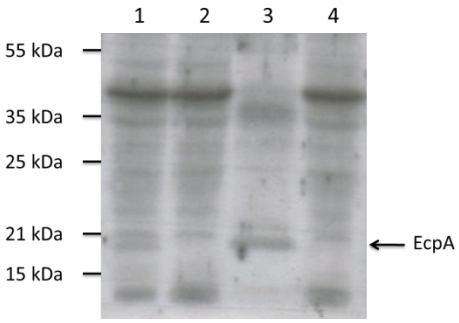


**Figure S14** Lane contents are as follows:
1 – EDL933 (14.7uL, 90ug total protein)
2 – EDL933 *ecpA*::Kan$^R$ (15.1uL, 90ug total protein)
3 – EDL933 *hns*::Kan$^R$ (66.8uL, 90ug total protein)
4 – EDL933 *uvrY*::Kan$^R$ (14.6uL, 90ug total protein)

**Overnight Growth Conditions:** LB; 37°C with shaking, no $CO_2$
**Subculture Conditions:** DMEM, 26°C static, 11 hours
**Sample Preparation:** boiled in water pH1.5, then boiled in Laemelli buffer
**SDS-PAGE:** 12.5% PAGE; 125V, 120 minutes
**Transfer:** 30V; 12 hours at 4°C
**Blocking:** TBST with 5% non-fat milk; overnight at 4°C
**1° Antibody:** Batch 2 α-EcpA (1:500 in TBST with 5% non-fat milk); 60 minutes at room temperature then overnight at 4°C
**2° Antibody:** Horseradish peroxidase conjugated goat-anti-rabbit (1:10,000 in TBST); 60 minutes at room temperature

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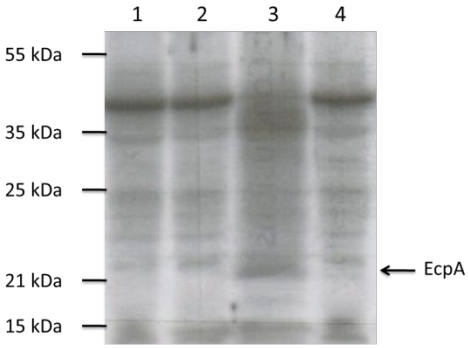


**Figure S15** Lane contents are as follows:
1 – EDL933 (24.6uL, 90ug total protein)
2 – EDL933 *ecpA*::Kan$^R$ (19.6uL, 90ug total protein)
3 – EDL933 *hns*::Kan$^R$ (70uL, 90ug total protein)
4 – EDL933 *uvrY*::Kan$^R$ (16.5uL, 90ug total protein)

**Overnight Growth Conditions:** LB; 37°C with shaking, no $CO_2$
**Subculture Conditions:** DMEM, 26°C static, 11 hours
**Sample Preparation:** boiled in water pH1.5, then boiled in Laemelli buffer
**SDS-PAGE:** 12.5% PAGE; 125V, 120 minutes
**Transfer:** 30V; 12 hours at 4°C
**Blocking:** TBST with 5% non-fat milk; overnight at 4°C
**1° Antibody:** Batch 2 α-EcpA (1:500 in TBST with 5% non-fat milk); 60 minutes at room temperature then overnight at 4°C
**2° Antibody:** Horseradish peroxidase conjugated goat-anti-rabbit (1:10,000 in TBST); 60 minutes at room temperature

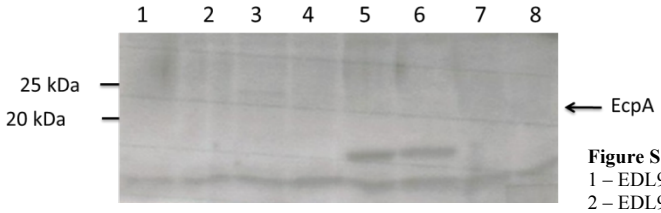


**Figure S16** Lane contents are as follows:
1 – EDL933 90mM SCFA stressed (18.1uL, 90ug total protein)
2 – EDL933 unstressed control (35uL, 90ug total protein)
3 – EDL933 *ecpA*::Kan$^R$ 90mM SCFA stressed (18.3uL, 90ug total protein)
4 – EDL933 *ecpA*::Kan$^R$ unstressed control (33.6uL, 90ug total protein)
5 – EDL933 *hns*::Kan$^R$ 90mM SCFA stressed (49.8uL, 90ug total protein)
6 – EDL933 *hns*::Kan$^R$ unstressed control (57.2uL, 90ug total protein)
7 – EDL933 *uvrY*::Kan$^R$ 90mM SCFA stressed (20.9uL, 90ug total protein)
8 – EDL933 *uvrY*::Kan$^R$ unstressed control (32.2uL, 90ug total protein)

**Overnight Growth Conditions:** LB; 37°C with shaking, no $CO_2$
**Subculture Conditions:** 90mM SCFA stress or unstressed control
**Sample Preparation:** boiled in water pH1.5, then boiled in Laemelli buffer
**SDS-PAGE:** 12.5% PAGE; 150V, 75 minutes
**Transfer:** 30V; 12 hours at 4°C
**Blocking:** TBST with 5% non-fat milk; overnight at 4°C
**1° Antibody:** Batch 2 α-EcpA (1:500 in TBST with 5% non-fat milk); 60 minutes at room temperature then overnight at 4°C
**2° Antibody:** Horseradish peroxidase conjugated goat-anti-rabbit (1:10,000 in TBST); 60 minutes at room temperature

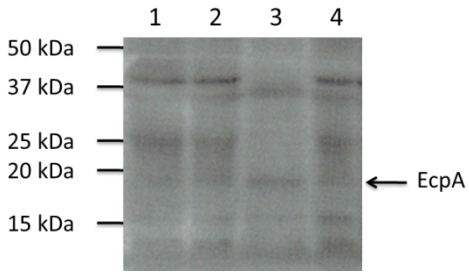


**Figure S17** Lane contents are as follows:
1 – EDL933 (14.7uL, 90ug total protein)
2 – EDL933 *ecpA*::Kan$^R$ (15.1uL, 90ug total protein)
3 – EDL933 *hns*::Kan$^R$ (55uL, 90ug total protein)
4 – EDL933 *uvrY*::Kan$^R$ (14.6, 90ug total protein)

**Overnight Growth Conditions:** LB; 37°C with shaking, no $CO_2$
**Subculture Conditions:** DMEM, 26°C static, 11 hours
**Sample Preparation:** boiled in water pH1.5, then boiled in Laemelli buffer
**SDS-PAGE:** 12.5% PAGE; 125V, 120 minutes
**Transfer:** 30V; 12 hours at 4°C
**Blocking:** TBST with 5% non-fat milk; overnight at 4°C
**1° Antibody:** Batch 3 α-EcpA (1:3000 in PBST with 5% non-fat milk); 60 minutes at room temperature then overnight at 4°C
**2° Antibody:** Horseradish peroxidase conjugated goat-anti-rabbit (1:10,000 in TBST); 60 minutes at room temperature

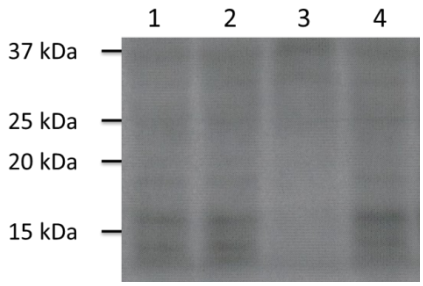


**Figure S18** Lane contents are as follows:
1 – EDL933 (14.7uL, 90ug total protein)
2 – EDL933 *ecpA*::Kan$^R$ (15.1uL, 90ug total protein)
3 – EDL933 *hns*::Kan$^R$ (55uL, 90ug total protein)
4 – EDL933 *uvrY*::Kan$^R$ (14.6, 90ug total protein)

**Overnight Growth Conditions:** LB; 37°C with shaking, no $CO_2$
**Subculture Conditions:** DMEM, 26°C static, 11 hours
**Sample Preparation:** boiled in water pH1.5, then boiled in Laemelli buffer
**SDS-PAGE:** 12.5% PAGE; 125V, 120 minutes
**Transfer:** 30V; 12 hours at 4°C
**Blocking:** TBST with 5% non-fat milk; overnight at 4°C
**1° Antibody:** α-ECP (1:3000 in PBST with 5% non-fat milk); 60 minutes at room temperature then overnight at 4°C
**2° Antibody:** Horseradish peroxidase conjugated goat-anti-rabbit (1:10,000 in TBST); 60 minutes at room temperature

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