Characterization of a Novel Promoter Region for the Enteropathogenic *Escherichia coli* Type III Secretion System Chaperone Gene *cesT*

by

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ABSTRACT

Enteropathogenic *Escherichia coli* (EPEC) is an enteric pathogen that causes potentially fatal infantile diarrhea. A type III secretion system is employed by EPEC to inject bacterial effector proteins directly into host intestinal epithelial cells. The multivalent chaperone, CesT, interacts with nine effectors and is a significant contributor to EPEC pathogenesis. A putative transcriptional promoter region was identified directly upstream of *cesT*. *In silico* analyses identified conserved elements that suggest the *cesT* promoter is recognized by σ^{70}. Using transcriptional fusions to *lux* reporter genes I showed that the *cesT* promoter region is active under conditions known to induce virulence-gene expression. I conclude that the *cesT* promoter is active early during an *in vitro* assay, and regulated by different mechanisms than those affecting the P_{tir} operon promoter.

LIST OF ABBREVIATIONS USED

A/E	Attaching and Effacing
Amp	Ampicillin
BLAST	Basic Local Alignment Search Tool
bp	Base pair
CAT	Chloramphenicol acetyltransferase
Cm	Chloramphenicol
CPS	Counts per second
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
EHEC	Enterohemorrhagic *Escherichia coli*
EIEC	Enteroinvasive *Escherichia coli*
EPEC	Enteropathogenic *Escherichia coli*
FBS	Fetal bovine serum
Hrs	Hours
HUS	Haemolytic renal failure
kb	Kilobases
Kn	Kanamycin
LB	Luria Broth
LEE	Locus of enterocyte effacement
MFP	Membrane fusion protein
Min	Minute
mRNA	Messenger ribonucleic acid

O/N	Overnight
OD	Optical density
OMF	Outer-membrane fusion
ORF	Open reading frame
PAI	Pathogenicity island
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
RNAP	RNA polymerase
SDS-PAGE	Sodium dodecyl sulphate – polyacrylamide gel electrophoresis
Sec	Second
St	Streptomycin
T3SS	Type III secretion system
TAT	Twin-arginine translocation
TBS-T	Tris-buffered saline with Tween
WCL	Whole cell lysate
X-gal	5-Bromo-4-chloro-4-indolyl-β-D-galactopyranoside

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CHAPTER 1. INTRODUCTION

1.1 Protein Secretion is an Essential Component of Bacterial Survival

Approximately 25% of all proteins in a cell must cross at least one membrane to reach their proper locations, highlighting the significance of protein translocation (Yuan *et al*., 2010). Protein translocation is an essential component for survival of both Gram-positive and Gram-negative bacteria for many reasons; proteins are fundamental components of bacterial cell membranes, and can be involved in cell-to-cell contact, nutrient uptake, and the evasion of host immune cells (Kusters and Driessen, 2011). Some pathogenic bacteria have evolved mechanisms to secrete proteins as a means of colonizing a host and causing disease.

Three different types of secretion system found in both Gram-positive and Gram-negative bacteria enable protein translocation across membranes: the Sec, TAT, and YidC pathways. The Sec pathway, which is responsible for a large portion of protein secretion, can transport unfolded proteins destined for insertion into the cell membrane, or translocation (Yuan *et al*., 2010). In Gram-negative bacteria, the Sec pathway is composed of SecA, a motor ATPase, SecYEG, which forms the translocation tunnel, and accessory proteins SecDFYajC (Driessen and Nouwen, 2008). Proteins are targeted for secretion through the Sec pathway by a signal peptide encoded in the N terminus of the protein, which is recognized by the signal-recognition particle (SRP) pathway, or by the cytosolic chaperone SecB. The Sec-dependent secretion pathway in Gram-positive bacteria is very similar to that found in Gram-negative bacteria, although recognition and transport of targeted proteins occurs only through the SRP pathway (Yuan *et al*., 2010).

The twin-arginine translocation (TAT) pathway has the capacity to transport fully folded proteins across cell membranes (Palmer *et al*., 2005). Similar to the Sec pathway, proteins are targeted to the TAT pathway by a signal peptide located within the N-terminal domain (Yuan *et al*., 2010). The YidC insertase pathway is a system that can operate in parallel with the Sec system, and is primarily responsible for insertion of small proteins into the cell membrane (Dalbey *et al*., 2011). Both the TAT and YidC pathways are present in Gram-positive and Gram-negative bacteria.

While Gram-positive bacteria have only one cell membrane, Gram-negative bacteria have an inner and an outer membrane. Gram-negative bacteria have evolved complex mechanisms involving structures known as "secretion systems" for transporting proteins across two membranes, either as a one-step or two-step process. There are at least four secretion systems in Gram-negative bacteria that can translocate proteins as a one-step process: type I secretion system, type III (T3SS), type IV, and type VI (Bonemann *et al*., 2010; Holland *et al*., 2005; Mattei *et al*., 2011; Waksman and Fronzes, 2010). The transport of proteins by these systems occurs directly from the bacterial cytoplasm, making transport independent of the Sec pathway (Economou *et al*., 2006). The secretion systems that transport proteins by a two-step mechanism, type II and type V secretion systems, deliver proteins from the periplasm to the extracellular milieu, making them Sec-dependent pathways (Campos *et al*., 2010; Dautin and Bernstein, 2007).

1.1.1 Sec-Dependent Systems

As mentioned above, there are two secretion systems that require the Sec pathway for transport of bacterial proteins into the periplasm, prior to translocation across the outer membrane. The type II secretion system is a protein complex comprised of 12 to 16 gene

products, depending on the species, which forms structures in both the inner and outer bacterial membranes. The outer-membrane protein forms a large, oligomeric ring which allows passage of folded protein from the periplasm to the extracellular milieu (Lybarger *et al*., 2009). It has recently been reported for *Vibrio cholerae* that the inner- and outer-membrane components interact, and this interaction transduces the energy required for translocation from the cytosolic ATPase to the outer-membrane complex (Campos *et al*., 2010; Lybarger *et al*., 2009). The type II secretion system is used by *Vibrio cholerae* for secretion of the cholera toxin.

The type V secretion system, also known as the autotransport system, was first identified in *Neisseria gonorrhoea*, the causative agent of the sexually transmitted disease gonnorhea (Centre for Disease Control, 2002). This system is formed by polypeptides composed of C-terminal translocator domains and N-terminal passenger domains. The autotransporter polypeptide is translocated across the inner membrane as an unfolded protein, *via* the Sec pathway. The C-terminal domain, or the β domain, forms a pore in the outer membrane which is then used to transport the passenger domain to the extracellular milieu (Dautin and Bernstein, 2007). The passenger domain may remain intact, or it may be cleaved from the translocator domain so that it can moderate its effector functions (Dautin and Bernstein, 2007).

1.1.2 Sec-Independent Systems

There are four secretion systems that have evolved as mechanisms to transport bacterial proteins across both inner and outer membranes in one continuous step: type I, type III, type IV, and type VI (Bonemann *et al*., 2010; Holland *et al*., 2005; Mattei *et al*., 2011; Waksman and Fronzes, 2010). The type I secretion system is comprised of three

components: an ABC-transporter, a membrane-fusion protein (MFP), and an outer-membrane factor (OMF). The ABC-transporter contains an integral membrane domain, which anchors it to the inner membrane, and a cytosolic domain with ATPase activity. The OMF spans the outer membrane and contains a periplasmic domain which is linked to the ABC-transporter *via* the MFP, thus generating a continuous tunnel for protein translocation (Holland *et al*., 2005).

The type IV secretion system is considerably more complex. This system has been implicated in not only monomeric protein transfer, but also transfer of multisubunit complexes, and is hypothesized to participate in DNA transfer during conjugation (Waksman and Fronzes, 2010). This system contains multiple cytosolic ATPases, proteins which span both the inner and outer bacterial membranes forming a continuous tunnel, and pilus proteins extending away from the outer membrane. Importantly, the pilus proteins can serve as a conduit which allows transfer of proteins or other bacterial substrates into the extracellular milieu or directly into neighbouring or host cells (Waksman and Fronzes, 2010).

The recently identified type VI secretion system allows protein translocation by a Sec-independent mechanism. Although little is known about this new system, certain structural components have an evolutionary relationship to the cell-puncturing mechanisms of bacteriophages (Bonemann *et al*., 2010). For example, a well-conserved protein, VgrG, shows structural similarities to the spike complex from T4 bacteriophages, and has the ability to puncture cells. The type VI secretion system has been implicated in protein translocation, biofilm formation and killing of other bacterial cells (Bonemann *et al*., 2010). It has recently been found that *Pseudomonas aeruginosa* uses this system to

inject lytic enzymes into the periplasm of recipient cells, which may enhance bacterial fitness (Russell *et al*., 2011).

The final Sec-independent secretion apparatus is the T3SS. Based on its relevance to the remainder of this thesis, it is discussed in more detail in the following section.

1.2 Type III Secretion Systems

1.2.1 Structure

The T3SS is a complex machine found in several Gram-negative human enteric pathogens including *Salmonella, Yersinia, Shigella,* and *Escherichia*. The T3SS, which is structurally conserved throughout these species, is comprised of at least 20 bacterial proteins which form a needle-like complex that spans both inner and outer bacterial membranes (Worrall *et al*., 2011). It is presumed that this apparatus functions as a conduit to allow the translocation of bacterial effectors directly into the cytosol of the host cell. For simplicity, I describe below the T3SS using *E. coli* nomenclature, as it is directly related to this thesis.

The T3SS complex can be separated into two components: the needle complex, and the export apparatus. The export apparatus is characterized by proteins forming the cytoplasmic complex, and the inner- and outer-membrane rings. It has been recently shown that three proteins, EscN, EscQ, and EscL, interact forming a complex that is localized at the base of the needle structure (Biemans-Oldehinkel *et al*., 2011). These interactions were first identified between the protein homologues YscN, YscQ, and YscL from *Yersinia pestis* through yeast two-hybrid procedures (Jackson and Plano, 2000). Importantly, EscN is the ATPase of the EPEC T3SS which generates the energy to drive effector translocation (Zarivach *et al*., 2007). The inner-membrane component of the

apparatus consists of several proteins, EscR, EscS, EscT, EscU, EscV (Aizawa, 2001). Interestingly, EscV contains a putative N-terminal signal sequence, suggesting that it is inserted into the inner-membrane via the Sec pathway (Gauthier *et al*., 2003). EscC belongs to a protein family of secretins, and forms the outer-membrane ring (Gauthier *et al*., 2003). The inner and outer membrane complexes are connected by a 24-subunit protein, EscJ, forming a conduit for protein translocation (Crepin *et al*., 2005; Yip *et al*., 2005).

The needle structure of the EPEC T3SS begins with EscF, which is presumed to form a filamentous needle structure which protrudes outward from the bacterium, and is believed to be anchored to the basal body of the T3SS through its interaction with EscJ (Gauthier *et al*., 2003). EspA is another major component of the filamentous structure, and has been shown to polymerize into an expandable sheath (Daniell *et al*., 2001). Interestingly, the extension to the T3SS formed by EspA elongates the structure to ~500 nm in length, which is 10 times longer than the filamentous needle found in *Shigella* and *Salmonella* (Daniell *et al*., 2001). The final two components of the EPEC injectisome are termed translocon components. EspD and EspB are adapter proteins that are secreted by the T3SS upon contact with host cells, and form the tip of the needle. It is presumed that these two proteins form a pore in the host cell to complete the conduit which allows EPEC proteins to be injected directly into a host cell.

1.2.2 Coorindate Regulation of Virulence Gene Expression

Expression of virulence genes is tightly regulated in pathogenic bacteria, as it is unfavorable to produce complex machinery under the wrong conditions. Virulence genes may be encoded in several locations in pathogenic organisms; genes may be encoded

chromosomally, either grouped as a pathogenicity island (PAI) or scattered throughout the chromosome, or plasmid encoded. Evidence suggests that bacterial pathogenesis is a process where the organism is constantly sensing its environment and regulating gene expression in response to these stimuli (Miller *et al*., 1989). Many pathogens have evolved regulatory cascades that control virulence-gene expression in a coordinated manner, which enables the activation of genes encoded in more than one location, in response to environmental stimuli (Mekalanos, 1992; Miller *et al*., 1989).

Many enteric pathogens including *E. coli*, *Shigella,* and *Salmonella*, rely on the histone-like nucleoid-structuring (H-NS) protein for repression of virulence genes, which is mediated by the formation of DNA-H-NS-DNA bridges that trap RNA polymerase (RNAP) and block transcription (Dorman, 2004). Binding of H-NS to DNA does not occur in a sequence-specific manner, but is dependent on DNA structure. H-NS has been shown to bind AT-rich sequences, which favour intrinsically bent DNA (Owen Hughes, 1992). Virulence genes are often subject to repression by H-NS since pathogenicity islands typically have a low GC content, presumably a result of DNA acquisition from a foreign source (Dorman, 2004).

Under optimal conditions, transcription of virulence genes can be initiated by de-repression of H-NS, a process that typically involves a cascade of transcriptional regulation or changes to DNA structure (Dorman, 2004). Expression of genes encoding the T3SS generally occurs once the bacteria have entered a host, or under conditions that mimic a host environment (Lara-Ochoa *et al*., 2010). Several environmental conditions have been identified which alter virulence gene expression during *in vitro* analyses. *In vitro* analyses, in this context, refers to experiments using whole organisms in a culture

system. Depending on the context, the term *in vitro* can have different meanings, which is why it is defined in this thesis. Environmental conditions that have been identified as factors that influence virulence gene expression include: temperature, pH, physiological osmolarity, and the presence of specific ions (Lara-Ochoa *et al*., 2010).

Temperature is a significant regulator of virulence-gene transcription in several enteric pathogens. When bacteria enter a mammalian host, they face a shift in temperature up to 37°C, which is typically higher than that of the external environment. In *Shigella flexneri*, this shift in temperature leads to expression of a transcriptional regulator, *virF*, which initiates a cascade of virulence gene expression (Falconi *et al*., 1998). Interestingly, expression of *virF* was shown to be repressed by H-NS when bacteria are grown at 30°C; however, this repression is alleviated when bacteria were grown at 37°C, possibly a result of altered DNA topology due to a change in termpature (Falconi *et al*., 1998). Growth of bacteria at 37°C has also been shown to be the optimal condition for protein secretion by T3SS in enteropathogenic *E .coli* (EPEC), in an *in vitro* model of infection (Kenny *et al*., 1997).

Virulence-gene expression has been shown to be influenced by growth conditions *in vitro* that mimic the host environment, such as pH, physiological osmolarity, and oxygen. When entering a mammalian host, enteric pathogens must first pass through the stomach, an environment with an acidic pH, before entering the neutral environment of the intestine. Expression of genes encoding the T3SS of *Salmonella typhimurium* is induced, and the secretion of EPEC effectors is optimal, when bacteria are grown at pH 7.0, in an *in vitro* model, a condition that mimics the neutral environment of the mammalian intestine (Gong *et al*., 2010; Kenny *et al*., 1997). However, virulence-gene

expression is repressed and protein secretion abolished when bacteria are grown at a pH of 6 or 8, conditions that may reflect the acidic environment of the stomach or the basic environment of the mammalian colon (Gong *et al*., 2010; Kenny *et al*., 1997).

The presence of sodium bicarbonate in media has also been shown to induce the expression of virulence genes. When enterohemorrhagic *E. coli* (EHEC) are grown in a non-inducing medium, Luria Bertani (LB) broth, with 44 mM of sodium bicarbonate, expression of virulence genes significantly increases, through activation of Ler, an important transcriptional regulator (Abe *et al*., 2002). The expression of virulence genes is also affected by the presence of oxygen. As oxygen levels are low in the human intestine, it has been shown that expression of virulence genes is induced when bacteria are grown in 5% CO_2, and repressed when grown in atmospheric air (Gong *et al*., 2010; Kenny *et al*., 1997).

There are also certain growth conditions that do not induce the expression of T3SS genes. The presence of oxygen has a negative effect on expression of virulence genes, and leads to decreased effector secretion. The presence of ammonium (18.4 mM) has also been shown to negatively impact effector secretion (Kenny *et al*., 1997). In the human intestine, ammonium is found at high concentrations in the colon, and this may act as a signalling mechanism to prevent colonization. The human intestine also contains low levels of calcium, a condition which may allow an organism to differentiate between environments; thus the addition of calcium has been shown to inhibit virulence gene expression *in vitro* (Deng *et al*., 2005). Bile salts are secreted by the gall bladder into the lumen of the human intestine, and may act as an environmental stimulus. Interestingly, the presence of bile salts was shown to induce expression of virulence genes in *Vibrio*

chloerae in vitro (Hung and Mekalanos, 2005), and to decrease the expression of several virulence genes in EHEC (Yin *et al*., 2011).

1.2.3 T3SS Function: Translocation of Virulence Proteins

The T3SS enables pathogenic bacteria to directly inject bacterial proteins into host cells, thereby facilitating colonization and disease progression. Similarly to the Sec pathway, bacterial effectors that are destined for secretion by the T3SS are targeted to the apparatus by a signal peptide that is typically encoded within the first 15-25 amino acids of the N-terminal domain (Dean, 2011). However, unlike the signal sequences of peptides targeted to the Sec pathway, the signal sequences of T3SS effectors are not cleaved.

Following injection into the host cell, effectors can disrupt cell processes to cause disease by a wide range of mechanisms, such as host cytoskeleton rearrangement, mitochondrial dysfunction, and disruption of signalling pathways that can up- or down-regulate an immune response. In mutant strains that produce non-functional T3SS, such as an EscN-deficient EPEC strain, effectors have been shown to accumulate in the bacterial cytoplasm and secretion is completely abolished *in vitro* (Gauthier *et al*., 2003; Thomas *et al*., 2005). The ability of an organism to cause disease is dependent on the successful translocation of effectors, highlighting the importance of a functional injectisome to bacterial pathogenesis.

Expression of bacterial effectors is under tight transcriptional control and often requires activation by a transcriptional regulator in response to environmental stimuli. For example, when *Salmonella typhimurium* are grown at 37°C a master regulator, *hilA*,

is expressed, which leads to the downstream activation of *invF* (Thijs *et al*., 2007). InvF is an AraC-like regulator which binds directly to DNA, and is required for expression of *Salmonella* effectors by de-repression of H-NS (Darwin and Miller, 2000). Similar regulatory cascades are present in *Shigella flexneri* and in EPEC, which employ MxiE and Ler transcriptional regulators, respectively, to activate expression of bacterial effectors. Regulatory cascades allow bacterial gene expression in a coordinated manner.

1.2.4 Chaperones of the T3SS

Prior to secretion, effectors must avoid degradation, improper or premature folding, and untimely interactions with other proteins in order to reach the needle complex (Page and Parsot, 2002). Many effectors accomplish this feat by interacting with a chaperone protein that maintains them in a partially unfolded state and escorts them to the T3SS (Page and Parsot, 2002). Strains which are deficient for T3SS chaperones exhibit altered effector secretion phenotypes, and have significantly reduced virulence, highlighting the importance of chaperones for bacterial pathogenesis (Deng *et al*., 2004; Page *et al*., 2002).

1.2.4.1 Chaperone Structure and Classification

Chaperones of the T3SS have been grouped into two classes based on their interactions with their cargo: Class I, which escort bacterial effectors, and Class II, which escort translocon components (Page and Parsot, 2002). Class I chaperones bind to effectors at the chaperone-binding domain (CBD), a sequence of ~50-100 amino acids encoded directly downstream of the N-terminal signal sequence (Galan and Wolf-Watz, 2006). These chaperones are small (15-20 kDa), leucine-rich cytoplasmic proteins, with

an acidic pI, that contain a predicted C-terminal amphipathic α-helical structure. Although Class I chaperones do not share sequence conservation, crystallization of several proteins has revealed structural similarities: they form homodimers, and each contain five β-strands and three α-helices, with the exception of the CesT chaperone in EPEC, which contains a sixth β-strand (Luo *et al*., 2001).

Class I chaperones have been further subdivided into two classes, Class IA and Class IB, based on the specificity of the chaperone to effectors. Class IA chaperones interact specifically with one effector, while Class IB chaperones have the capacity to interact with multiple effectors, and have been recently termed multivalent chaperones (Page and Parsot, 2002). CesT, the chaperone under investigation is a Class IB chaperone.

1.2.4.2 Class IB Chaperones: Importance for Bacterial Pathogenesis

There are several examples of multivalent chaperones in enteric pathogens including *Shigella flexneri, Salmonella enteric* serovar *typhimurium*, and EPEC. The chaperones and their effectors are summarized in Table 1.1. Initially, it was observed that a chaperone is generally encoded next to its cognate effector; however, this feat is virtually impossible for multivalent chaperones, as the different effectors may be encoded in more than one location. For example, the EPEC chaperone CesT, which is discussed in more detail below, has been shown to interact with effectors encoded within the PAI encoding the T3SS, and also effectors encoded elsewhere on the chromosome (Thomas *et al*., 2005; Thomas *et al*.. 2007).

Table 1.1 Class IB chaperones interact with multiple effectors in EPEC, *S. flexneri*, and *S. enterica*

Organism	Chaperone	Effectors	Source
EPEC	CesT	Tir	(Abe *et al*., 1999; Elliott *et al*., 1999)
		Map	(Creasey *et al*., 2003)
		NleA	(Thomas *et al*., 2005)
		NleH	(Thomas *et al*., 2005)
		NleF	(Thomas *et al*., 2005)
		EspH	(Thomas *et al*., 2005)
		EspZ	(Thomas *et al*., 2005)
		NleI	(Li *et al*., 2006)
		EspG	(Thomas *et al*., 2007)
Shigella flexneri	Spa15	IpaA	(Page *et al*., 2002)
		IpgB1	(Page *et al*., 2002)
		OspC2	(Page *et al*., 2002)
		OspC3	(Page *et al*., 2002)
		OspB	(Page *et al*., 2002)
		OspD1	(Parsot *et al*., 2005)
		IpgB2	(Hachani *et al*., 2008)
		OspC1	(Schmitz *et al*., 2009)
		OspD2	(Schmitz *et al*., 2009)
Salmonella enterica	InvB	SipA	(Bronstein *et al*., 2000)
		SopE	(Lee and Galan, 2003)
		SopE2	(Ehrbar *et al*., 2003)
		SopA	(Ehrbar *et al*., 2004)
	SrcA	SseL	(Cooper *et al*., 2010)
		PipB2	(Cooper *et al*., 2010)

The importance of these multivalent chaperones can be appreciated when examining the altered pathogenicity in chaperone-deficient bacterial strains. Deletions of CesT, Spa15, or SrcA have been shown to severely alter effector secretion profiles *in vitro*, highlighting the requirement of these chaperones for efficient secretion (Cooper *et al*., 2010; Page *et al*., 2002; Thomas *et al*., 2005). Attenuated virulence has also been observed in mouse models of infection. Deletion of *cesT* from *Citrobacter rodentium*, a close relative of EPEC, and SrcA from *Salmonella eneterica* leads to significantly reduced colonization of infected mice (Cooper *et al*., 2010; Deng *et al*., 2004), and deletion of Spa15 from *Shigella* leads to severely attenuated virulence in a murine pulmonary model of shigellosis, and also a Sereny model of infection (Hachini *et al*., 2008). These results clearly demonstrate the importance of chaperones in virulence for these pathogens.

1.2.4.3 Chaperones May Participate in Transcriptional Regulation

Currently there are three examples which implicate T3SS chaperones in regulating transcription: SicA in *Salmonella typhimurium*, and IpgC and Spa15 in *Shigella flexneri*. The Class II chaperone SicA interacts with translocon components SipB and SipC, preventing their degradation and premature interaction (Tucker and Galan, 2000). A transposon mutagenesis screen revealed that SicA is required for the expression of several T3SS effectors, through its interaction with an AraC-family transcriptional activator InvF (Darwin and Miller, 2000; Darwin and Miller, 2001). As noted above, InvF is the first gene encoded in the *Salmonella* Pathogenicity Island (I) and activates the expression of T3SS.

Interestingly, the SicA homologue in *Shigella flexneri* is IpgC, which has been shown to interact with IpaB and IpaC (Menard *et al*., 1994). During conditions of non-secretion, IpgC is sequestered in the cytoplasm by IpaB and IpaC. However, during conditions of secretion IpaB and IpaC are secreted by the T3SS, freeing IpgC to interact with MxiE, an AraC-family regulator, and activate the expression of several *Shigella* effectors (Mavris *et al*., 2002; Pilonieta and Munson, 2008).

The class IB chaperone from *Shigella flexneri*, Spa15, has also been shown to play a role in transcriptional regulation of T3SS genes. During conditions of non secretion, MxiE interacts with an effector, OspD1, and its chaperone Spa15, hindering the expression of T3SS effectors, thus giving Spa15 a role as a co-antiactivator (Parsot *et al*., 2005).

Even more recently, secretion of Spa15 in a T3SS-dependent manner has been observed, and this secretion is sufficient to prevent staurosporine-induced apoptosis (Faherty and Maurelli, 2009). Staurosporine is a protein kinase inhibitor, isolated from *Streptomyces staurospores*, that can induce apoptosis by activation of caspase-3; however, this activation can be prevented by Spa15 (Faherty and Maurelli, 2009). This is the first demonstration of translocation of a T3SS chaperone and is not understood mechanistically; however, this observation gives light to the exciting new possibility that chaperones may have an even greater role in bacterial pathogenesis than originally thought.

1.2.5 Importance of a Functional T3SS in Bacterial Pathogenesis

There is no debate regarding the importance of the T3SS in bacterial pathogenesis. Mutational analyses performed *in vitro* on components of the EPEC T3SS led to an accumulation of effectors in the bacterial cytoplasm, and completely abolished effector secretion (Gauthier *et al*., 2003; Thomas *et al*., 2005). To further indicate the importance of a functional T3SS for bacterial pathogenesis, expression of genes encoding the T3SS in nonpathogenic bacteria such as *E. coli* K-12 can be engineered to induce a pathogenic phenotype *in vitro* (McDaniel and Kaper, 1997). Mutants for T3SS components in *Citrobacter rodentium* display significantly reduced virulence in a mouse model of infection, highlighting the importance of a functional T3SS for bacterial pathogenesis. These data indicate that rendering the injectisome non-functional disrupts the transport of disease-causing effectors and the ability of an organism to cause disease.

1.3 Pathogenic *Escherichia coli*

E. coli is a gram-negative facultative anaerobe belonging to the Enterobacteriaceae family of bacteria (Garrity *et al*., 2001). This organism is found commensally in the lower intestine of humans and warm-blooded animals. Although *E. coli* is present in the normal intestinal flora of humans, several strains have acquired the ability to cause disease, with symptoms ranging from mild diarrhoea, to meningitis, urinary tract infections, septicaemia and death. As reported by the World Health Organization, several millions of people die each year from pathogenic *E .coli*, most of which are children under the age of five in developing countries. Pathogenic strains include, EHEC, EPEC, enteroinvasive *E. coli* (EIEC), enterotoxigenic *E. coli*, enteroaggregative *E. coli*, uropathogenic *E. coli*, and diffusely adherent *E. coli*.

One of the most dangerous forms of pathogenic *E. coli* is EHEC, which can cause bloody diarrhoea, with 10-15% of cases developing into potentially fatal haemolytic uraemic syndrome (HUS), a form of acute renal failure (Tarr *et al*., 2005). The factor which makes EHEC so dangerous to humans is the production of Shiga-like toxin, which has the capability of destroying red blood cells. Recently, an outbreak of EHEC in Germany resulted in 810 patients with HUS, and 39 deaths (Bielaszewska *et al*., 2011). In May 2000, drinking water in Walkerton, Ontario, Canada, was contaminated with cattle feces, which led to 2300 EHEC-caused illnesses and 7 deaths (Walkerton Inquiry and O'Connor, 2002). Although it does not produce the Shiga-like toxin, EPEC shares 97% sequence conservation with EHEC, making it an excellent model organism to study (Perna *et al*., 1998). Pathogenic strains of *E. coli* are of global significance since they pose a serious threat for individuals in both developing and developed countries.

1.3.1 Enteropathogenic *E. coli*

EPEC was first discovered as the causal agent of infant diarrheal outbreaks in the 1940s to 1950s, and is now considered one of the main agents causing diarrhoea in children less than six months old, primarily in developing countries (Lara-Ochoa *et al*., 2010). The strain of EPEC that is currently studied in many laboratories is E2348/69 (O127:H6), which was isolated from an outbreak in Taunton, England, in 1969 (Levine *et al*., 1978).

1.3.2 EPEC LEE Encodes a T3SS

Pathogenic bacteria have acquired genes that encode a repertoire of factors that cause damage to the hosts. In the case of EIEC, these genes are carried on a large 230-kb plasmid (Small and Falkow, 1988). In the case of EPEC, these genes are encoded

primarily on a PAI. In 1995, McDaniel and colleagues found that pathogenic strains of *E. coli* contained a 35.6-kb region of DNA that is absent in commensal *E. coli* (McDaniel *et al*., 1995). They termed this PAI the locus of enterocyte effacement (LEE), as the encoded genes are responsible for effacement of infected intestinal epithelial cells (McDaniel *et al*., 1995). This group also found that the LEE is not only found in EPEC, but also EHEC and other diarrheal pathogens such as *C. freundii, H. alvei,* and rabbit EPEC (McDaniel *et al*., 1995).

Based on amino acid sequence similarities, the LEE PAI was found to encode a type III secretion system, and sequencing revealed a strikingly low GC content, far lower than that of the EPEC chromosome. This implies that the PAI was acquired by horizontal gene transfer; the process whereby genetic information is spread between organisms (Elliott *et al*., 1999; Jarvis *et al*., 1995; McDaniel *et al*., 1995). It was predicted that the LEE contains 41 open reading frames (ORF) which are organized into at least five polycistronic operons (LEE1-LEE5) encoding a type III secretion system ,including the needle apparatus, translocon components, effectors, chaperones, and transcriptional regulators (Elliott *et al*., 1998). Interestingly, it was later shown that non-pathogenic bacteria, such as lab strain *E. coli* K-12 DH5α, can induce an attaching and effacing phenotype by expression of the LEE on a plasmid (McDaniel *et al*., 1995). These data are in accordance with similar findings by Falkow and coworkers, who introduced the virulence plasmid of EIEC into *E. coli* K-12, which was sufficient to confer the invasive phenotype (Small and Falkow, 1988).

Table 1.2 Expression of *ler* is influenced by several transcriptional regulators, generating a hierarchy of T3SS gene activation

Regulator	Regulation (Pos./Neg.) (+/-)	Gene location	Cues/Stimuli	Source
IHF	+	Chromosome	Environmental	(Friedberg *et al*., 1999)
PerC	+	EAF plasmid	Environmental	(Mellies *et al*., 1999)
GrlA	+	LEE PAI	Activated by Ler	(Deng *et al*., 2004; Barba *et al*., 2005)
QseE	+	Chromosome	Quorum sensing	(Sharp and Sperandio, 2007)
Fis	+	Chromosome	Bacterial growth phase	(Goldberg *et al*., 2001)
H-NS	-	Chromosome	Non-secretion environment, thermoregulation	(Umanski *et al*. 2002)
GrlR	-	LEE PAI	Activated by Ler	(Deng *et al*., 2004; Barba *et al*., 2005)
Ler	-	LEE PAI	Autoregulatory	(Berdichevsky *et al*., 2005)

1.3.3 Regulation of the LEE pathogenicity island

Tight transcriptional regulation of the LEE PAI prevents EPEC from transcribing genes encoding components of the T3SS unnecessarily. The primary regulator of the LEE is the LEE-encoded regulator (Ler), a 15-kDa protein encoded as the first gene in the PAI (Mellies *et al*., 1999). The expression of *ler* is tightly controlled by a cascade of several positive and negative regulators which are summarized in Table 1.2. Ler activates expression of LEE-encoded genes by binding to DNA sequences that are rich in AT, displacing H-NS and thereby alleviating transcriptional repression (Bustamante *et al*., 2001). Ler has been shown to promote the transcription of genes encoded in LEE1, LEE2, LEE3, LEE4 and LEE5, suggesting that it is an important mediator for virulence-gene expression in acoordinated fashion (Bustamante *et al*., 2001; Haack *et al*., 2003; Mellies *et al*., 1999).

Ler forms a regulatory feedback loop with Global Regulator Ler Repressor and Activator (GrlRA), a bicistronic operon encoded in the LEE between LEE1 and LEE2. To begin the feedback loop, Ler activates the expression of the GrlRA operon by displacing H-NS at the promoter, thereby alleviating transcriptional repression. GrlA can bind directly to DNA in close proximity to the Ler promoter, activating its expression, which persumably occurs by alleviating H-NS repression (Barba *et al*., 2005). This positive feedback loop is moderated by GrlR, which can bind directly to GrlA and prevent Ler activation through this mechanism (Barba *et al*., 2005). Ler has been shown to autoregulate its own expression, which also feeds into this regulatory loop (Berdichevsky *et al*., 2005).

1.3.4 Hierarchy of Protein Secretion

The tight regulation of *ler* suggests that T3SS gene expression occurs in a coordinated manner, which likely ensures that the needle apparatus is expressed under conditions which promote EPEC colonization. Protein translocation into host cells is dependent on the presence of the translocon components EspB and EspD, which persumably form a pore in the host cell. These data suggest that translocon components are secreted prior to effector proteins. It has been hypothesized that a hierarchy of effector translocation also exists at a posttranscriptional level. In EPEC, it has been shown that the secretion of an effector, Tir, is required for the secretion of other effectors, indicating that a hierarchy exists (Thomas *et al*., 2007).

A mechanism for protein sorting for secretion was recently identified in *Salmonella enterica*. A protein complex which was found to localize at the cell membrane was shown to preferentially bind translocon components, over effectors (Lara-Tejero *et al*., 2011). However, in the absence of the translocon proteins, the complex bound effectors, which suggests that the newly identified complex acts as a sorting platform that mediates the hierarchy of protein delivery. This finding provides the first piece of evidence to support the hypothesis that assembly of the T3SS and effector translocation occurs by an ordered mechanism.

1.3.5 EPEC Hallmark of Infection: Attachment and Effacement

The presence of the LEE pathogenicity island confers the ability to cause attaching and effacing (A/E) lesions on infected intestinal epithelial cells, a hallmark of EPEC infection (Staley *et al*., 1969). The A/E lesions are characterized by the organism's ability to intimately adhere to the human intestinal epithelium, followed by the

effacement of infected cells. The effacement of intestinal epithelium cells is characterized by the loss of the structure and function of these cells (Knutton, Lloyd, and McNeish, 1987). A/E lesions were first observed by Staley (1969) while studying rabbits infected with EPEC, and first observed in human biopsies in 1980 (Staley *et al*., 1969; Ulshen and Rollo, 1980). As non-pathogenic strains of *E. coli* do not form A/E lesions, this response became a tool for diagnosis of EPEC and EHEC.

The first stage in A/E lesion formation is localized adherence of bacteria to the epithelial cell, a process that requires expression of an EPEC-adherence factor (EAF) plasmid. The EAF plasmid encodes the bundle-forming pilus (*bfp*), a type IV pilus that is responsible for microcolony formation and bacterial aggregation. The EAF plasmid also encodes the *per* (plasmid-encoded regulator) operon which is important for activation of the *bfp* operon, and of Ler (Gomez-Duarte and Kaper, 1995). This regulatory cascade is an example of coordinated regulation of the expression of plasmid-encoded virulence genes, and chromosomally encoded virulence genes.

The second stage of A/E lesion formation is intimate adherence, which requires proteins encoded in the fifth operon of the LEE (LEE5): intimin, an outer membrane protein, and the translocated intimin receptor (Tir), an effector. Initially it was believed that intimin binds a phosphorylated host protein, Hp90; however, this protein was later identified as a bacterial effector, Tir, which is translocated in a T3SS-dependent manner (Kenny, 1999). Following injection into the host cell, Tir is embedded into the cell membrane in a horse-shoe conformation, which enables it to interact with intimin (Luo *et al*., 2000). Within the C terminus of Tir, residue Y474 gets phosphorylated by a host cell tyrosine kinase, an action which ultimately leads to actin polymerization in an N-WASP-

dependent manner (Kenny, 1999; Lommel *et al*., 2001). Polymerized actin then accumulates at the site of intimate adherence, leading to the formation of pedestals. Like many other effectors, efficient translocation of Tir requires interaction with a chaperone, CesT (Abe *et al*., 1999; Elliott *et al*., 1999).

1.3.6 CesT: a Major Contributor to EPEC Pathogenesis

1.3.6.1 Identification of CesT-Effector Interactions

The chaperone *E. coli* secreted Tir (CesT) is a multivalent chaperone which interacts with at least nine bacterial effectors (Table 1.1). Initially CesT was found to interact with Tir, by two research groups simultaneously (Abe *et al*., 1999; Elliott *et al*., 1999). Both groups found that, although Tir transcription was not affected, intracellular levels of Tir were severely reduced in a *cesT* mutant strain, implying that CesT is important for Tir stabilization (Abe *et al*., 1999; Elliott *et al*., 1999). It was also observed that minute amounts of Tir were still secreted in a *cesT* mutant, suggesting that CesT is not absolutely required (Elliott *et al*., 1999).

CesT was later identified as a bivalent chaperone when a yeast two-hybrid screen revealed an interaction with Map (Creasey *et al*., 2003). Then, during a column binding assay using secreted proteins from a *sepD* mutant, a strain which hypersecretes several EPEC effectors, CesT was identified as a multivalent chaperone as it was shown to interact with EspH, EspZ, EspF, NleA, NleH, NleH2 and NleF (Thomas *et al*., 2005). Two other EPEC effectors NleI and EspG, were also shown to interact with CesT by similar approaches (Li *et al*., 2006; Thomas *et al*., 2007). *In vitro* assays examining the secretion profiles of a *cesT* mutant indicated that effectors are not efficiently secreted or

translocated in the absence of this protein. Furthermore, *C. rodentium*, a close relative of EPEC, has severely attenuated virulence in a mouse model of infection when *cesT* is deleted (Deng *et al*., 2004). The reduced virulence of a *cesT* mutant strain can be explained by its inability to secrete disease-causing effectors, highlighting the significance of this protein as a major contributor to EPEC pathogenesis.

The ability of CesT to interact with nine effectors, some of which are encoded outside of the LEE PAI, shows the versatility of this chaperone. Although the mechanism is not well understood, it has been shown that secretion of Tir is necessary for the secretion of the remaining effectors, suggesting that a hierarchy exists (Thomas *et al*., 2007).

1.3.6.2 CesT Production

The *cesT* ORF is encoded in the fifth operon of the LEE PAI between *tir* and *eae* (intimin). In terms of organization, based on the coupled action of Tir and Intimin along with the requirement of CesT for efficient secretion of Tir, the grouping of these three genes into an operon appears to be biologically relevant. Similar to the other operons of the LEE PAI, transcription of LEE5 is controlled by a main operon promoter, or P_{tir}, and expression from this promoter generates a multigene transcript encoding Tir, CesT, and Intimin (Elliott *et al*., 1999; Sanchez-SanMartin *et al*., 2001). Expression from the P_{tir} promoter is repressed by H-NS in conditions of non-secretion, and can be activated by Ler (Sanchez-SanMartin *et al*., 2001).

In 2001, during characterization of the P_{tir} promoter using a choramphenicol acetyltransferase (CAT) reporter, activity was reported for a fusion containing the *tir-cesT*

intergenic region, suggesting the presence of a promoter element directly upstream of *cesT* (Sanchez-SanMartin *et al*., 2001). Interestingly, a fusion containing both the *tir-cesT* and *cesT-eae* intergenic regions exhibited minimal activity in a CAT reporter assay, indicating the presence of a terminating element encoded between *cesT* and *eae* (Sanchez-SanMartin *et al*., 2001). Further characterization of the *tir-cesT* intergenic region was performed using primer-extension analysis, and revealed the presence of two putative transcriptional start sites, P1 and P2, positioned 15 and 87 nucleotides upstream of the *cesT* start codon, respectively (Sanchez-SanMartin *et al*., 2001). Sequence analysis allowed prediction of a promoter region directly upstream of the putative P1 transcriptional start site which showed sequence conservation to a σ^{70} promoter; no such element was observed upstream of the putative P2 transcriptional start site. These data suggest that a promoter region exists within the *tir-cesT* intergenic region, and this promoter is responsible for the expression of *cesT* specifically; for this thesis is named P_{cesT}.

1.4 *E. coli* σ^{70} promoters

Gene expression is regulated at the transcriptional level by RNAP. RNAP consists of five subunits ($\beta\beta'\alpha_2\omega$) which are sufficient for the elongation of the mRNA message and termination of transcription. However, this complex is not able to initiate transcription (Paget and Helmann, 2003). Another element, a σ factor, binds directly to RNAP forming a holoenzyme, which recognizes a promoter region and can initiate transcription. There are seven σ factors found in *E. coli* which are responsible for regulating the transcription of specific genes (Ghosh *et al*., 2010). For example, σ^{70} is responsible for regulation of 'house-keeping' genes, σ^{54} is responsible for nitrogen-

assimilation genes, and σ^{32} is responsible for heat-shock response genes (Mooney *et al*., 2005).

As it is responsible for regulation of the vast majority of genes, the σ^{70} factor is present in the cell in high copy number. There are four conserved regions within σ^{70} factors: $\sigma_{1,}$ $\sigma_{2,}$ σ_3, and σ_4. The interaction between the σ factor and the RNAP is mediated primarily by $\sigma_{2,}$ σ_3, and σ_4 (Paget and Helmann, 2003). Subregions $\sigma_{2.4}$ and $\sigma_{4.2}$ mediate the interaction between the holoenzyme and the promoter region by recognizing specific elements of a promoter, centred at 10 and 35 nucleotides upstream of the transcriptional start site (Campbell *et al*., 2002; Severinova *et al*., 1996). Interaction of the holoenzyme with the promoter region initiates the formation of the 'open complex' of DNA, a confirmation that enables transcription to begin (Mekler *et al*., 2002).

A bioinformatics study of 554 *E. coli* σ^{70} promoters revealed a conserved consensus sequence. The consensus sequence for the -10 element, also known as the TATA box, is TATAAT (5'→3'), and 95% of promoters have at least three out of six nucleotides conserved (Mitchell, Zheng, Busby, and Minchin, 2003). The consensus sequence of the -35 element has been identified as TTGACA (5'→3'), and 83% of promoters have at least three of six nucleotides conserved (Mitchell *et al*., 2003). The -35 element is less well-conserved, and it was observed that the poorer the -35 element, the better conserved the -10 element (Mitchell *et al*., 2003).

The sequence of the spacer region linking the -10 and -35 elements also influences the activity of a promoter. Bioinformatics analysis revealed that the most frequent length of a spacer region is 17 nucleotides, which is present in 44% of all promoters analyzed

(Mitchell *et al*., 2003). The presence of an extended 'TG' motif located 14 and 15 nucleotides upstream of the transcriptional start site, has been shown to be an important component of σ^{70} promoters. The TG motif is recognized by domain $\sigma_{3.1}$, and is present in 20% of *E .coli* promoters (Mitchell *et al*., 2003). It was observed that when an extended TG motif is present, the -10 and -35 sequences are less conserved and deviate from the consensus sequence. Interestingly, it was shown that promoter activity could be increased by the addition of the TG motif, and reduced by its removal (Mitchell *et al*., 2003). Another important feature of σ^{70} promoters is the nucleotide at position -18 relative to the transcriptional start site. This nucleotide is well-conserved as a T, and has been found to be important for DNA topology, and thus promoter recognition by the σ factor (Singh *et al*., 2011).

1.5 Objectives

My objectives are to further characterize the activity of the promoter encoded within the *tir-cesT* intergenic region, to identify conditions under which the promoter is active, and to identify possible regulatory mechanisms. Also, it is my goal to generate an unbiased tool that can report the activity of P_{cesT} directly from the chromosome, which can be used in future applications to study this promoter.

CHAPTER 2. MATERIALS AND METHODS

2.1 Bioinformatic Analysis of P_{cesT}

Computational analysis of the *cesT* promoter region (P_{cesT}) was performed using the Basic Local Alignment Search Tool (BLAST) on the National Center for Biotechnology Information (NCBI) website (National Centre for Biotechnology Information, http://www.ncbi.nlm.nih.gov/). Briefly, a 660-basepair (bp) nucleotide sequence of DNA from EPEC strain E2348/69 serotype O127:H6 encompassing the *tir-cesT* intergenic space with 250 bp upstream and downstream was entered into BLAST. Strains which showed high sequence conservation to E2348/69 were aligned by the Multiple Sequence Alignment tool CLUSTALW (Kyoto University Bioinformatics Centre, http://www.genome.jp/tools/clustalw/).

2.2 Growth Conditions of Bacterial Strains

The bacterial strains used in this study are listed in Table 2.1. Bacteria were routinely grown in LB (10 g tryptone, 5 g yeast extract, 10 g NaCl, per litre of dH_2O) (Sigma), shaking at 200 rpm with a constant temperature maintained at 37°C. Antibiotics were added to a final concentration of 75 μg/ml for kanamycin (Kn75), 50 μg/ml for streptomycin (St50), 30 μg/ml for chloramphenicol (Cm30), 10 μg/ml for tetracycline (Tet10), or 100 μg/ml for ampicillin (Amp100), where applicable. Bacteria were grown statically on LB agar (Sigma), MacConkey agar (50 g per litre of dH_2O) (Sigma), and LB agar with 5-Bromo-4-chloro-4-indolyl β-D-galactopyranoside (X-gal) (0.2 μg/ml) (Sigma) at 37°C, overnight (O/N).

Table 2.1. Strains and plasmids used in this study

Strain	Description	Reference
Wild-type EPEC	EPEC strain E2348/69, serotype O126:H7 streptomycin resistant	(Levine *et al*., 1978)
Δ*cesT*	*cesT* deletion mutant	(Abe *et al*., 1999)
Δ*escN*	*escN* deletion mutant	(Gauthier *et al*., 2003)
Δ*grlA*	*grlA* deletion mutant	J. Puente
Δ*grlR*	*grlR* deletion mutant	J. Puente
Δ*ler*	*ler* deletion mutant	J. Puente
EPECΔ-10	E2348/69 with a chromosomal mutation in the *cesT* promoter region	This study
EPECΔintg	E2348/69 with a chromosomal deletion of the *tir-cesT* intergenic space	This study
EPECΔ*lacZ*	E2348/69 with a chromosomal deletion of the *lacZ* ORF	This study
EPECΔ*lacZ*/pRK*lacZ*	EPECΔ*lacZ* complementation strain expressing the lacZ ORF on plasmid pRK415	This study
EPECΔ*lacZ*::*cesT* VIK	EPECΔ*lacZ* containing a chromosomally encoded *lacZ* reporter at the *cesT* promoter region	This study
NH4	E2348/69Δ*hsdR*. Contains a mutation in the EPEC type-I restriction modification system. Used as a 'helper' strain.	(Hobson *et al.,* 2008)
DH5α	General strain for cloning purposes	Invitrogen life technologies
MT616	Used as a 'helper' strain during bacterial	Invitrogen life

	conjugation	technologies
Sm10λ*pir*	Conjugative strain of pRE112	(Miller and Mekalanos, 1988)
DH5αλ*pir*	Cloning strain of pRE112	Invitrogen life technologies
Plasmids		
pJW15	Vector containing *luxCDABE* reporter genes and kanamycin-resistance cassette	(Macritchie *et al*., 2008)
P1*lux*	*cesT* promoter (P1) in pJW15	This study
P2*lux*	*cesT* promoter (P2) in pJW15	This study
P3*lux*	*cesT* promoter (P3) in pJW15	This study
P_{tir} *lux*	LEE5 promoter in pJW15	This study
P3Δ10*lux*	P3l*ux* containing the Δ-10 base pair substitution mutation	This study
P3Δintg*lux*	P3l*ux* containing the Δintg deletion mutation	This study
pRK415	Conjugatable vector containing a tetracycline resistant cassette	
pRK*lacZ*	pRK415 backbone *lacZ* ORF	This study
pVIK112	β-galactosidase reporter plasmid; suicide plasmid containing a kanamycin-resistance cassette	(Kalogeraki and Winans, 1997)
*cesT*VIK	pVIK112 expressing P_{cesT}	This study
pRE112	Suicide plasmid that requires *pir* for replication, and contains *sacB1*	(Edwards *et al*., 1998)

Table 2.2. Oligonucleotides used in this study. Restriction endonuclease sites have been underlined.

Name	Oligonucleotide Sequence 5' →3'	Restriction Site
EB1	CCGCTCGAGTACTAGCGTTAATGTTTCAG	XhoI
EB2	CGGGATCCCCAACACCAATTTTTTCCGC	BamHI
EB3	CCGCTCGAGTCGACACACTGGTCAGATTCC	XhoI
EB4	CGGGATCCTTCTCTGAAACATTAACGC	BamHI
EB5	GCCTCGAGGCAGGATACTCGGCTGTAGG	XhoI
EB6	CCGGATCCCCAATAGGCATACATATATCC	BamHI
EB7	CCGAATTCTCGACACACTGGTCAGATTCC	EcoRI
EB8	CCGGTACCGGACGTTGATACGCGTTC	KpnI
EB9	GGCTCGAGACAGAAGTTCAAGAGTTA	XhoI
EB10	GGCTCGAGAAATCAATTAAGAGAA	XhoI
EB11	CCGAGCTCCTGCTGACCAGGCGCGGCC	SacI
EB12	GGCTCGAGATACTCACAGATATATTAA	XhoI
EB13	GGCTCGAGGAACTTCTGTTATTATAAATC	XhoI
EB14	GGTCTAGACACAGGTATGGCCCACC	XbaI
EB15	CCGGTACCTCCGCTGGATGACCAGGATG	KpnI
EB16	GGCTCGAGCATAGCTGTATCCTGTGTGAA	XhoI
EB17	GGCTCGAGTAATAATAACCGGGCAGGCC	XhoI
EB18	CCGAGCTCCCACCAGCAGGAACGGTAC	SacI
EB19	CCAAGCTTAATGCAGCTGGCGCGACA	HindIII
EB20	CCTCTAGATTATTTTGACACCAGACC	XbaI
EB21	ACACCGGAACCGGATGATCC	
EB22	GGACGACGACAGTATCGGCG	
EB23	/5Biosg/TCGACACACTGGTCAGATTCCT	
EB24	/5Biosg/GCAGGATACTCGGCTGTAGG	

2.3 Isolation of DNA

Plasmid DNA was isolated from bacterial strains grown overnight in LB, using a Qiaprep Spin Miniprep kit (Qiagen) or the HiSpeed Plasmid MIDI kit (Qiagen), following the manufacturers' instructions. Genomic DNA was isolated from strains using the Puregene Core kit A (Qiagen).

2.4 Generation of EPEC Mutants

2.4.1 EPECΔ-10

The EPECΔ-10 mutant was generated using homologous recombination to substitute the six base pairs that comprise the -10 element of the P_{cesT} promoter region with a XhoI restriction site. To begin, two fragments corresponding to the DNA regions directly upstream (Fragment A) and downstream (Fragment B) of the -10 hexamer were amplified using EPEC genomic DNA as a template in a polymerase chain reaction (PCR) using Phusion High-Fidelity DNA Polymerase (New England Biolabs (NEB)). The typical reaction conditions for PCR, unless otherwise stated, were as follows: 5 min at 95°C, 30 cycles of 30 sec at 95°C, 30 sec at 50°C, and 1 min/kb of expected product at 72°C, followed by a final 5-min step at 72°C. Fragment A was amplified using primers EB8 and EB9, which contained a 5' KpnI and 3' XhoI restriction site, respectively. Fragment B was amplified using primers EB10 and EB11 which contained a 5' XhoI and 3' SacI restriction site, respectively.

PCR products were purified using the Qiaquick PCR purification kit (Qiagen). Fragments A and B were treated with the XhoI restriction endonuclease (NEB) and used in a ligation reaction, generating Fragment A:B. A typical ligation reaction had the following recipe: 1µl of T4 DNA ligase (NEB), 1 µl of T4 DNA ligase buffer, purified

PCR product and vector, added at a 1:1 ratio, and dH_2O added to obtain a final volume of 10 µl, which was incubated at 16°C for 18 hrs. The resulting plasmid was heat-shocked into DH5αλpir chemically competent cells, and plated on LB agar with Cm30. Resulting colonies were screened for the correct plasmid by restriction digest.

The pRE112 suicide plasmid containing the correct insert was heat-shocked into cells of the conjugatable strain Sm10λpir, followed by transfer into EPEC *via* conjugation. Sm10λpir harbouring pRE112 and WT EPEC were grown O/N with shaking in LB with Cm30 or St50, respectively. One millilitre of culture was pelleted by centrifugation (13,200 rpm for 1 min), then resuspended in 200 µl of LB. Both strains (25 µl) were plated in the centre of an LB plate, which was then incubated at 37°C for 5-6 hrs. Following incubation, the bacterial emulsion was scraped off the surface of the solid medium with a sterile toothpick, smeared into the bottom of a sterile centrifuge tube, and resuspended in 200 µl of LB. The resuspension was plated onto LB agar with St50 and Cm30, and incubated O/N at 37°C. It is important to note that EPEC does not have the machinery to allow replication of the pRE112 plasmid; thus, resistance to Cm30 is strictly mediated by integration of the plasmid into the EPEC chromosome by a single cross-over event *via* homologous recombination. Following the O/N incubation, colonies were picked from the St50/Cm30 plate and streaked onto 5% sucrose solid medium (10 g tryptone, 5 g yeast, 50 g sucrose, 15 g agar per litre) and incubated at 30°C O/N. Resulting colonies were patched onto LB agar with St50 and screened for correct integration of the mutation in the chromosome by PCR. PCR products were treated with XhoI (NEB) to ensure integration of a novel restriction site into the chromosome. This

chromosomal mutation was sequenced at McGill University and Génome Quebec (McGill University, Montreal, Canada).

2.4.2 EPECΔintg

The EPECΔintg mutant was generated by deletion of 83-bp of the *tir-cesT* intergenic region. Two fragments were amplified by PCR using EPEC genomic DNA as a template. Fragment 1 was amplified using EB8 and EB12 which contain 5' KpnI and 3' XhoI restriction sites, respectively. Fragment 2 was amplified using primers EB13 and EB11 which contain a 5' XhoI and 3' SacI restriction site, respectively. Each fragment was treated with XhoI (NEB), ligated, and cloned into pRE112 by treatment with KpnI and SacI (NEB). Integration of this mutation into the EPEC chromosome was performed as described above (Section 2.4.1). This chromosomal mutation was sequenced at McGill University and Génome Quebec (McGill University, Montreal, Canada).

2.4.3 EPECΔ*lacZ*

The EPECΔ*lacZ* mutant was generated by deletion of the *lacZ* ORF, excluding the start and stop codons, from the EPEC chromosome. Two fragments, which correspond to the DNA directly upstream and downstream of the *lacZ* ORF, were amplified by PCR using EB15 and EB16, containing a 5' KpnI and 3' XhoI restriction site, and EB17 and EB18, containing a 5' XhoI and 3' SacI restriction site. Each fragment was digested with XhoI (NEB), ligated, and cloned into pRE112. Integration of this mutated DNA into the EPEC chromosome was performed as described above, generating EPECΔ*lacZ*. EPECΔ*lacZ* was also grown on MacConkey Agar (Sigma) containing St50 and LB agar with X-gal.

2.4.3.1 Complementation of EPECΔ*lacZ*

The *lacZ* ORF was amplified using EPEC genomic DNA as a template in a PCR using primers EB19 and EB20, containing HindIII and XbaI restriction sites, respectively. The PCR product was cloned into pRK415, generating pRK*lacZ*, and heat-shocked into cells of strain DH5α. Plasmid pRK*lacZ* was moved into EPECΔ*lacZ* by conjugation, using helper strain MT616. The conjugation reaction was performed as described (Section 2.4.1) by incubating the strains of interest, DH5α harbouring pRK*lacZ*, EPECΔ*lacZ*, and MT616, on LB agar for 5-6 hrs at 37°C. Following incubation, bacteria were grown on LB agar with St50 and Tet10 at 37°C O/N to select for EPECΔ*lacZ*/pRK*lacZ*. Resulting colonies were tested for *lacZ* complementation by growth on MacConkey agar and LB agar with X-gal.

2.5 Luciferase Reporter Assay

The luciferase reporter assay employs the use of plasmid-encoded *luxCDABE* genes from *Photorhabdus luminescens*. The *luxAB* genes encode the luciferase enzyme, which catalyzes the oxidation of reduced flavin mononucleotide and a long-chain fatty aldehyde resulting in bacterial bioluminescence (Engebrecht and Silverman, 1984). The *luxCDE* genes are required for the conversion of fatty acids into the long-chain aldehyde which acts as an intermediate in this reaction (Meighen, 1993). The rate-limiting step in this reaction is the production of luciferase, as the enzyme will only undergo a single turnover (Meighen, 1993). The rate of decay of luminescence occurs quickly, making this assay highly sensitive, and a useful tool for real-time analyses.

2.5.1 Generation of the *lux* Transcriptional Fusions

Generation of the *lux* transcriptional fusions was performed by amplifying DNA of interest by PCR, using EPEC genomic DNA as a template. P1*lux*, P2*lux*, and P3*lux* were amplified using primers EB1 and EB2, EB3 and EB4, and EB3 and EB2, respectively (Table 2.2, Figure 2.1). These primers were engineered to encode a 5' XhoI restriction site and a 3' BamHI restriction site. The plasmid backbone, pJW15 (Figure 2.2), was isolated from DH5α using a MIDI kit and treated with the XhoI and BamHI (NEB). Each digestion reaction was visualized on a 1.0% agarose (UltraPure Agarose, Invitrogen) gel, and the appropriate band was purified by gel extraction using a QIAEX II Gel Extraction Kit (Qiagen), or by column purification. Purified pJW15 vector was mixed in equal parts with the treated PCR fragments in a ligation reaction, which was then incubated for 18 hrs at 16°C. Following the 18-hour incubation, each ligation reaction was heat-shocked into DH5α chemically competent cells, and the resultant cells were grown on LB agar with Kn75, at 37°C, O/N. The resulting colonies were screened by digestion and sequenced at McGill University and Génome Quebec (McGill University, Montreal, Canada).

Cells of strain DH5α harbouring the *lux* transcriptional fusions were grown O/N in 50 ml of LB containing Kn75, and plasmid DNA was isolated using a MIDI kit (Qiagen), and transferred to a helper strain, NH4. Briefly, 2 µl of plasmid DNA was added to 50 µl of electrocompetent NH4 cells and the mixture was subjected to electroporation using the BioRad Gene Pulser Xcell Microbial System (0.2-cm cuvette, 3 kV, 25 µF, 200 Ω) (BioRad). Following electroporation, cells were immediately transferred to 1 ml of LB

ATGcctattggtaaccttggtaataatgtaaatggcaatcatttaattccccctgcgccgccactaccttca
caaacagacggcgcggcacggggaggaactggtcatctaattagctctacaggagcattaggatctcgttca
ttgttttctcccctgagaaattctatggctgattctgtcgattccagagatattccaggacttcctacaaac
ccatcgaggcttgctgcagctacatctgagacatgcttgcttggaggatttgaagttctccatgataagggg
ccacttgatattctcaatacgcaaattggaccctctgcatttcgtgttgaagtgcaggcagatggtactcat
gccgctattggagaaaaaaatggtttggaggttagcgttacattaagtcctcaagaatggagcagcttgcaa
tctattgatactgagggtaaaaacagatttgtttttaccgggggacgtggcggtagtgggcatccgatggtc
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agtggtggcgagagcgcagtaagttctgtaaatgccgcaccaacgccgggaccagtacgtttcgtttaaata
tatctgtgagtatttagttgaggttggggtggggtgggggggcgtt**T**tactagcgttaatgtttcagagaac
aacgttgcagcatgggtaactcttgaacttctgttattataaatcaa**T**taagagaaattata**ATG**tcatcaa
gatctgaacttttattagataggtttgcggaaaaaattggtgttggatctatttccttttaatgaaaatagat
tgtgttcttttgctattgatgaaatttattatatttcgttatctgatgccaatgacgaatatatgatgattt
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ttgcagagaatggtgggccatacctgtgttatgagtcaggagcacaatcgctgttgttagcgttacgtttcc
cgctcgatgatgctacccctgaaaaacttgagaatgaaatagaagtcgtcgttaagtcgatggaaaatctgt
atttggtattacataatcagggaataacattagaaaacgaacatatgaaaatagaggaaattagctcaagcg
ataataaacattattacgccggaagataa

EB3
EB1
EB4
EB2

Figure 2.1 Nucleotide sequence of the *tir* and *cesT* ORF. The nucleotide sequence of the *tir* (red) and *cesT* (blue) ORF of E2348/69 (O127:H6) obtained from NCBI. Primer sequences of EB1, EB2, EB3 and EB4 are boxed. The translational start site of *tir* and *cesT* are capitalized, and the putative P1 and P2 transcriptional start sites within the intergenic region are capitalized and bold.

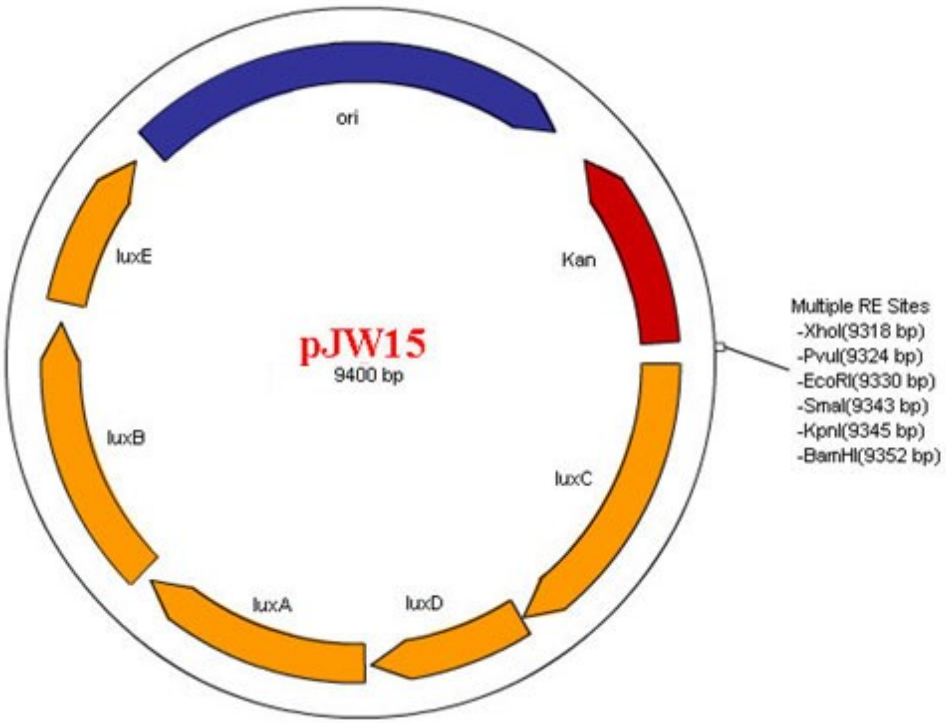


Figure 2.2 A representation of the *luxCDABE* genes encoded in the pJW15 reporter plasmid. The pJW15 plasmid (9.4 kb) encodes *luxCDABE* from *P. luminescens*, an origin of replication (ori), and a kanamycin resistant cassette (Kan). The multiple cloning site contains a XhoI, PvuI, EcoRI, SmaI, KpnI, and BamHI restriction site, respectively.

and incubated for 1 hr with shaking at 37°C for recovery. Cells were then plated onto LB agar with Kn75, and grown at 37°C O/N. Plasmid DNA was subsequently isolated from NH4 cells and electroporated into EPEC electrocompetent cells by the technique described above. NH4 was used as a 'helper' strain since DNA preparations from DH5α did not move readily into EPEC; however, passing the DNA through NH4 cells first dramatically increased efficiency.

A transcriptional fusion encoding the P_{tir} operon promoter was generated by the technique described above. A fragment encoding P_{tir} was amplified using primers EB5 and EB6, engineered to contain a 5' XhoI and 3' BamHI site. This fragment was cloned into pJW15, generating P_{tir} *lux*, and introduced into EPEC cells.

Transcriptional fusions to the *lux* reporter genes were made to measure the activity of P_{cesT} which contain mutations. EPECΔ-10 genomic DNA was used as template in a PCR using primers EB7 and EB2, which contained a 5' EcoRI site, and a 3' BamHI site, respectively. PCR products were purified, treated with EcoRI and BamHI, and cloned into pJW15, generating P3Δ10*lux*, and introduced into EPEC cells. EPECΔintg genomic DNA was used as a template in a PCR using primers EB7 and EB2; the resulting product was cloned into pJW15, generating P3Δintg*lux*, and introduced into EPEC cells.

2.5.2 Luciferase Reporter Assay

Each strain investigated by a luciferase reporter assay was streaked for isolated colonies using a sterile loop on LB agar containing St50 and Kn75. A single bacterial colony was used to inoculate a 5-ml LB culture supplemented with St50 and Kn75 and grown at 37°C O/N. The resulting cultures were gently vortexed for 10 sec, after which a

1:2 dilution was made into PBS, generating a working stock of culture. The OD of each stock culture was measured using the Biophotometer Plus, which was then used to equalize the number of bacteria being added to the assay for each, using EPEC P3*lux* as a standard.

Strains of interest were inoculated into colourless Dulbecco's Modified Eagle Medium (DMEM) (Hyclone), M9 minimal medium (10x M9 salts (42 mM Na_2HPO_4, 22 mM KH_2PO_4, 18.7 mM NH_4Cl_2, 8.6 mM NaCl), 1 mM $MgSO_4$, 1 mM $CaCl_2$, 1 mM Thiamine-HCl, glucose), or LB, prewarmed at 37°C. Briefly, tubes containing the working stock of culture were vortexed gently for 10 sec and the required volumes, as calculated above, were added to 3 ml of the appropriate media which had been aliquoted into sterile test tubes, to obtain a final dilution of 1:100. Medium was added to obtain a final volume of 4 ml. Each culture was vortexed gently and aliquoted into two 96-well plates, an Optiplate-96 (Perkin Elmer), and a clear 96-well plate (Costar), 200 µl per well, 8 wells per strain. Bacterial bioluminescence was captured as counts per second (CPS), measured from the Optiplate-96 using a Victor XLight 2030 Luminescence Reader (Perkin Elmer), while the OD was captured simultaneously from the 96-well plate using a Victor X5 2030 Multilabel Reader (Perkin Elmer). CPS and OD measurements were taken every 30 min for the first 4 hr of the assay, and every subsequent hour following. Between readings the Optiplate-96 and the 96-well dish were incubated at 37°C with 5% CO_2.

The data generated were analyzed using Microsoft Excel. The average CPS and OD values were calculated for each strain at each time point, and subtracted from the average of the medium-only blank. The adjusted OD values were multiplied by a

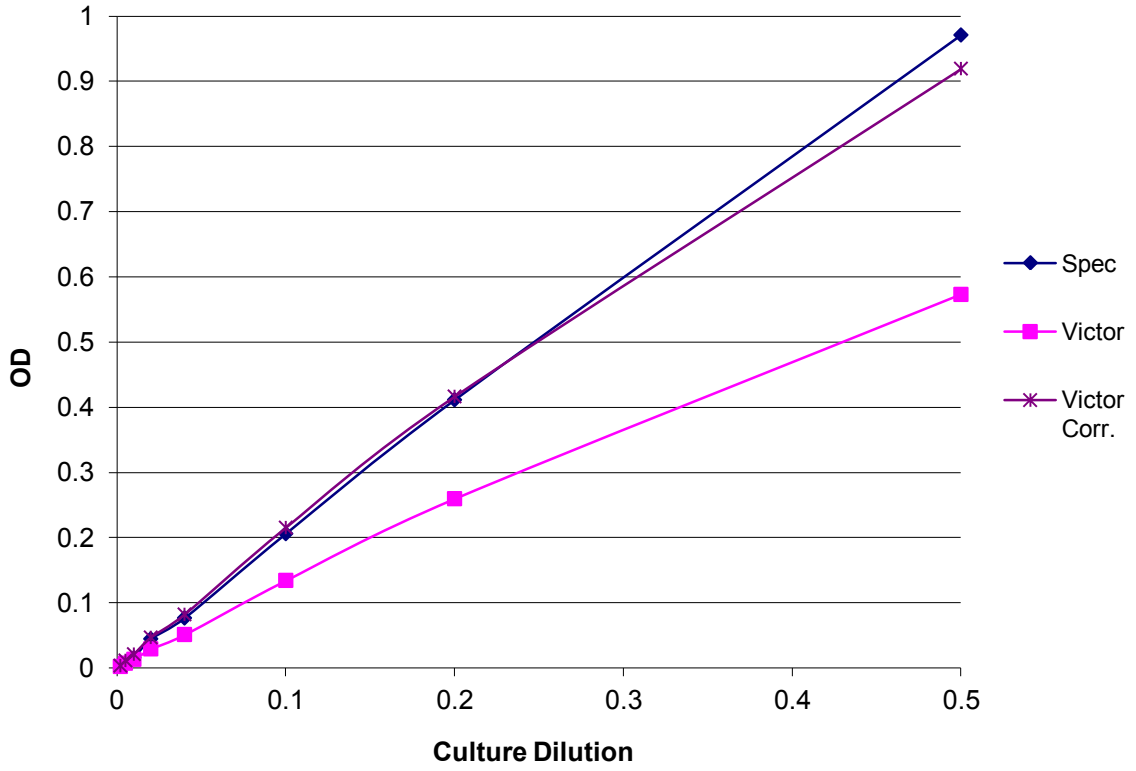


Figure 2.3 Determination of the optical density correction factor. An O/N bacterial culture of WT EPEC grown in LB was diluted in PBS. The OD was measured using a spectrophotometer and the Victor X Light, and the correction value was determined by taking the ratio of the two values.

correction factor, which was generated to account for differences in OD between the Victor X5 plate reatder and the Biophotometer. The correction factor was determined by dilution of an O/N bacterial culture into PBS, and measuring the OD600 in the Biophotometer Plus, and the Victor X5 plate reader (Figure 2.3). For each time point, the adjusted CPS was normalized for the corresponding adjusted corrected OD (CPS/Corr OD).

2.5.3 Statistical Analysis

To determine statistical significance between two data points, a one-tailed Student's T-test was performed using Microsoft Excel software. A p-value of less than 0.05 ($p<0.05$) was considered statistically significant.

2.6 *In vitro* Secretion Assays

Bacteria were grown in 5 ml of LB (37°C, 200 rpm) O/N. Prewarmed DMEM containing phenol red (Gibco) (2 ml) was inoculated with 40 µl of O/N culture and grown statically for 3 hr at 37°C and 5% CO_2. Whole cell lysates (WCL) were prepared by centrifugation (13,200 rpm) of 1 ml of culture for 1 min, and resuspended in 100 µl of 2x ESB (0.0625 M Tris-HCl (pH 6.8), 1% (w/v) SDS, 10% glycerol, 2% 2-mercaptoethanol, 0.001% bromophenol blue). Samples were boiled for 5 min and stored at -20°C. A time-course secretion assay was performed whereby WCL were boiled immediately at every time point of interest.

2.7 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Protein samples were separated by SDS-PAGE as described by Laemmli (1970). The electrode buffer consisted of 25 mM Tris, 192 mM glycine, 0.1% sodium dodecyl

sulphate (SDS). Electrophoresis was carried out using the mini-protean system (1.5 hours, 100 volts) (BioRad). Proteins were visualized using Coomassie blue staining.

2.8 Western Blotting

Proteins separated by SDS-PAGE were transferred to Immobilon-P Polyvinylidene Fluoride membranes (Millipore) using a blotting apparatus (Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell (BioRad); 15V, 30 mins). The buffer used for the semi-dry transfer was composed of 48 mM Tris, 39 mM glycine, 1.3 mM SDS and 20% methanol (v/v).

Following the protein transfer, the membrane was washed twice for 5 min in Tris-buffered saline with Tween (TBS-T; 145 mM NaCl, 100 mM Tris-HCl (pH 7.4), 0.15% (v/v) Tween-20) followed by a 1 hr incubation with 5% (w/v) skim milk in TBS-T for blocking. Following this incubation, the membrane was washed twice for 5 min with 10 ml TBS-T and then incubated with primary antibodies for 1 hr (Table 2.3). The membrane was washed twice in 10 ml of TBS-T for 10 min, and then incubated with secondary antibodies for 1 hr (Table 2.3). Following this incubation, the membrane was washed twice with 10 ml of TBS-T for 5 min. After the final wash, the membrane was reacted with ECL Plus (GE healthcare Bio-Science), or Immun-star WesternC kit (BioRad) as per manufacturer's instructions. Images were captured with the VersaDoc MP 5000 Imaging System (BioRad)

2.8.1 Protein Quantification

Images of western blots captured by the VersaDoc, as explained above, were subjected to densitometry analysis using Image Lab 3.0 software (BioRad), which can

Table 2.3 Antibodies used in this study

Antibody	Dilution	Source
Rabbit Anti-CesT	1:10000	Pacific Immunology
Mouse Anti-RNA polymerase	1:10000	Santa Cruz Biotechnology
Goat anti-rabbit HRP	1:5000	Rockland Immunochemicals
Goat anti-mouse HRP	1:5000	Rockland Immunochemicals
Horse anti-mouse HRP	1:5000	Cell Signalling Technology

measure the density of any particular band on a membrane. Band densities were recorded and analyzed in Microsoft Excel. The relative quantity of CesT was normalized for the loading control, RNA polymerase, by taking the ratio of the two densities. The linear range at which the software could accurately quantify band density was determined by performing a dilution series of a WCL sample obtained from a 6 hr secretion assay. The WCL dilutions were subjected to SDS-PAGE followed by western blotting, as described above.

2.9 Generation of a β-Galactosidase Reporter

A fragment of DNA consisting of the P_{cesT} promoter region was amplified by PCR using primers EB3 and EB14, containing 5' XhoI and 3' XbaI restriction sites, respectively. The fragment of DNA was treated with XhoI and XbaI (NEB), and cloned into the pVIK112 suicide reporter plasmid and heat-shocked into DH5αλ*pir* cells. The plasmid was transferred into EPECΔ*lacZ* cells *via* conjugation using strain MT616 as a 'helper', and selected on LB agar with Kn75. Correct integration of this plasmid into the Δ*lacZ* chromosome was confirmed by PCR and by blue/white screening on LB agar containing X-gal.

2.10 Biotin Pulldown Assay

2.10.1 DNA Containing Biotin was Bound to Streptavidin-Coated Beads

Two fragments of DNA corresponding to P_{tir} and P_{cesT} were amplified by PCR using primer EB24 and EB6, and EB23 and EB2, respectively. Primers EB23 and EB24 contained 5' biotin tags (Integrated DNA Technology). PCR products were visualized on a 1% agarose gel and purified by gel extraction (Qiagen Kit).

Beads coated with streptavidin (Dynabeads M-270 Streptavidin) (Invitrogen) were resuspended and 4 mg was distributed equally into two sterile centrifuge tubes. The P_{tir} and P_{cesT} amplified DNAs were bound to the beads as per the manufacturer's instructions. To determine the quanitity of DNA bound to the streptavidin beads, DNA was eluted off (2 µl of each sample), as per the manufacturer's instructions, and visualized on a 1% agarose gel. The relative quantity of DNA captured on the beads was determined by measuring band density, as compared to the band density of similarly sized bands from the 100-bp DNA ladder (NEB). It was calculated that 0.540 µg/mg of the P_{cesT} fragment was captured by the beads, and 1.70 µg/mg of the P_{tir} fragment was captured. DNA-containing beads were stored at 4°C until used.

2.10.2 EPEC Fractionation

WT EPEC cells were grown O/N in 5 ml of LB with St50, shaking at 37°C. The resulting culture (3 ml) was pelleted by centrifugation (13,200 rpm, 1 min) in a sterile centrifuge tube. The pellet was resuspended in 1 ml of PBS and the resulting resuspension was plated onto MacConkey agar and LB agar. Briefly, 100 µl of the bacterial resuspension was added to the centre of five MacConkey and five LB agar plates, both containing St50. Bacteria were spread to cover the entire plate and grown at 37°C O/N.

The resulting lawn of bacteria was scraped off each plate and resuspended in 25 ml of chilled PBS. Cells were pelleted by centrifugation at 5000 rpm at 4°C for 15 min. The supernatant was removed and the resulting pellet was washed in 25 ml of chilled PBS, followed by centrifugation. Cells were then resuspended in 10 ml of room temperature PBS containing lysozyme (100 µg/ml) (Sigma), and incubated for one hour

at room temperature with gentle shaking. A sample of 50 µl was taken at this time and pelleted by centrifugation (13,200 rpm, 1 min) in a centrifuge tube. The bacterial pellet was resuspended in 2x ESB and boiled immediately for 5 min; this sample was representative of WCL.

Following treatment with lysozyme, bacteria were pelleted by centrifugation, the supernatant was removed, and the remaining pellet was incubated at -80°C for 30 min. Pellets were thawed quickly by incubation in a 37°C water bath, followed by resuspension in 5 ml of chilled column binding buffer (20 mM Tris (pH 8.0), 1 mM EDTA, 1 mM DTT) (TED). Cells were subjected to lysis by sonication (6 x, 30 sec, setting 3), followed by high-speed centrifugation (20,000 rpm, 30 min, 4°C) in a Beckman Avanti Centrifuge with a fixed angle rotor. The supernatant was collected as the soluble protein fraction, and the resulting pellet was resuspended in 5 ml of TED and saved as the insoluble protein fraction. Proteins isolated from EPEC cells grown on MacConkey agar and LB agar were separated by SDS-PAGE and visualized by Coomassie Blue Staining.

2.10.3 Heparin-Sepharose Column Binding

Soluble proteins isolated from bacteria grown on LB agar or MacConkey agar were passed over heparin-sepharose resin (15 ml) (Sigma). Briefly, the column was washed with 30 ml column binding buffer containing 1 M NaCl (Sigma), followed by equilibration with 30 ml TED. Soluble proteins were passed over the column; the resulting flow-through was collected as the 'unbound' fraction. The column was washed with 30 ml of TED; the flow-through was saved as the 'wash' fraction. Proteins were eluted off the column by washing with column-binding buffer containing varying

concentrations of NaCl. Briefly, the first elution was performed using 15 ml of buffer with 0.1 M NaCl. The flow through containing the eluted proteins was saved in 10 sterile centrifuge tubes. This process was repeated 4 more times using buffer containing 0.2 M, 0.3 M, 0.4 M and 1 M NaCl. In total, there were 52 samples for both proteins isolated from LB agar and MacConkey agar corresponding to one unbound fraction, one wash fraction, and 10 samples for each elution fraction.

2.10.4 Determination of Samples Which Contained Nuclease Activity

To determine which protein fractions described above contain nucleases, protein fractions were incubated for 1 hr at 37°C with 1-kb DNA ladder (NEB). Each reaction contained 2 µl of the protein fraction of interest, 0.5 units of 1-kb ladder, 10x NEB Buffer 2 diluted to a 1x final concentration, and dH_2O to make the final volume to 5 µl. Each digestion was visualized on a 1% agarose gel and imaged using the VersaDoc (Figure 3.21).

Protein fractions were pooled according to the resulting band pattern and the concentration of NaCl in their respective buffer. The protein fractions which represented bacteria grown on LB agar were pooled into six fractions (labelled 1 through 6), and the fractions which represented bacteria grown on MacConkey agar were pooled into eight fractions (labelled 1, 2, 3, 4a, 4b, 5a, 5b, 6). Fractions 4b and 5b were excluded for subsequent experiments based on the hypothesized presence of DNA nucleases.

Table 2.4 Protein fractions were pooled prior to incubation with biotin-tagged DNA. Pooled protein fractions were mixed with TGED containing 50mM NaCl followed by incubation for 10 min at room temperature with biotin-tagged DNA.

Sample	A	B	C	D	E	F
Initial [NaCl]	0.1M	0.2M	0.3M	0.4M	1.0M	1.0M
Sample (ml)	0.75	0.375	0.5	0.375	0.3	0.3
TGED (ml)	0.75	1.125	1.0	1.125	1.2	1.2
Final [NaCl]	0.05	0.05	0.09	0.1	0.2	0.2

2.10.5 Protein-DNA Interaction

Beads which contained the bound P_{cesT} and P_{tir} DNA fragments were divided equally among six sterile centrifuge tubes. The tubes were placed on a magnetic rack for 1 min, and the supernatant was removed. Tubes were removed from the magnetic rack and beads were washed once in 0.5 ml of TGED buffer (20 mM Tris (pH 8.0), 1 mM EDTA, 10% glycerol, 1 mM DTT, 0.01% Triton-X 100). Beads were incubated twice in 1.5 ml of TGED buffer with 50 mM NaCl and protein samples (Table 2.4) for 10 min at room temperature. Following incubation, samples were placed on the magnetic stand for 1 min and the supernatants were collected and saved as the 'unbound' fractions.

The beads were washed once with 0.5 ml of TGED with 50 mM NaCl, followed by two washes with competitor DNA. The competitor DNA used was salmon sperm, which was added at a 10x concentration to the DNA captured on the streptavidin beads. Proteins which bound specifically to the P_{cesT} or P_{tir} sequences were eluted from the DNA by washing the beads with 50 µl of TGED with 1 M NaCl. Following elution, proteins were subjected to SDS-PAGE and visualized by Coomassie blue staining.

2.11 Pedestal Assay

Pedestal assays were performed by infection of HeLa cells with EPEC cells, and actin-enriched pedestals were visualized by immunofluorescence. Briefly, HeLa cells were seeded onto 12-mm glass coverslips at a density of 10^5/ ml in DMEM containing 10% fetal bovine serum (FBS), and grown O/N at 37°C and 5% CO_2. EPEC strains of interest were grown O/N in LB with St50, shaking at 37°C. EPEC O/N cultures were diluted 1:2000 into DMEM with FBS, added to the cells (1 ml), and incubated for 3 hr at 37°C and 5% CO_2. Following this incubation, cells were washed three times with PBS,

and fixed for 15 min at room temperature using 2.5% paraformaldehyde (Alfa Aesar). Cells were then washed three times with PBS, and permeabilized by incubation for 5 mins in PBS with 0.1% Triton X-100. Cells were stained with Alexa Fluor Phalloidin 568 (Invitrogen Life Technologies) and DAPI (Sigma). Briefly, phallodin 568, which stains actin red, was diluted 300 fold into PBS with 0.1% Triton X-100; 15 µl was added to the cells which were incubated at room temperature for 30 min in the dark. DAPI, which stains nucleic acid blue, was diluted 20,000 fold into PBS; 500 µl was added to the cells and incubated for 1 min at room temperature. Cells were washed twice with PBS and mounted onto microscope slides using ProLong Gold reagent. Actin-rich pedestals were visualized using the Axiovert 200M inverted microscope (Zeiss).

CHAPTER 3. RESULTS

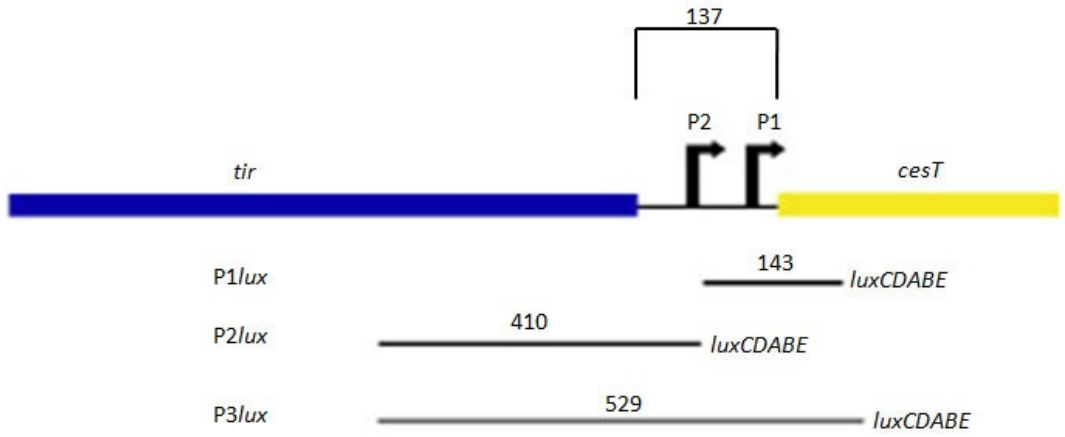
3.1 Characterization of the *tir-cesT* Intergenic Region

3.1.1 The *tir-cesT* Intergenic Region Contains Active Promoter Elements

The initial characterization of the *cesT* promoter (P_{cesT}) was performed by Puenté and coworkers (Sanchez-SanMartin *et al*., 2001). These investigators used transcriptional fusions to CAT genes to identify the presence of a putative promoter encoded within the *tir-cesT* intergenic region (Sanchez-SanMartin *et al*., 2001). Primer-extension analysis revealed two putative transcriptional start sites within this intergenic region, suggesting that two transcriptional promoters may exist, one directly upstream of each transcriptional start site. My objective was to further characterize the transcriptional elements encoded within the *tir-cesT* intergenic region and to understand factors that may influence *cesT* gene expression.

To further analyze promoter activity within the *tir-cesT* intergenic region, transcriptional fusions to promoterless *P. luminescens* luciferase genes, *luxCDABE*, were generated in the pJW15 reporter plasmid (Figure 3.1A). As shown in Figure 3.1A, fusions were designed to measure the activity of each hypothesized promoter region (P1*lux* and P2*lux*) individually, as well as together (P3*lux*). The P1*lux* insert was 143 bp in length and designed to encode the DNA upstream and downstream of the predicted P1 transcriptional start (Sanchez-SanMartin *et al*., 2001). The P2*lux* insert was 410 bp in length and designed to contain only the predicted P2 transcriptional start site (Sanchez-SanMartin *et al*., 2001). The P3*lux* insert was 529 bp in length, and was designed to encompass both P1 and P2. These fusions were introduced into EPEC O127:H6 E2348/69 (WT) cells, which were grown in DMEM at 5% CO_2 and 37°C,

A



B

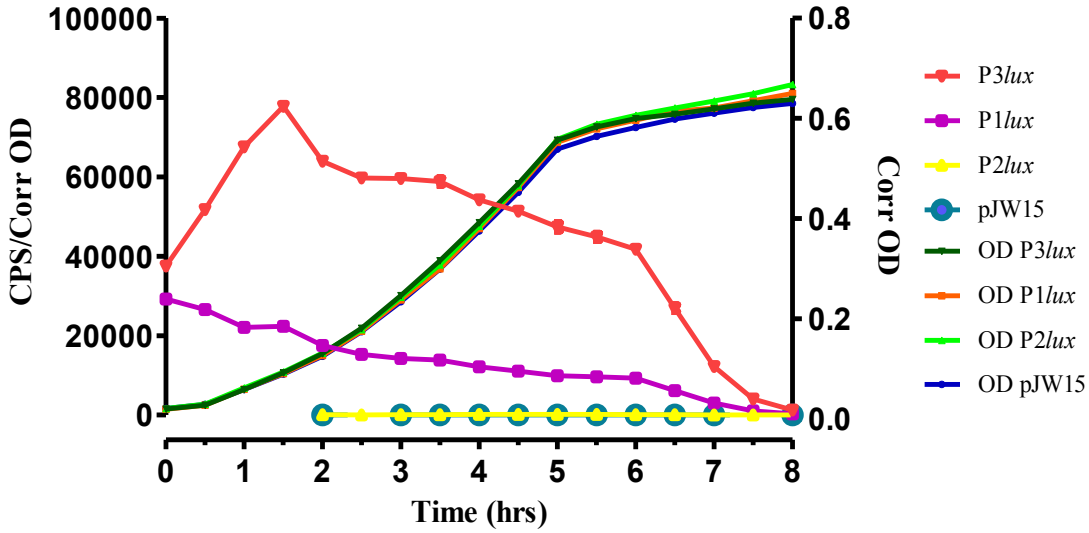


Figure 3.1. The *tir-cesT* intergenic region contains an active promoter region. A. WT genomic DNA was used as a template in PCR to amplify regions of DNA of interest (Figure 2.1). PCR products were cloned into the multiple cloning site of the promoterless pJW15 luciferase reporter plasmid (Figure 2.2). The size of each insert is indicated (bp). **B.** WT cells harbouring the *lux* reporter plasmids were grown in DMEM, 5% CO_2 at 37°C for 8 hrs, and CPS and OD were measured every 30 mins. Mean CPS of eight replicates was normalized by the corrected OD. This is a representative figure of three repeated experiments; error bars represent standard deviation from one experiment. Transcriptional fusions which exhibited less activity than the medium only blank were excluded.

conditions known to induce the expression of EPEC virulence genes (Kenny *et al*., 1997). Bacterial bioluminescence as a result of *luxCDABE* expression was measured as counts per second (CPS), and OD was observed during an 8-hr *in vitro* assay.

The P1*lux* fusion reported activity which was highest at the first time point measured (t=0), then gradually decreased over the course of the 8-hr assay. The P2*lux* fusion reported activity similar to the empty pJW15 vector, suggesting that a promoter does not exist within this region of DNA. The P3*lux* construct reported activity which increased until reaching maximal activity at 1.5 hrs post inoculation, then decreased for the remaining 8 hrs. Interestingly, the activity of the P3*lux* fusion was significantly ($p<0.005$) greater at every time point measured than the summation of the two fragments (P1 and P2) that were encoded, for the first 7 hrs of the assay. From these results, I conclude that the transcriptional start site as hypothesized by Sanchez-SanMartin and colleagues is P1 (Figure 3.1A), and that P_{cesT} is encoded in the sequence directly upstream. These results also indicate that the sequence of DNA consisting of -473 to -88 nucleotides upstream of P_{cesT} contributes to maximal activity from this promoter (Figure 3.1B (P3*lux*)), possibly due to the existence of regulatory elements within this upstream sequence. For the purpose of this thesis, P_{cesT} will refer to the σ^{70} promoter located directly upstream of the P1 transcriptional start site and the DNA upstream which has been shown to influence promoter activity, as represented by the P3*lux* transcriptional fusion. For clarification, the P3*lux* transcriptional fusion was generated by amplification of DNA by PCR using primers EB3 and EB2 (Table 2.2, Figure 2.1), and cloned into the pJW15 reporter plasmid.

3.1.2 Bioinformatics Reveals a σ^{70} Promoter

To investigate the elements encoded in the predicted *cesT* promoter region, a bioinformatics analysis was performed using software provided by the National Centre of Biotechnology Information. A sequence of 660 nucleotides, containing the *tir-cesT* intergenic region with 250 nucleotides upstream and downstream, was entered into the BLAST software (National Centre for Biotechnology Information, http://www.ncbi.nlm.nih.gov/). Strains which showed sequence conservation were further analyzed using CLUSTALW, a sequence alignment tool (Kyoto University Bioinformatics Centre, http://www.genome.jp/tools/clustalw/). As shown in Figure 3.2, the region of DNA directly upstream of the *cesT* ORF is well conserved among several different strains of bacteria containing the LEE (*tir-cesT*), from the family *Enterobacteriaceae*, including *Escherichia, Hafnia,* and *Citrobacter*. The sequence alignment also revealed conservation of nucleotides comprising a σ^{70} promoter. A σ^{70} promoter contains two conserved hexamers, the -10 element, or TATA box, and the -35 element, relative to the location the transcriptional start site (denoted +1) (Singh *et al*., 2011). A bioinformatics analysis of 554 *E. coli* σ^{70} promoter regions determined the consensus sequence of the -10 element to be TATAAT, and the sequence of the -35 hexamer to be TTGACA (Mitchell *et al*., 2003). As shown in Figure 3.2, the predicted P_{cesT} from E2348/69 has five out of six nucleotides of the -10 hexamer conserved, and three out of six nucleotides matched to the -35 hexamer, which strongly supports the hypothesis that this region of DNA encodes a σ^{70} promoter (Sanchez-SanMartin *et al*., 2001).

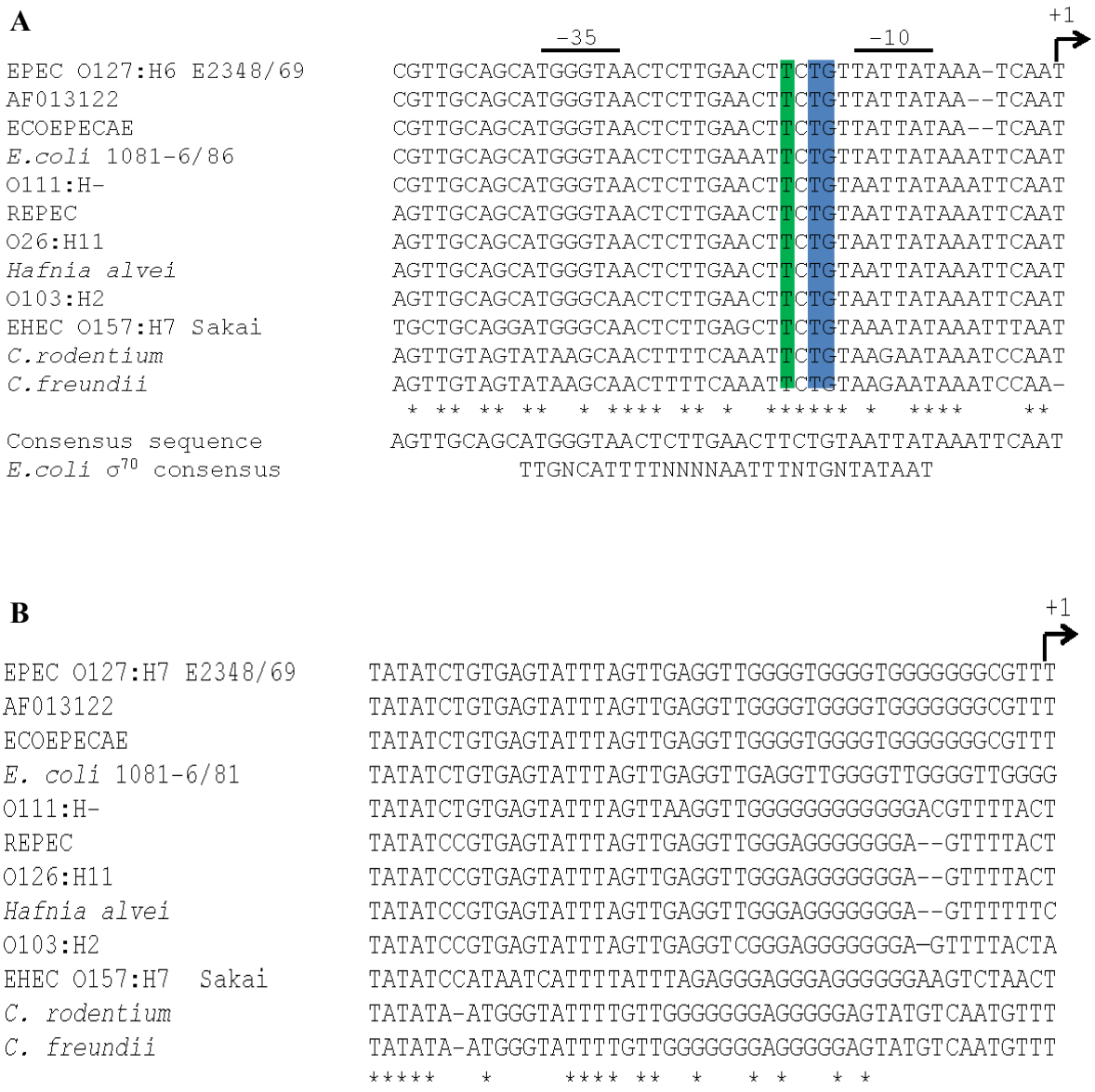


Figure 3.2. The *PcesT* region of DNA is conserved among *E. coli* species, and shows sequence homology to an *E. coli* σ70 consensus sequence. **A.** A 660 nucleotide sequence encoding the tir-cesT intergenic region with 250 nucleotides upstream and downstream was entered into BLAST software. Strains of interest were aligned using CLUSTALW. The nucleotide sequence located directly upstream of the putative P1 transcriptional start site was aligned and compared to the *E. coli* σ70 consensus as determined by Mitchell and colleagues (2003). The predicted location of the -10 and -35 elements of this promoter are identified (Sanchez-SanMartin et al., 2001). Highlighted in green is nucleotide at position -18 within the spacer region, which has been shown to be well conserved among *E. coli* σ70 promoters, and highlighted in gray is a well conserved extended 'TG' motif at position -15 and -14 (Mitchell et al., 2003). **B.** The nucleotide sequence located directly upstream of the putative P2 transcriptional start site was also aligned. The transcriptional start codon is denoted by +1. The letter N represents any nucleotide, and asterisks indicate nucleotides conserved among all strains aligned.

The presence of conserved elements within the spacer region between the -10 and -35 hexamers also supports the hypothesis of a σ^{70} promoter upstream of the *cesT* ORF. As found during the analysis of 554 *E. coli* σ^{70} promoters, the most frequent length of the spacer region, the DNA between the -10 and -35 hexamers, is 17 bp, which is present in 44% of all promoters studied (Mitchell *et al*., 2003). Based on the predicted location of the P_{cesT} -10 and -35 hexamers, the spacer region of this promoter is only 16 nucleotides in length (Figure 3.2). Within the spacer region, nucleotides at positions -15 and -14 respective to the transcriptional start are always a T and a G, respectively. The 'TG' motif was conserved in all P_{cesT} promoters examined (Figure 3.2 (gray)). The nucleotide at position -18, identified as a T, is also a strongly conserved element among σ^{70} promoters (Mitchell *et al*., 2003). This nucleotide is conserved in 100% of the P_{cesT} promoters examined in this analysis (Figure 3.2 (green)). Based on this *in silico* assay, it can be concluded that the region of DNA encoded by the *tir-cesT* intergenic region of EPEC strain E2348/69 contains a σ^{70} promoter which is conserved among other pathogenic and non-pathogenic strains of bacteria from the family *Enterobacteriaceae*.

The nucleotide sequence located directly upstream of the putative P2 transcriptional start site shows limited sequence conservation among the strains included in the alignment (Figure 3.2B). This analysis did not reveal conserved elements that would indicate that a σ^{70} promoter region is located upstream of the putative P2 transcriptional start site. It was also observed during this analysis that the *tir* ORF was less conserved between strains than the *tir-cesT* intergenic region and the *cesT* ORF. This observation suggests that the *cesT* promoter is important for bacterial pathogenesis since the sequence has remained well conserved among pathogenic strains of *E. coli*.

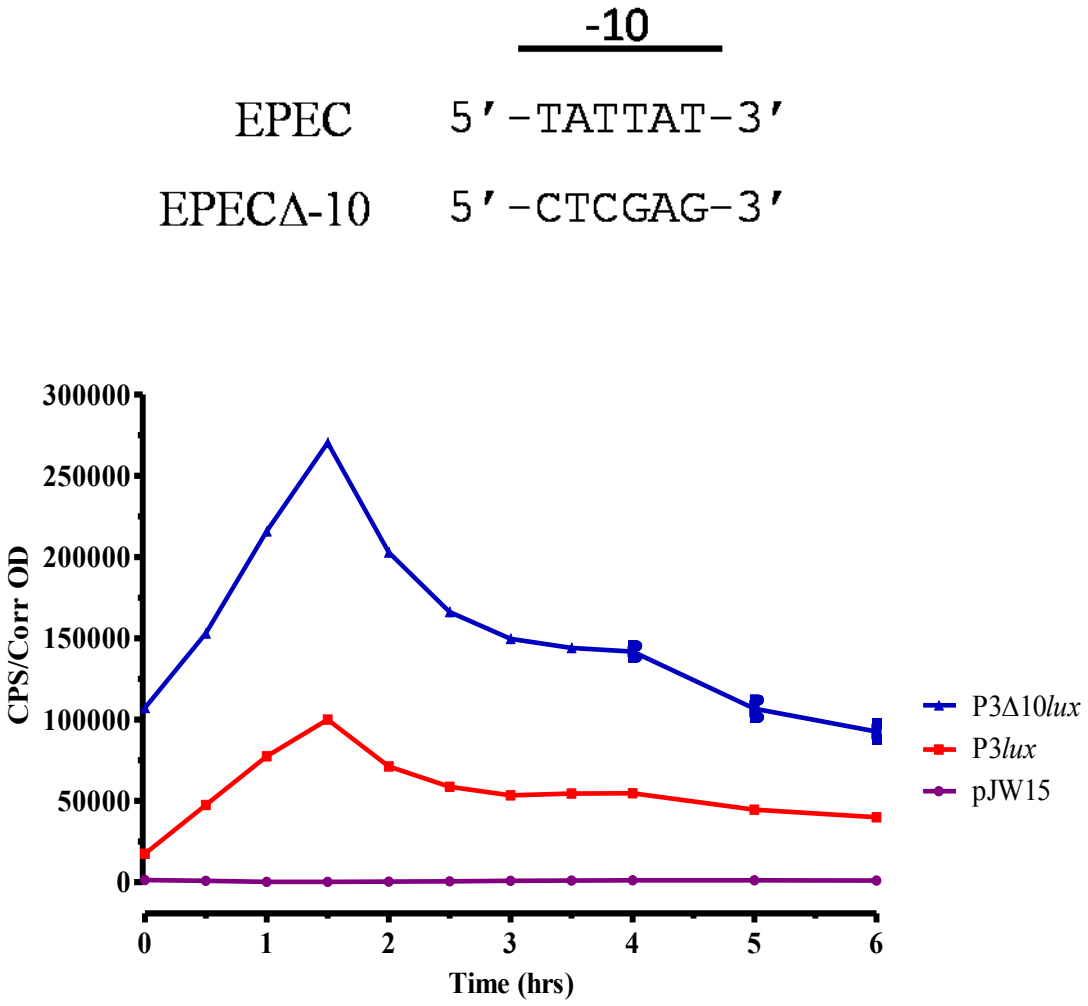
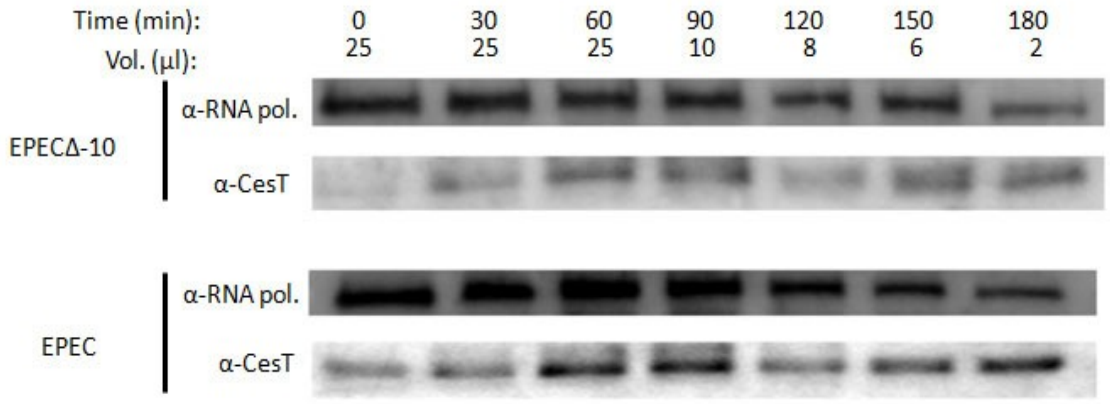


Figure 3.3. A mutation of the -10 hexamer of p*cesT* yields increased promoter activity. A chromosomal mutation of the -10 element of the *cesT* promoter was made by basepair substitution, from TATTAT to CTCGAG, generating EPECΔ-10. Genomic DNA from EPECΔ-10 was used in a PCR with primers EB7 and EB2; the amplicon was cloned into pJW15 generating P3Δ10*lux*. WT E2348/69 cells harbouring the P3*lux* or P3Δ10*lux* reporter fusions, or the empty plasmid, pJW15, were grown in DMEM at 5% CO2 and 37°C, and bacterial bioluminescence (CPS) and OD were measured over 6 hr. Mean CPS of eight replicates was normalized by mean corrected OD. This is a representative figure of three repeated experiments; error bars represent standard deviation from one experiment.

A



B

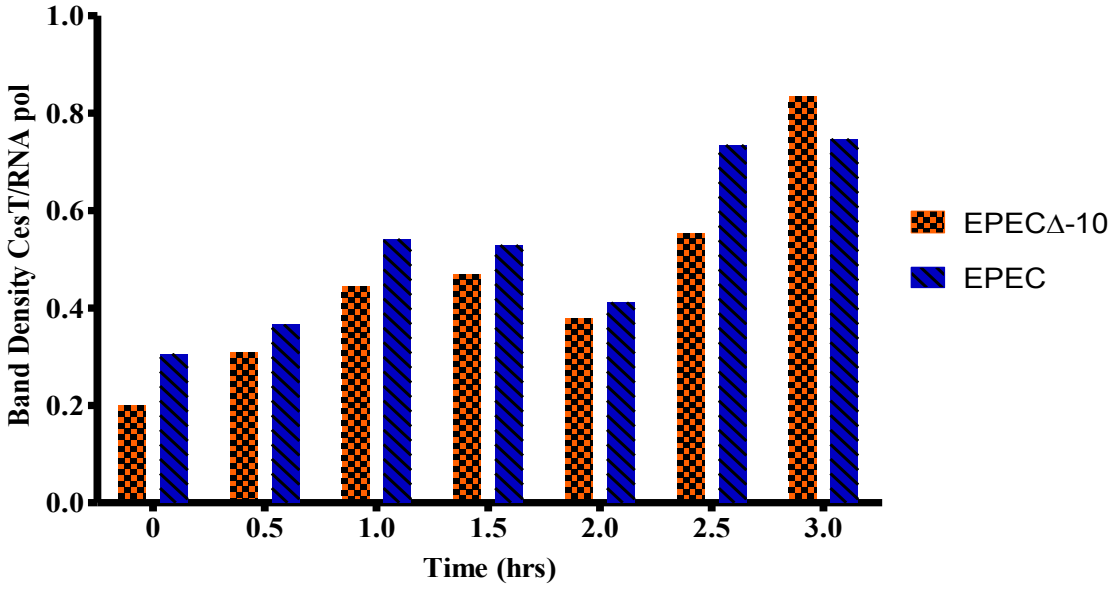


Figure 3.4. Densitometry analysis was performed on EPEC and EPECΔ-10 proteins to determine differences in CesT levels. A. WT EPEC cells and EPECΔ-10 cells were grown in DMEM at 5% CO2 and 37°C for 3 hrs. Every 30 min, 1 ml of bacteria were pelleted by centrifugation and resuspended in 100 µl of 2x ESB. Samples were boiled immediately, separated by SDS-PAGE, and blotted against RNA polymerase and CesT (Table 2.4). The resulting blots were visualized using ECL Plus Western Blotting Detection System (GE Healthcare). **B.** Densitometry analysis was performed using Image Lab software. Band density was measured, and CesT was normalized by RNA polymerase. The experiment was repeated three times, and data for one representative experiment is shown.

3.1.3 The TATA box of P_{cesT} is Amenable to Change

To further characterize the function of the P_{cesT} promoter I sought to make a loss-of-function mutation that would inactivate the region, and to study the resulting consequences to gain insight on the role of this promoter to EPEC pathogenesis. This task was performed by basepair substitution of the -10 hexamer (TATTAT) to CTCGAG *via* homologous recombination, introducing a XhoI restriction enzyme site into the EPEC chromosome, generating EPECΔ-10. This strategy was selected because it had been previously used to abolish promoter activity of the P_{tir} promoter using a CAT reporter assay (Sanchez-SanMartin *et al*., 2001).

Genomic DNA isolated from EPECΔ-10 was used as a template in a PCR and the resulting amplicon was cloned into pJW15, generating P3Δ10*lux*. The reporter plasmid containing the P3Δ10*lux* fusion was introduced into WT and used in a luciferase reporter assay to determine the effect of such a mutation on P_{cesT} activity. As shown in Figure 3.3, the P3Δ10*lux* fusion reported a significantly ($p<0.005$) higher level of activity than the WT P3*lux* fusion. This result was unexpected; however, similar results have been previously described while studying mutations to the -10 element of a σ^{70} promoter, and it is possible that elements within the spacer region, such as the 'TG' motif, compensate for the -10 mutation (Niedziela-Majka and Heyduk, 2005).

Following the observation of increased promoter activity seen using the luciferase reporter assay, I sought to determine if the EPECΔ-10 chromosomal mutation alters intracellular CesT protein levels. To answer this question, a time-course assay was performed where WT EPEC cells or EPECΔ-10 cells were grown in DMEM and WCL were isolated every 30 min for 3 hr. Collected proteins were separated by SDS-PAGE

and probed with antibodies against RNA polymerase, which was used as a loading control, and CesT (Figure 3.4A). The blots were then subjected to analysis by measuring band density using Image Lab software; the relative amount of CesT was normalized by RNA polymerase. This densitometry analysis revealed that there was no apparent difference between CesT protein levels between EPEC and EPECΔ-10 at the time points measured (Figure 3.4B). The increase in CesT protein levels in both EPEC cells and EPECΔ-10 cells observed at 3 hrs post inoculation likely corresponds to CesT production from the P_{tir} promoter.

The effect of the Δ-10 mutation was also examined by pedestal assay, a technique which uses immunofluorescence to visualize actin-rich pedestals as a measure of Tir translocation. Briefly, HeLa cells were infected with EPEC or EPECΔ-10 for 3 hrs; cells were washed, fixed, stained with phallodin-568 and DAPI, and visualized using the Axiovert 200M Inverted Microscope (Zeiss). Immunofluorescence revealed that HeLa cells infected with EPECΔ-10 cells were able to form pedestals (Figure 3.5). Taken together, these results suggest that the mutation that I introduced to the P_{cesT} TATA element was not detrimental to the activity of the promoter, and did not diminish the ability of EPECΔ-10 to induce pedestal formation.

3.1.4 Deletion of the *tir-cesT* Intergenic Region Reduced P_{cesT} Activity

A second mutation was generated in the EPEC chromosome in an attempt to inactivate the *cesT* promoter. This mutation was generated by deletion of 83-bp of the *tir-cesT* intergenic region, and was introduced by a homologous-recombination strategy, and was named EPECΔintg (intg: intergenic). A luciferase reporter assay was performed

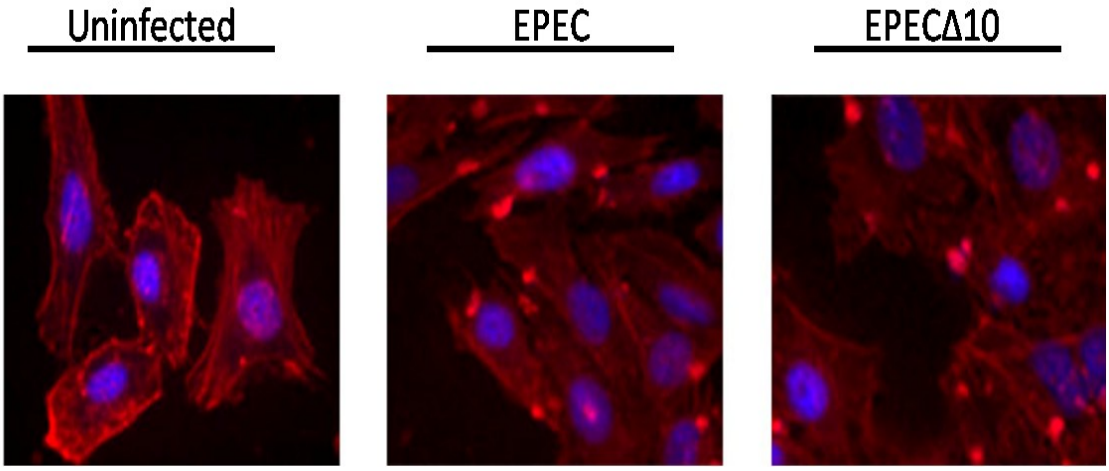


Figure 3.5. EPECΔ-10 induces pedestal formation of infected HeLa cells, similarly to WT. HeLa cells infected with EPEC cells or EPECΔ-10 cells were incubated for 3 hrs at 37°C and 5% CO2. Cells were washed with PBS, fixed using ProLong Gold Antifade Reagent, and stained with phalloidin-568 and DAPI, and visualized using an Axiovert 200M Inverted Microscope (Zeiss). White arrows indicate examples of pedestal formation.

to measure the promoter activity of P_{cesT} following the deletion of the intergenic region. Genomic DNA was isolated from EPECΔintg cells and used as a template in a PCR. The PCR product was cloned into pJW15, generating P3Δintg*lux*, and introduced into WT. Compared to the activity reported by P3*lux*, P3Δintg*lux* exhibited significantly ($p<0.005$) less activity while maintaining a similar temporal profile, suggesting that the intergenic space is important for P_{cesT} activity (Figure 3.6). Although 60% of the intergenic space is deleted, the -10 element and part of the spacer region remained intact, which may explain why the P3Δintg*lux* fusion still reported some activity.

To determine the effect that the Δintg chromosomal mutation had on the production of CesT in the bacteria, WCL were collected every 15 mins for 3 hrs during a time-course assay, as described above. Proteins were separated by SDS-PAGE and probed with antibodies against RNA polymerase and CesT (Figure 3.7A), followed by densitometry analysis to determine the relative amount of intracellular CesT (Figure 3.7B). Measuring band density revealed that EPECΔintg had less CesT than WT during this *in vitro* assay. Therefore it may be concluded that deletion of the 60% *tir-cesT* intergenic region leads to reduced expression of P_{cesT}.

3.2 Identification of Transcriptional Regulators That Influence P_{cesT} Activity

3.2.1 P_{cesT} Exhibits Activity Independently of the P_{tir} Promoter Activity

The activity of P_{cesT} was examined in comparison to the activity of P_{tir} promoter to determine temporal differences in promoter activity. As the regulation of the P_{tir} promoter is well characterized, it can also be used as a comparative tool to gain insight on the regulation of P_{cesT} (Macritchie *et al*., 2008; Sanchez-SanMartin *et al*., 2001). A PCR

EPEC

TAAATATATCTGTGAGTATTTAGTTGAGGTTGGGGTGGGGTGGGGG
GGCGTT**T**TACTAGCGTTAATGTTTCAGAGAACAACGTTGCAGCATG
GGTAACTCTTGAACTTCTGTTATTATAAATCAA**T**TAAGAGAAATTA
TA**ATG**

EPECΔintg

TAAATATATCTGTGAGTATCTCGAGGAACTTCTGTTATTATAAATC
AA**T**TAAGAGAAATTATA**ATG**

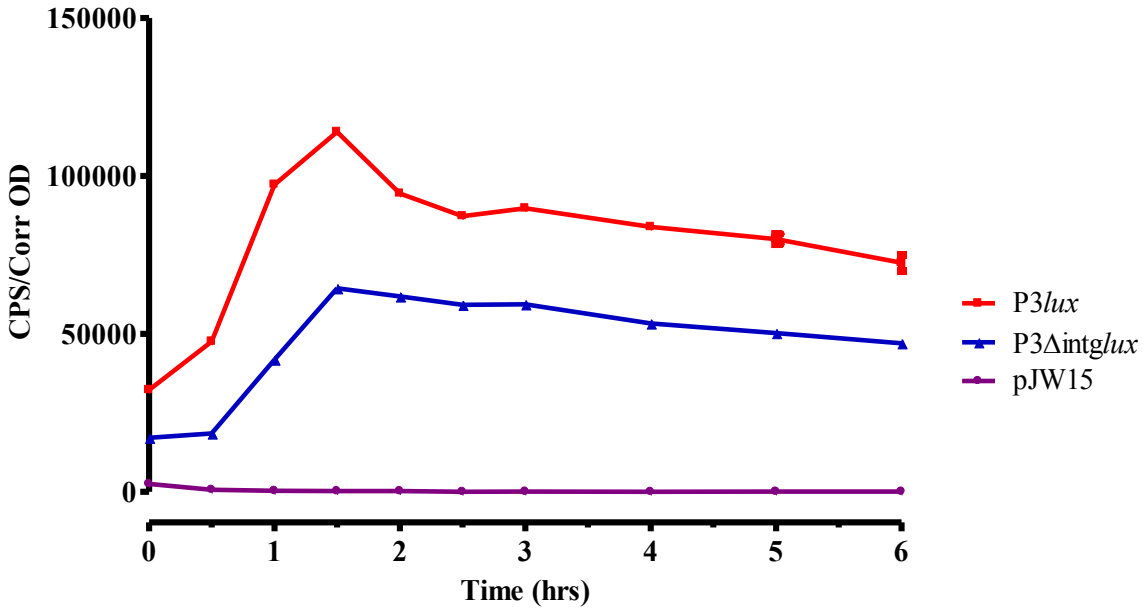


Figure 3.6. Deletion of the *tir-cesT* intergenic region reduces the activity of the *cesT* promoter. The sequence of the *tir-cesT* intergenic region is shown including the *tir* stop codon (TAA) (bold), and the *cesT* start codon (ATG) (bold). The nucleotide sequence deleted from WT EPEC (underlined) was replaced by a XhoI restriction enzyme scar (CTCGAG) (underlined), generating EPECΔintg. Genomic DNA from EPECΔintg was used in a PCR using primers EB7 and EB2. The resulting amplicon was cloned into pJW15, generating P3Δintg*lux*. WT E2348/69 cells harbouring the P3*lux* or P3Δintg*lux* reporter fusions were grown in DMEM at 5% CO2 and 37°C CPS and OD were measured. Mean CPS of eight replicates was normalized by mean corrected OD. This is a representative figure of three repeated experiments; error bars represent standard deviation from one experiment.

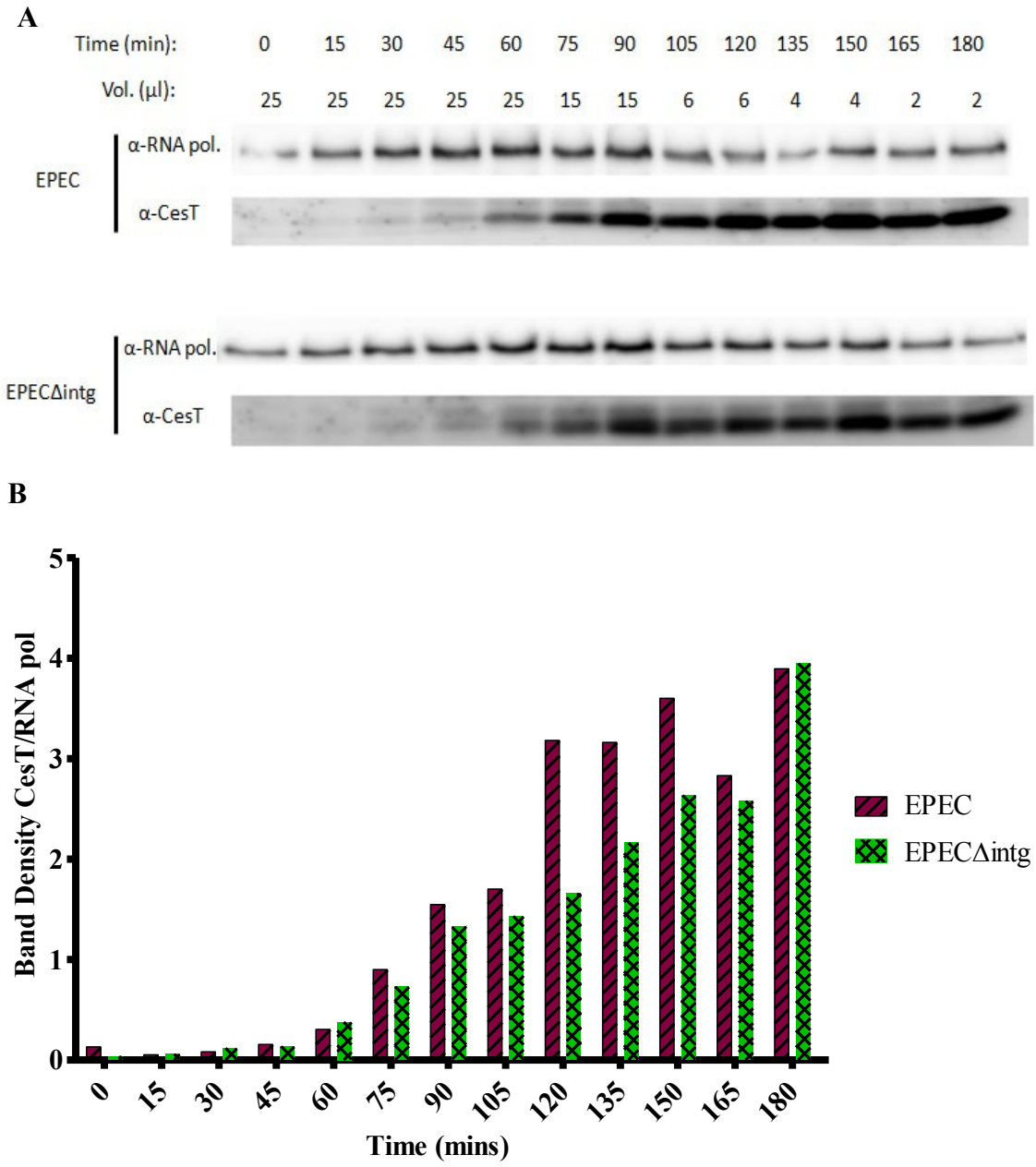


Figure 3.7. Deletion of the *tir-cesT* intergenic region results in decreased CesT levels. A. WT EPEC cells and EPECΔintg cells were grown in DMEM at 5% CO_2 and 37°C for 3 hrs. Every 15 min, 1 ml of bacteria were pelleted by centrifugation and resuspended in 100 µl of 2x ESB. Samples were boiled immediately, separated by SDS-PAGE, and blotted against RNA polymerase and CesT (Table 2.3). The resulting blots were visualized using Immun-Star WesternC Kit (BioRad). **B.** Densitometry analysis was performed using Image Lab software. Band density was measured, and CesT was normalized by RNA polymerase. The experiment was repeated three times, and data for one representative experiment is shown.

product designed to encode the P_{tir} promoter was amplified using EPEC genomic DNA as a template. The resulting PCR product was cloned into the pJW15 reporter plasmid, generating P_{tir}*lux* (Figure 3.8A) (Sanchez-SanMartin *et al*., 2001). The P_{tir}*lux* transcriptional fusion was introduced into WT cells and promoter activity was evaluated during an *in vitro* luciferase assay (Figure 3.8B). The P_{tir}*lux* fusion reported minimal activity within the first hour post inoculation; however, activity then dramatically increased until reaching maximal activity at 4 hrs. Following the peak exhibited at 4 hrs the activity of P_{tir}*lux* steadily decreased for the remainder of the assay. In contrast, P3*lux* showed increasing activity early during an *in vitro* assay, when P_{tir}*lux* exhibits minimal activity, suggesting that P_{cesT} activity is independent of P_{tir} activity, and that the temporal activity of these promoters is different.

3.2.2 The *cesT* Promoter is not Influenced by the Presence of CesT

Transcriptional regulation of virulence genes can be highly specialized and involve a complex network of signaling proteins. Of particular interest, T3SS chaperones are known to affect virulence-gene expression through interaction with a signalling network. For example, two T3SS chaperones from *Shigella*, the translocator chaperone IpgC and the multivalent effector chaperone Spa15, have been shown to influence virulence gene expression through their interaction with MxiE, an AraC-family activator (Mavris *et al*., 2002, Parsot *et al*., 2005). To determine whether a similar network of transcriptional regulation may be present in EPEC cells, the P3*lux* fusion was introduced into a CesT mutant strain, Δ*cesT*, and bacterial bioluminescence was measured during a luciferase reporter assay (Figure 3.9A). The P_{tir}*lux* fusion was also studied in the Δ*cesT* strain to determine the influence of functional CesT on the P_{tir} promoter (Figure 3.9B).

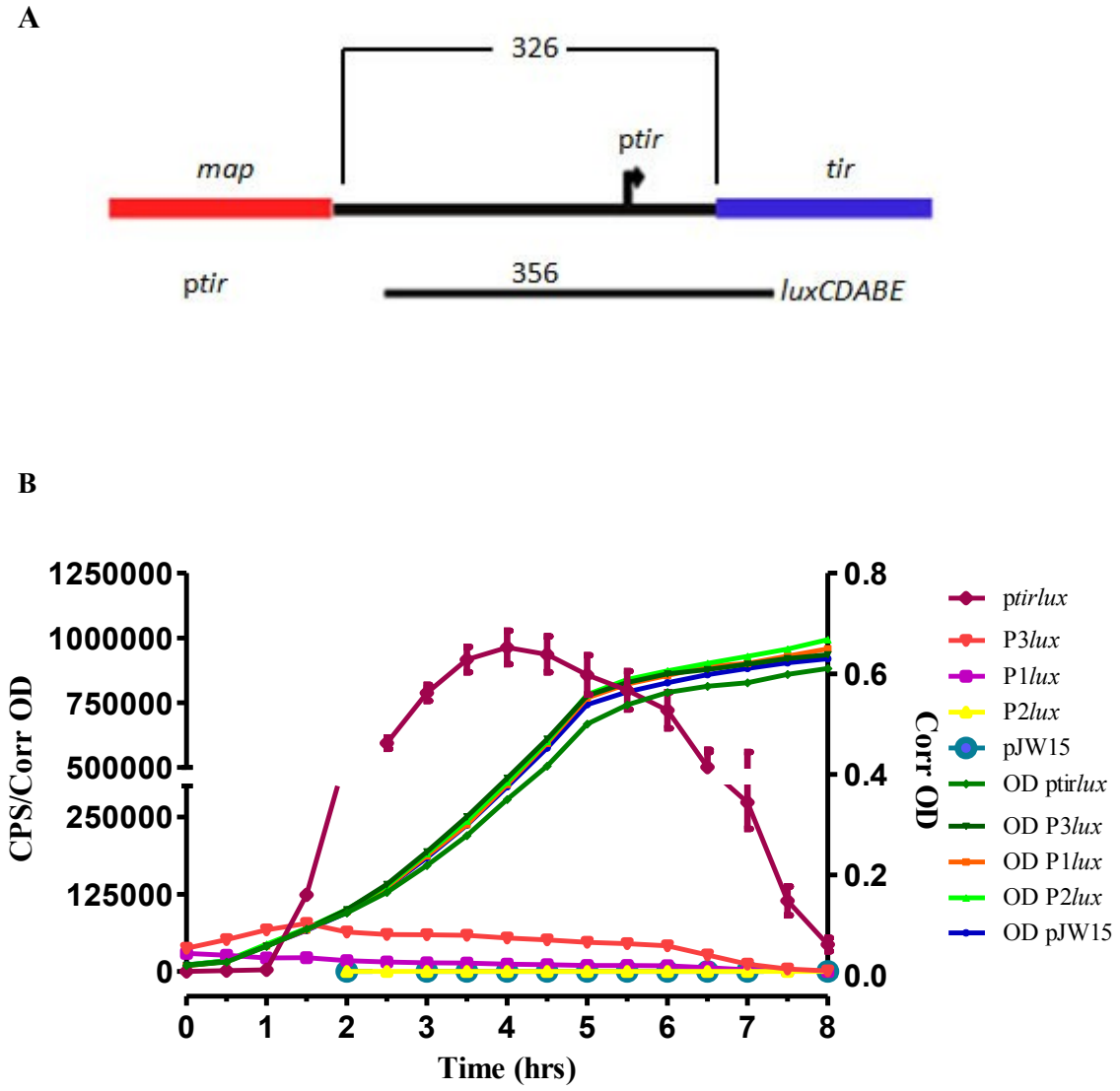


Figure 3.8 P_{cesT} activity occurs early during an *in vitro* assay, prior to P_{tir}. A. The P_{tir} promoter was amplified by PCR and cloned into the *lux* reporter plasmid. **B.** WT E2348/69 cells harbouring the *lux* reporter fusions were grown in DMEM, 5% CO_2 at 37°C, for 8 hrs, and CPS and OD were measured every 30 mins. Mean CPS of eight replicates was normalized by mean corrected OD. This figure is representative of three repeated experiments; error bars represent standard deviation from one experiment. Note that the y-axis has been segmented. The data for P1*lux*, P2*lux*, and P3*lux* has been shown previously (Figure 3.1B).

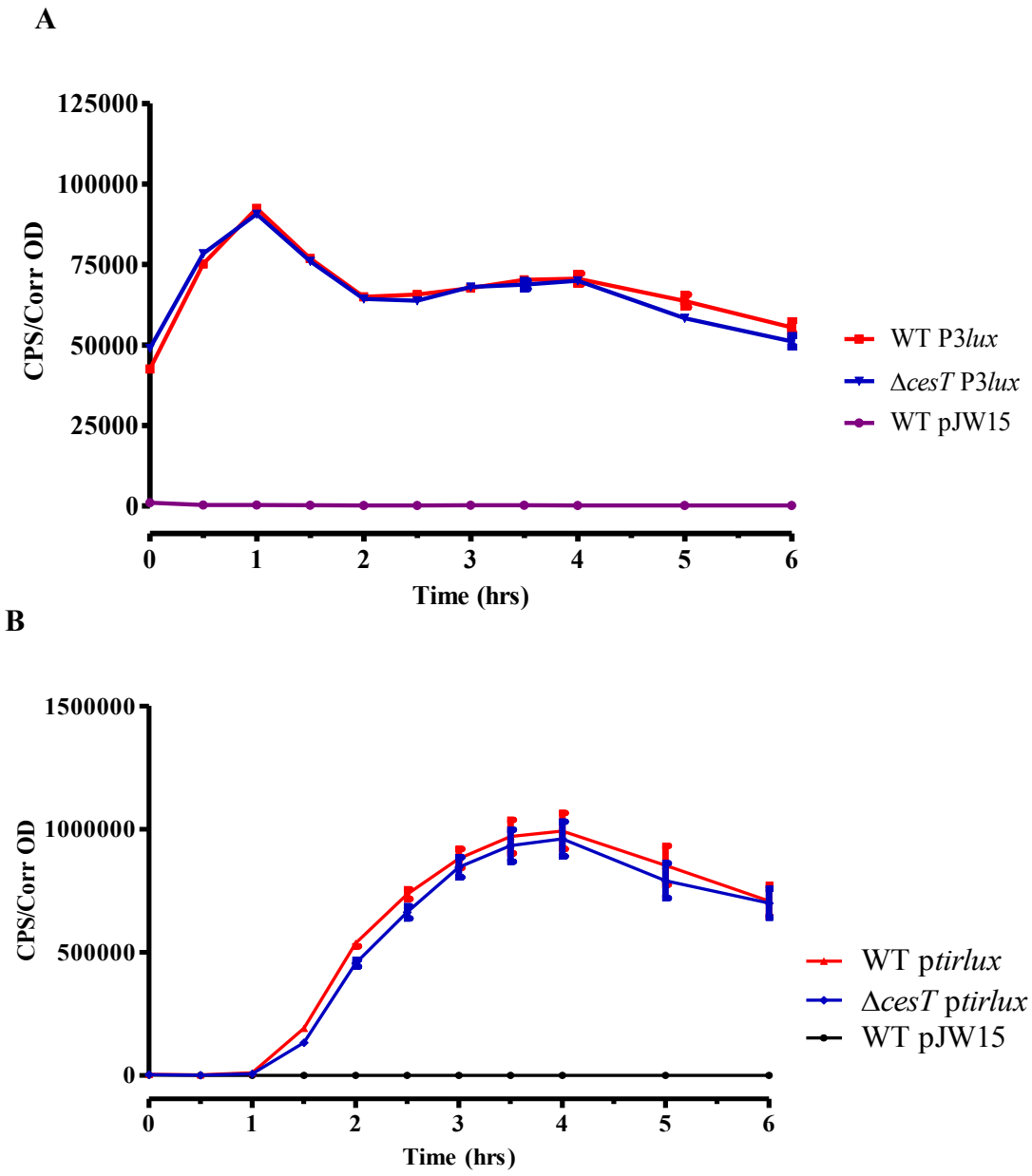


Figure 3.9 The activity of P_{cesT} is not influenced by the presence of CesT. WT E2348/69 cells or E2348/69 Δ*cesT* cells harbouring the P3*lux* **(A)** or P_{tir}*lux* **(B)** reporter fusions were grown in DMEM at 5% CO_2 and 37°C, and bacterial bioluminescence and OD were measured over 6 hr. Mean CPS of eight replicates was normalized by mean corrected OD. This is a representative figure of three repeated experiments; error bars represent standard deviation from one experiment.

The P3*lux* transcriptional fusion expressed similar levels of activity when introduced into Δ*cesT,* compared to WT (Figure 3.9A). Activity of the P_{tir}*lux* fusion also remained similar when introduced into Δ*cesT* (Figure 3.9B). These results suggest that the presence of CesT does not influence the activity of either P_{cesT} or P_{tir}. Although there is not conclusive evidence to rule out that a transcriptional regulatory feedback loop involving CesT does not exist, these data suggest that CesT does not regulate its own expression from P_{cesT}, or the expression of P_{tir}.

3.2.3 P_{cesT} is not Dependent on a Functional T3SS

The ability of the T3SS apparatus to successfully translocate effector proteins to the host cell is dependent on the functionality of the ATPase which provides the energy required for secretion. In EPEC, the T3SS ATPase is EscN, and in a Δ*escN* mutant, effector translocation is completely abolished (Thomas *et al*., 2005). As many effectors require CesT for stability, I hypothesized that P_{cesT} activity may be influenced by the accumulation of effectors within the bacteria, through a positive feedback loop. To test the hypothesis that P_{cesT} is governed by the activity of the T3SS, the P3*lux* transcriptional fusion was studied in a Δ*escN* mutant. As shown in Figure 3.10A, the P3*lux* fusion reported similar activity in Δ*escN* as in WT cells, which indicated that the *cesT* promoter does not require the presence of EscN. These data also suggest that there is no regulatory feedback loop that exists under the condition of an inactive T3SS apparatus.

The activity of the P_{tir} promoter, as represented by P_{tir}*lux*, increased significantly ($p<0.005$) when introduced into Δ*escN* (Figure 3.10B). This result suggests that the intracellular accumulation of effectors, which has been previously reported for Δ*escN*,

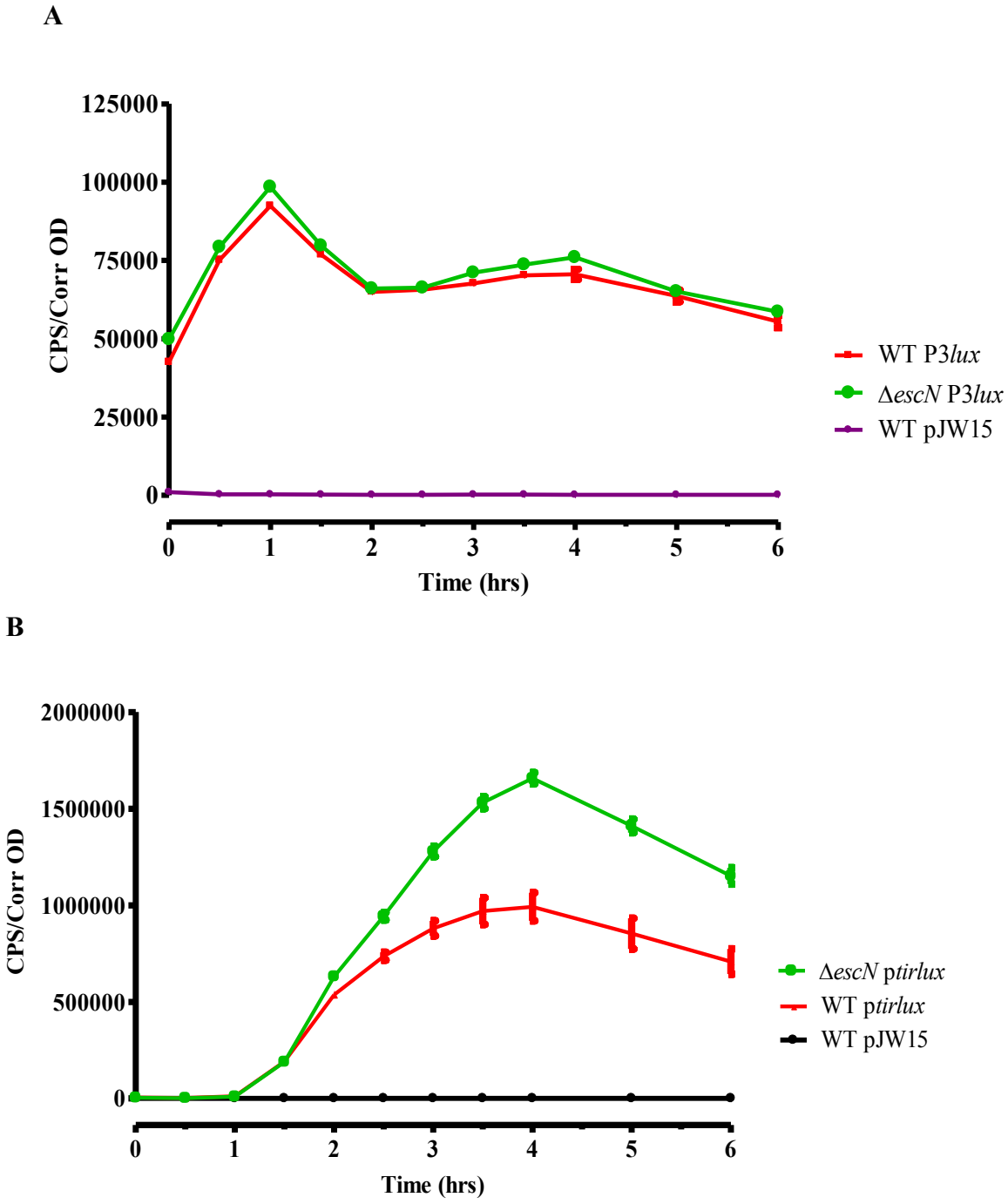


Figure 3.10 The P_{cesT} promoter is not affected by disruption of the T3SS. WT E2348/69 cells and Δ*escN* cells harbouring the P3*lux* (**A**) or P_{tir}*lux* (**B**) reporter fusions were grown in DMEM at 5% CO_2 and 37°C, and bacterial bioluminescence and OD were measured over 6 hr. Mean CPS of eight replicates was normalized by mean corrected OD. This is a representative figure of three repeated experiments; error bars represent standard deviation from one experiment.

can influence P_{tir} expression (Thomas *et al*., 2005). As Tir translocation is necessary for intimate adherence of bacteria to host cells, and effectors are not secreted in Δ*escN*, P_{tir} expression may be influenced by factors which upregulate the expression of genes required for intimate adherence.

3.2.4 P_{cesT} Activity is Decreased in Δ*ler* Mutant

The LEE Encoded Regulator, Ler, has been shown to de-repress the activity of H-NS at every characterized operon within the LEE pathogenicity island (Barba *et al*., 2005; Bustamante *et al*., 2001; Haack *et al*., 2003). Since Ler is an important regulator in expression of EPEC virulence genes, I hypothesized that Ler could also influence the activity of P_{cesT}. This hypothesis was tested by expression of the P3*lux* and P_{tir}*lux* fusions in a Δ*ler* background (Figure 3.11).

The P3*lux* fusion reported significantly ($p<0.005$) less activity when introduced into a strain lacking the Ler protein than in WT EPEC cells during the first 4 hrs of an *in vitro* assay (Figure 3.11A). Interestingly, the temporal trend of activity remained similar within the different strains tested. Consistent with published reports, the P_{tir}*lux* fusion exhibited minimal activity, up to a ~300-fold decrease, when introduced into Δ*ler* (Figure 3.11B) (Macritchie *et al*., 2008). In contrast to the change in activity exhibited by the P_{tir}*lux*, P_{cesT} reported a modest two-fold decrease in expression (Figure 3.11A). I conclude that the activity of P_{cesT} does not strictly require the presence of Ler. However, it was clear that the presence of Ler influenced P_{cesT} activity, although this may have been through downstream or indirect effects of the Δ*ler* mutation, for example, the down regulation of all LEE genes.

3.2.5 The Activity of P_{cesT} is Influenced by GrlRA

The Global Regulator Ler Repressor (GrlR) and Activator (GrlA) proteins are encoded between LEE1 and LEE2, and co-transcribed as an operon (Deng *et al*., 2004). A regulatory feedback loop between Ler and GrlRA exists, as Ler activates expression of GrlRA and, in turn, GrlRA moderates Ler expression (Barba *et al*., 2005; Berdichevsky *et al*., 2005). As this regulatory feedback loop influences *ler* expression, and Ler is required for the activation of every LEE operon, I hypothesized that GrlR and GrlA would influence the activity of P_{cesT}.

The activity of the P3*lux* fusion was significantly increased when introduced into the *grlA* mutant strain, compared to expression in WT (Figure 3.12A). This result was unexpected, as I had hypothesized P_{cesT} activity would be reduced in the Δ*grlA* strain, if GrlA influenced this promoter. As expected, the P_{tir}*lux* fusion reported significantly less activity (Figure 3.12B) when studied in the Δ*grlA* background, a trend consistent with the literature (Deng *et al*., 2004). This effect was likely due to interference with the GrlRA-Ler regulatory feedback loop, as GrlA does not activate P_{tir} promoter expression in a Ler-independent mechanism in EHEC cells (Russell *et al*., 2007).

Through a yeast two-hybrid screen, GrlR was shown to interact with itself, forming a homodimer, and also with GrlA (Creasey *et al*., 2003). It has been proposed that the GrlR-GrlA interaction moderates expression of LEE operons by preventing *ler* expression in a GrlA-dependent manner (Creasey *et al*., 2003). To determine the influence of GrlR on the activity of the *cesT* promoter, transcriptional *lux* fusions were introduced into a GrlR-deficient strain, Δ*grlR*, and measured by luciferase reporter assay.

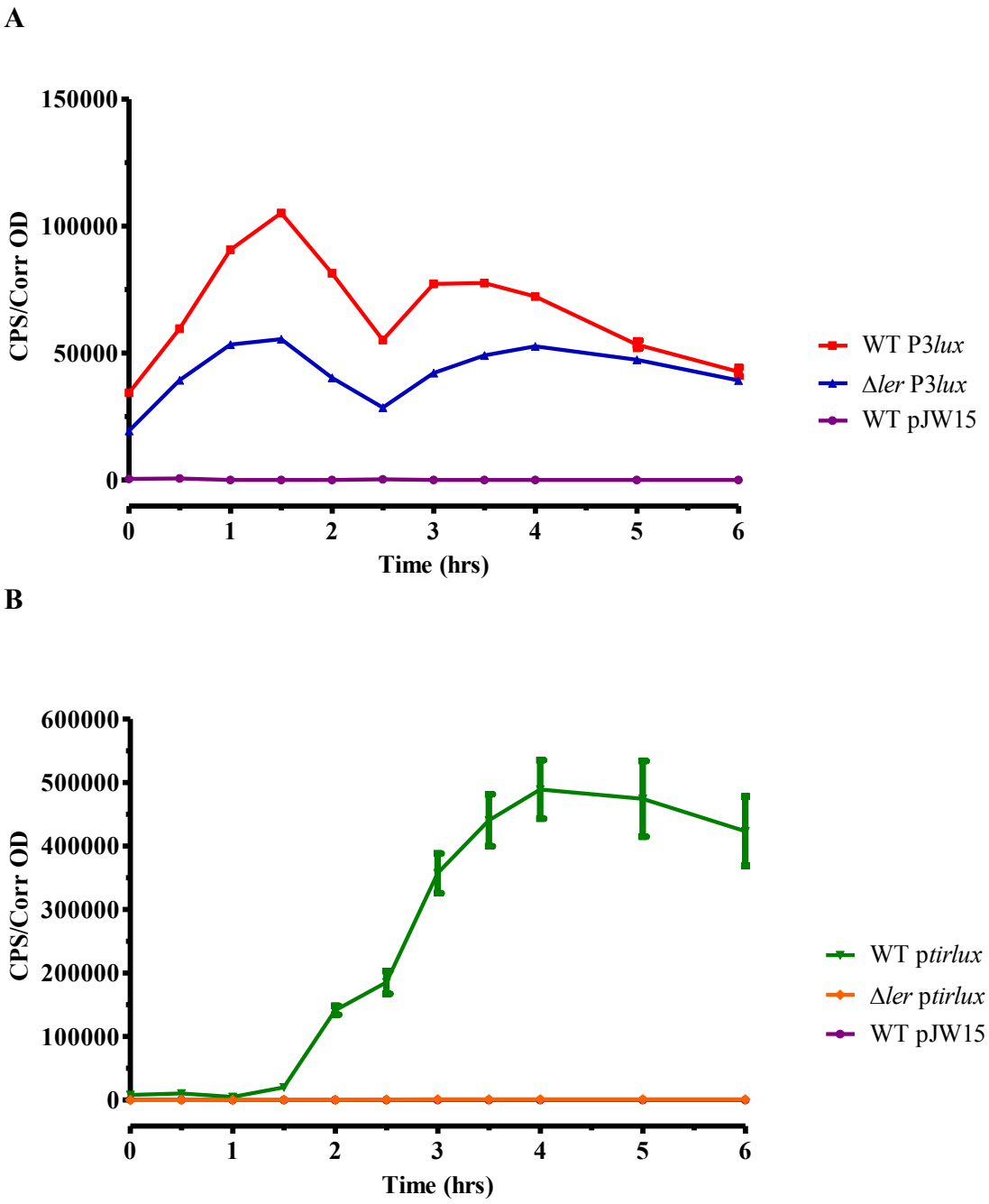


Figure 3.11 Ler regulates activity of P_{cesT} and P_{tir} promoters. WT cells or Δ*ler* cells harbouring the P3*lux* **(A)** or P_{tir}*lux* **(B)** reporter fusions were grown in DMEM at 5% CO_2 and 37°C. Bacterial bioluminescence and OD were measured over 6 hr. Mean CPS of eight replicates was normalized by mean corrected OD. This is a representative figure of three repeated experiments; error bars represent standard deviation from one experiment.

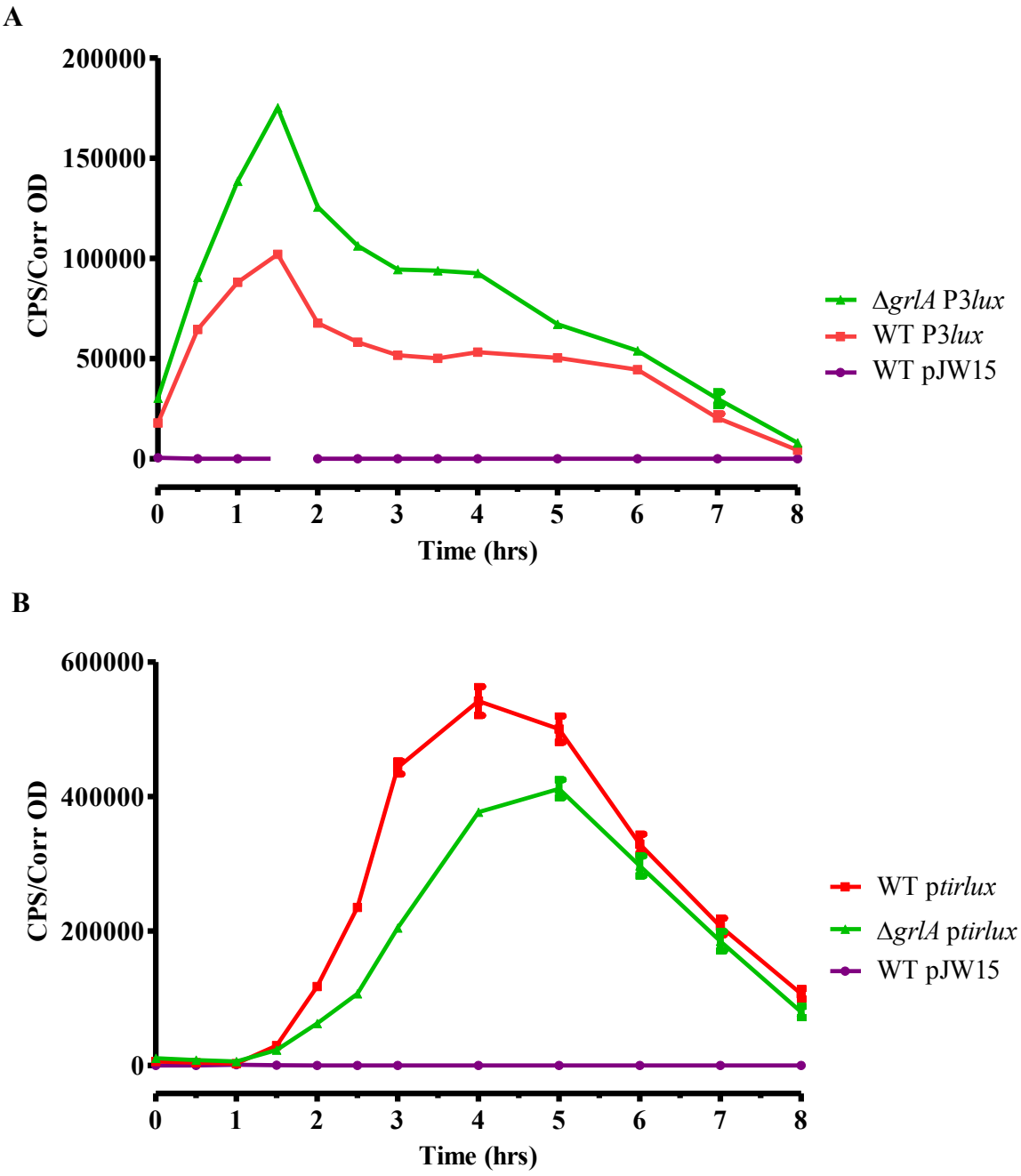


Figure 3.12 The GrlA transcriptional regulator influences the activity of the P_{cesT} and P_{tir} promoters. WT cells or Δ*grlA* cells harbouring the P3*lux* **(A)** or P_{tir}*lux* **(B)** reporter fusions were grown in DMEM at 5% CO_2 and 37°C, and bacterial bioluminescence and OD were measured over 8 hr. Mean CPS of eight replicates was normalized by mean corrected OD. This is a representative figure of three repeated experiments; error bars represent standard deviation from one experiment. Transcriptional fusions which exhibited less activity than the medium only blank were excluded.

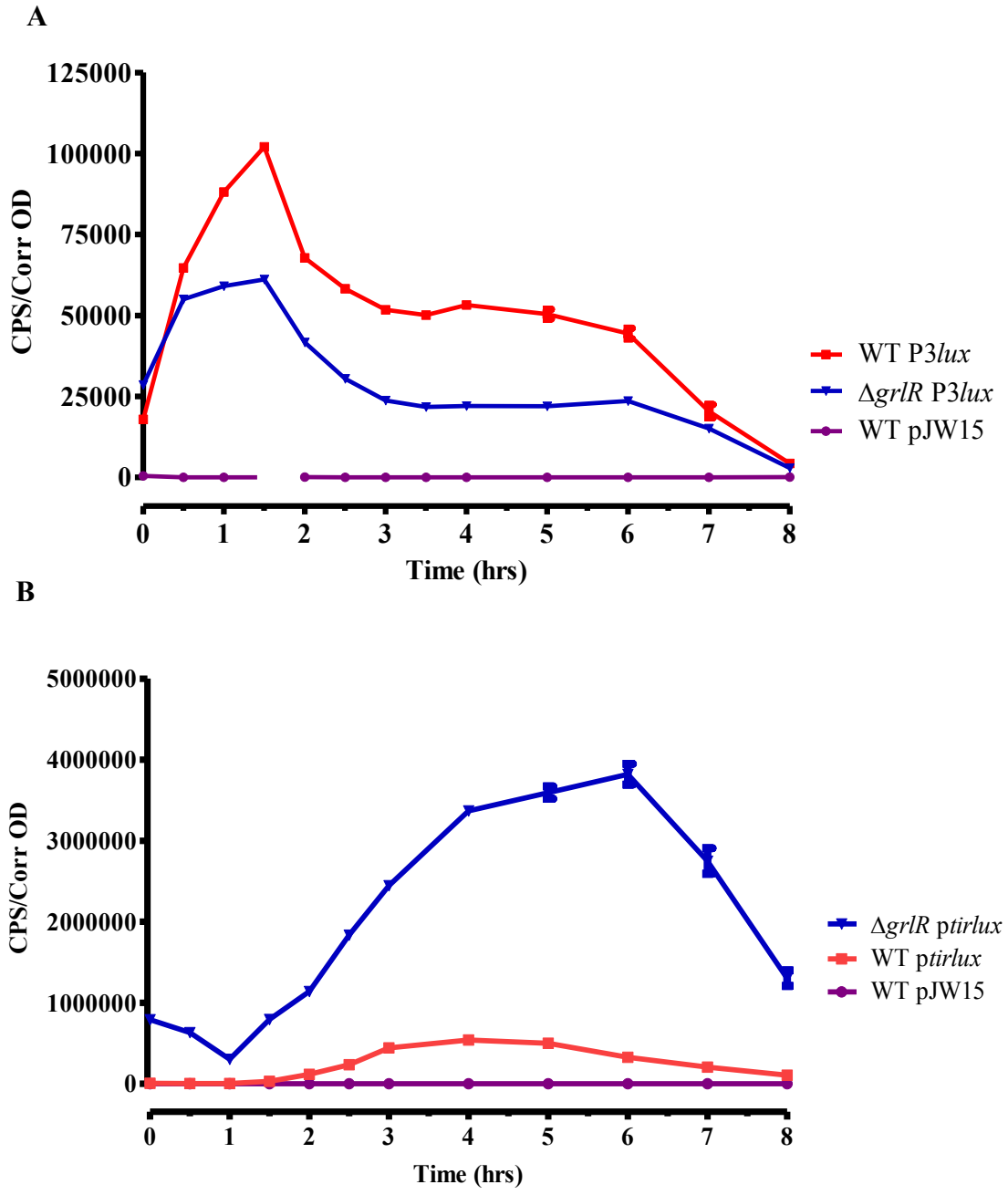


Figure 3.13 The presence of GrlR influences the activity of the *cesT* and *tir* promoters. WT cells or Δ*grlR* cells harbouring the P3*lux* **(A)** or P_{tir}*lux* **(B)** reporter fusions were grown in DMEM at 5% CO_2 and 37°C, and bacterial bioluminescence and OD were measured over 8 hr. Mean CPS of eight replicates was normalized by mean corrected OD. This is a representative figure of three repeated experiments; error bars represent standard deviation from one experiment. Transcriptional fusions that exhibited less activity than the medium only control were excluded.

As shown in Figure 3.13A, the activity of the P3*lux* fusion is significantly reduced when introduced into Δ*grlR* compared to WT cells. Consistent with literature, the activity of P_{tir}*lux* was significantly increased in the Δ*grlR* background (Figure 3.13B), likely a result of increased *ler* activation through GrlA (Deng *et al*., 2004).

3.2.6 The P_{cesT} Promoter is Active in a Lab Strain *E. coli*, DH5α

To gain insight on the basic transcriptional machinery required for P_{cesT} expression, and to determine if EPEC-specific factors are needed for activation of this promoter, *lux* transcriptional fusions were introduced into a lab strain of *E. coli*, DH5α (*E. coli* K-12), and used in a luciferase reporter assay. As shown in Figure 3.14, P1*lux* and P3*lux* reported activity in the DH5α background. As DH5α does not possess the LEE PAI, these data suggest that P_{cesT} does not strictly require the products of genes encoded within the LEE for activity.

As described above, expression of P_{tir} requires Ler, which is encoded in the LEE PAI. Consistent with published reports which used a CAT reporter to study P_{tir} activity in another lab strain of *E. coli,* MC4100 (Sanchez-SanMartin *et al*., 2001), P_{tir}*lux* exhibited reduced activity when introduced into DH5α (Figure 3.14) The reduction in promoter activity was explained by the absence of a regulatory element specific to EPEC cells, which was then determined to be Ler (Sanchez-SanMartin *et al*., 2001). As P_{cesT} maintained activity when the *lux* transcriptional fusions were introduced into DH5α, this suggetsthat regulators encoded within the LEE are not required for P_{cesT} expression.

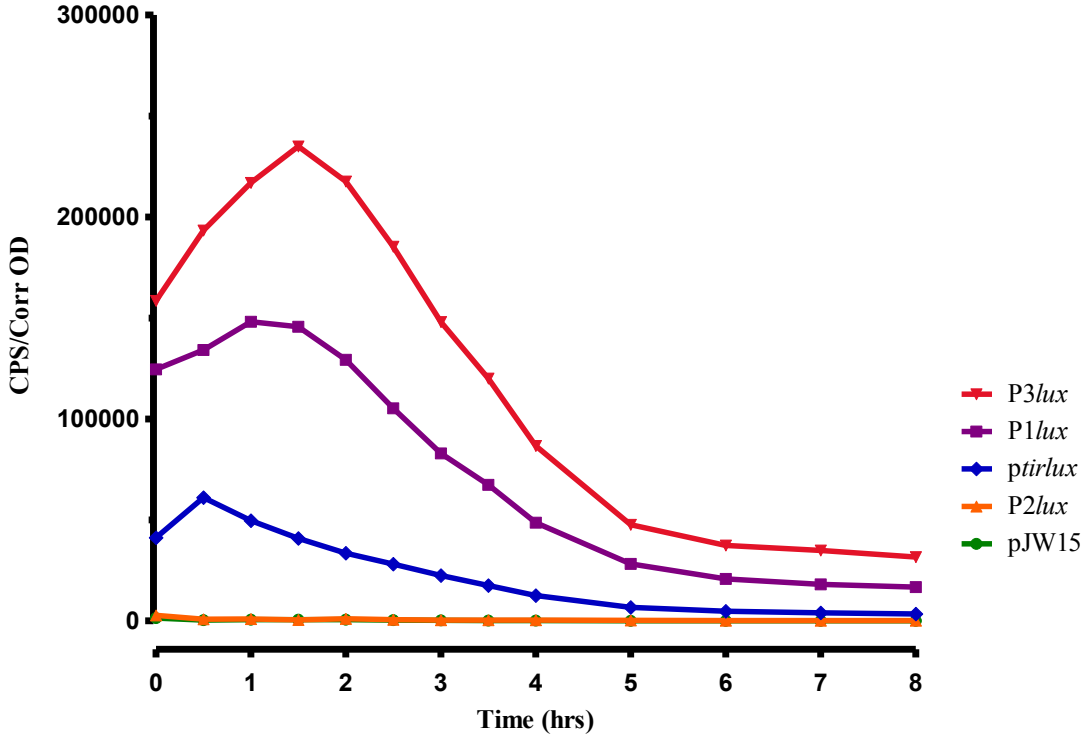


Figure 3.14 The activity of P_{cesT} is increased when fusions are introduced into lab strain *E .coli*, DH5α. DH5α cells harbouring the reporter fusions were grown in DMEM at 5% CO_2 and 37°C, and bacterial bioluminescence and OD were measured over 8 hr. Mean CPS of eight replicates was normalized by mean corrected OD. This is a representative figure of three repeated experiments; error bars represent standard deviation from one experiment.

3.2.7 The Δintg Mutation Reports Higher Activity When Introduced into DH5α

As shown above (Figure 3.6) a P3Δintg*lux* construct, which has an 83-bp deletion of the *tir-cesT* intergenic region, exhibited less activity than an intact fragment (P3*lux*) when introduced into WT cells. To investigate the effect of this deletion in a lab strain *E. coli*, DH5α, a luciferase reporter assay was performed using DH5α cells that harboured the *lux* transcriptional fusions. In DH5α, P3Δintg*lux* reported higher activity than P3*lux* (Figure 3.15). This information once again demonstrates that DH5α cells possess the basic machinery required for σ^{70} promoter recognition and transcription initiation at P_{cesT}. However, this finding also demonstrates that the factors influencing P_{cesT} recognition and transcription initiation differ between DH5α and EPEC cells.

3.3 Environmental Conditions Influence the Activity of P_{cesT}

3.3.1 P_{cesT} Activity is Reduced in Rich Medium

Expression of the LEE virulence genes can be induced *in vitro* when bacteria are grown under conditions that mimic the host environment, such as temperature, pH, and components of the medium (Kenny *et al*., 1997). When grown in a rich medium which contains an abundance of peptides and carbohydrates for energy, such as LB, reduced virulence-gene expression has been reported (Bustamante *et al*., 2001; Macritchie *et al*., 2008)

. To determine if the activity of P_{cesT} is also downregulated when grown in rich medium, WT cells harbouring *lux* transcriptional fusions were grown in LB, at 37°C and 5% CO_2, and promoter activity was measured by a luciferase reporter assay. As shown in Figure 3.16, the P_{cesT} transcriptional fusions were active when bacteria were grown in rich medium and followed the same trends as when the assay was performed in DMEM

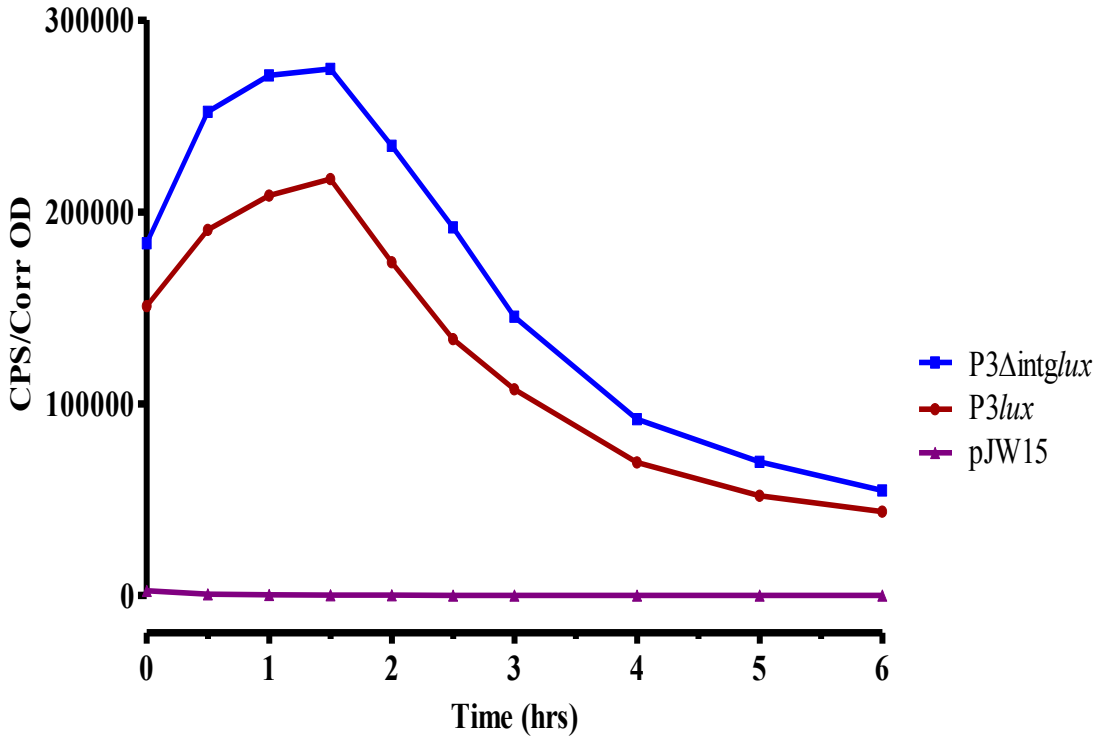


Figure 3.15. The activity of P3Δintg*lux* is significantly increased in DH5α cells. DH5α cells harbouring the P3*lux* or P3Δintg*lux* reporter fusions were grown in DMEM at 5% CO_2 and 37°C, and bacterial bioluminescence and OD were measured over 6 hr. Mean CPS of eight replicates was normalized by mean corrected OD. This is a representative figure of three repeated experiments; error bars represent standard deviation from one experiment.

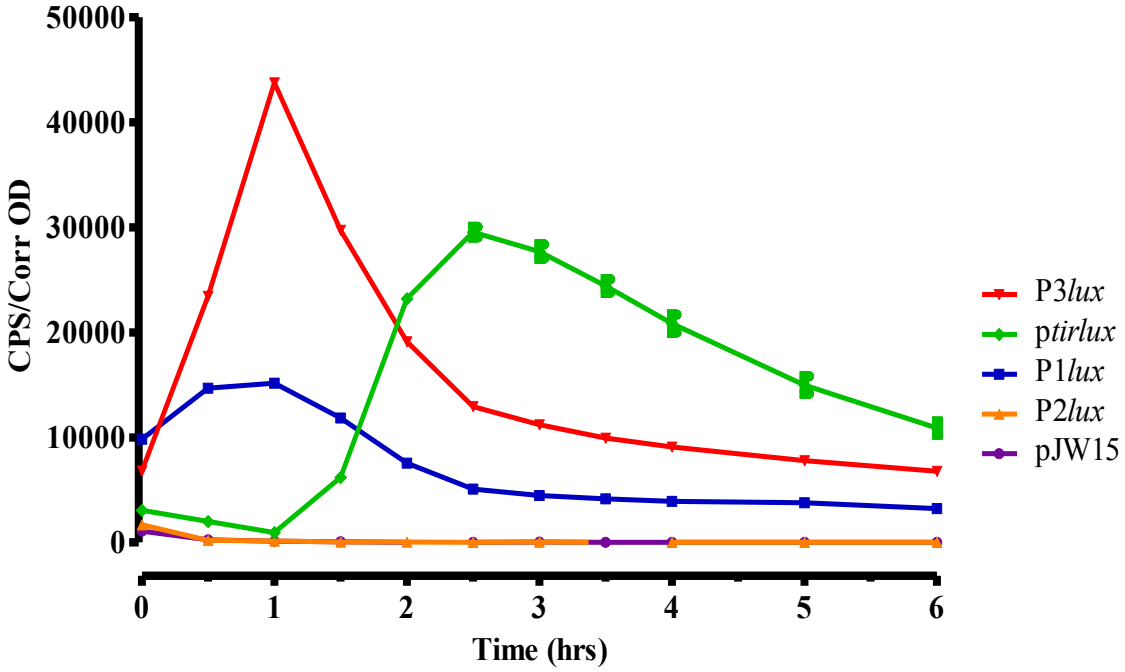


Figure 3.16 The activity of P_{cesT} was decreased when bacteria are grown in LB medium. EPEC cells harbouring the *lux* reporter fusions were grown in LB, incubated at 5% CO_2 at 37°C for 6 hrs, and CPS and OD were measured. Figure is representative of three repeated experiments; error bars represent standard deviation within one experiment. Transcriptional fusions which exhibited less activity than the medium only blank were excluded.

medium. However activity was reduced approximately four fold when bacteria were grown in LB (Figure 3.16). Interestingly, maximal activity of the P3*lux* fusion occurred at 1 hr post inoculation, while it had occurred at 1.5 hr when bacteria were grown in DMEM (Figure 3.1B). The P_{tir}*lux* transcriptional fusion also exhibited activity when bacteria were grown in rich medium, but, in accordance with the literature, this activity was decreased compared

to that found in bacteria grown in DMEM medium (Figure 3.16) (Macritchie *et al*., 2008). The P_{tir}*lux* fusion reported minimal activity within the first hour post inoculation, but then began to steadily increase until reaching maximal activity at 3 hrs. Similar to the trend seen in DMEM, activity of the P3*lux* fusion was significantly ($p<0.005$) higher than P_{tir}*lux* activity within the first 1.5 hrs of the assay. These data demonstrate that the mechanisms controlling P_{cesT} activity can be influenced by components of the growth medium, and support the conclusion that P_{cesT} activity is independent of P_{tir} activity.

3.3.2 P_{cesT} Exhibits Activity in M9 Minimal Medium

To further investigate the activity of the *cesT* promoter under different growth conditions, EPEC cells harbouring transcriptional *lux* fusions were grown in M9 minimal medium and bioluminescence was measured during a luciferase reporter assay (Figure 3.17). M9 has been shown to induce the expression of EPEC virulence genes, as it is a low-calcium medium (Deng *et al*., 2005). The P3*lux* fusion was active when bacteria were grown in M9 medium, with maximal activity reached 2.5 hrs post inoculation. The P_{tir}*lux* fusion reported minimal activity during the first 2 hrs of the assay, with maximal activity reached at 7 hrs (Figure 3.17). The shift in maximal promoter activity along the

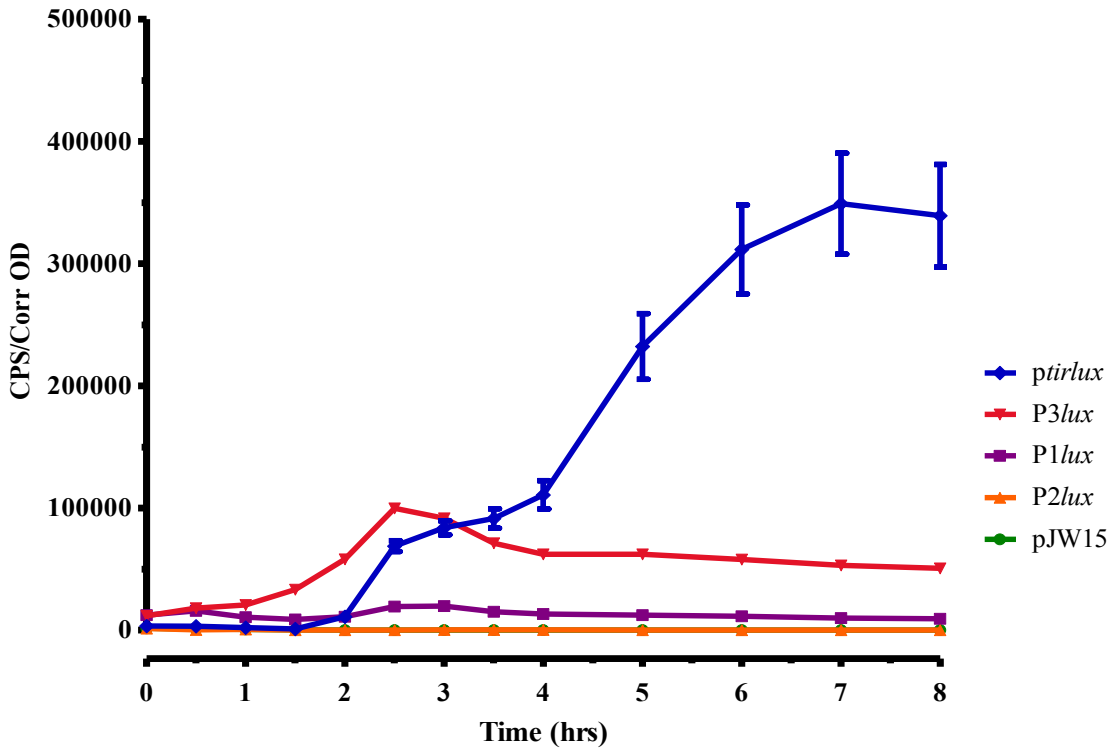


Figure 3.17 The P_{cesT} promoter maintains activity in M9 minimal medium. A luciferase reporter assay was performed on EPEC cells harbouring the transcriptional *lux* fusions, grown in M9 mediuim at 5% CO_2 at 37°C for 8 hrs, and CPS and OD were measured. Mean CPS of eight replicates was normalized by mean corrected OD. This is a representative figure of three repeated experiments; error bars represent standard deviation from one experiment.

x-axis of every transcriptional fusion may be explained by a slower growth rate of the bacteria in M9 medium, which was observed throughout the assay.

3.4 Generation of a Chromosomally Encoded *lacZ* Reporter

3.4.1 P_{cesT} Drives the Expression of a Chromosomally Encoded Reporter

To confirm the results of the luciferase reporter assay by a complementary technique, a chromosomally encoded *lacZ* reporter was generated which can measure the activity of P_{cesT} by a colourmetric assay. Activity of P_{cesT} can be assessed by a colormetric assay when bacteria are grown on differential media which can indicate the production of β-galactosidase. Of particular interest is MacConkey agar and LB agar containing X-gal. MacConkey agar contains a neutral red dye and the production of β-galactosidase (Lac^+) leads to acidification of the medium, resulting in the formation of red colonies. Bacteria which do not produce β-galactosidase (Lac^-) form white colonies when grown on this medium. When grown on LB agar with X-gal, the expression of β-galactosidase results in the breakdown of X-gal into two products, resulting in the formation of blue colonies. Bacteria which are Lac^- form white colonies when grown on this medium.

An EPECΔ*lacZ* strain was generated by deletion of the *lacZ* ORF from WT cells. When grown on MacConkey agar, EPECΔ*lacZ* grew white, while WT grew red (Figure 3.18A), indicating that the mutant had lost *lacZ*. When grown on LB agar with X-gal, EPECΔ*lacZ* grew white, while WT grew blue (Figure 3.18B), confirming that *lacZ* had been lost. Complementation of *lacZ* was performed by expression of *lacZ* on plasmid pRK415 (EPECΔ*lacZ*/pRK*lacZ*) which formed blue and red colonies when grown on LB agar with X-gal and on MacConkey agar, respectively (Figure 3.18).

To measure the activity of P_{cesT} from a chromosomally encoded reporter, a plasmid containing a β-galactosidase cassette was integrated into the Δ*lacZ* chromosome, at P_{cesT}, generating EPECΔ*lacZ::cesT*VIK cells. When grown on MacConkey agar, EPECΔ*lacZ*::*cesT*VIK cells formed white colonies (Figure 3.18A), suggesting that P_{cesT} is unable to drive the expression of this β-galactosidase reporter. An alternative explanation for the lack of colour change is that either the Δ*lacZ* mutation or the chromosomal integration of the β-galactosidase cassette (EPEC*lacZ*::*cesT*VIK) led to downstream effects which have disrupted the cells' ability to metabolize lactose. If such an event occurred the lack of colour change would not be reflective of an inactive promoter, but of a downstream effect of the mutation on lactose metabolism.

When grown on LB agar with X-gal, the bacteria formed blue colonies (Figure 3.18B). Taken together, these results demonstrate that P_{cesT} can drive the expression a chromosomally integrated reporter, and that P_{cesT} expression is repressed when cells are grown on MacConkey agar and active when grown on LB agar. Moreover, these data identify conditions whereby P_{cesT} is differentially regulated.

3.4.2. Pull-Down Assay Reveals Putative Binding Proteins

The observation that the P_{cesT} promoter could drive the expression of a β-galactosidase reporter and induce a color change when cells are grown on Xgal, but LB agar with X-gal but could not induce such a color change when grown on MacConkey agar, provided the means to analyze further the *cesT* promoter. To identify proteins that bind to the *cesT* promoter and regulate its activity, a pull-down assay was performed using streptavidin-coated beads coupled with DNA-tagged biotin. Such techniques have been used to identify DNA-protein interactions (Gabrielsen and Huet, 1993).

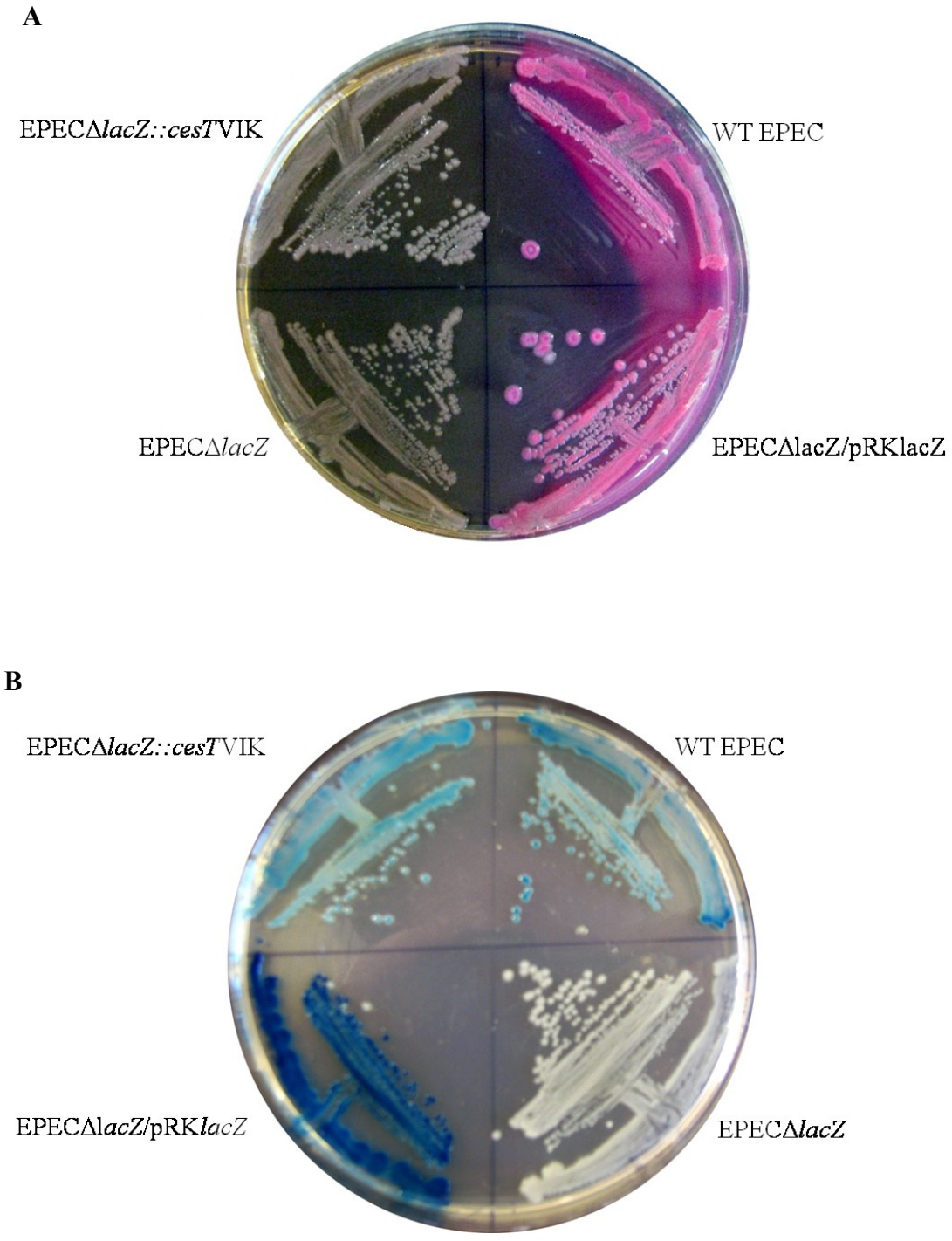


Figure 3.18. The *cesT* promoter can drive the expression of a chromosomally encoded β-galactosidase reporter when grown on LB agar with X-gal. EPEC strains were streaked for isolated colonies on MacConkey agar **(A)** and LB agar with X-gal **(B)**, and incubated at 37°C O/N.

PCR was used to amplify DNA encoding P_{cesT} and P_{tir}, using EPEC genomic DNA, which was bound to streptavidin-coated beads and washed with EPEC proteins (Figure 3.19). Proteins from EPEC cells grown on MacConkey agar and on LB agar were isolated by sonication. Following a high-speed centrifugation, proteins collected from the supernatant (soluble) and proteins collected from the pellet (insoluble) were resolved by SDS-PAGE and visualized by Coomassie stain (Figure 3.20). WCL were included to show the relative abundance of proteins in each fraction (soluble *vs.* insoluble). The Coomassie stain revealed differences in the protein profile between soluble and insoluble fractions, indicating that the sonication procedure was successful. Although loading volumes remained equal between samples, based on band intensity, it appeared that the lysis of bacteria grown on MacConkey agar was more successful than bacteria grown on LB agar.

Soluble proteins isolated from EPEC cells grown on MacConkey or LB agar were then passed through a heparin-sepharose column. Heparin is a polyanionic structure thought to mimic the phosphate backbone of DNA (Gadgil and Jarrett, 1999). Based on this feature, heparin is used as a tool to enrich for proteins that bind DNA (Gadgil and Jarrett, 1999). The flow-through from the column was saved as an 'unbound' fraction, representing proteins that did not bind to heparin, followed by a washing step which was saved as the 'wash' fraction. Proteins were eluted off the column into fractions using increasing concentrations of NaCl.

To determine which protein fractions contained nucleases, each fraction was incubated with 1-kb ladder (NEB) for 1 hr at 37°C, and visualized on a 1% agarose gel. The digestion reactions for protein fractions isolated from EPEC cells grown on LB are

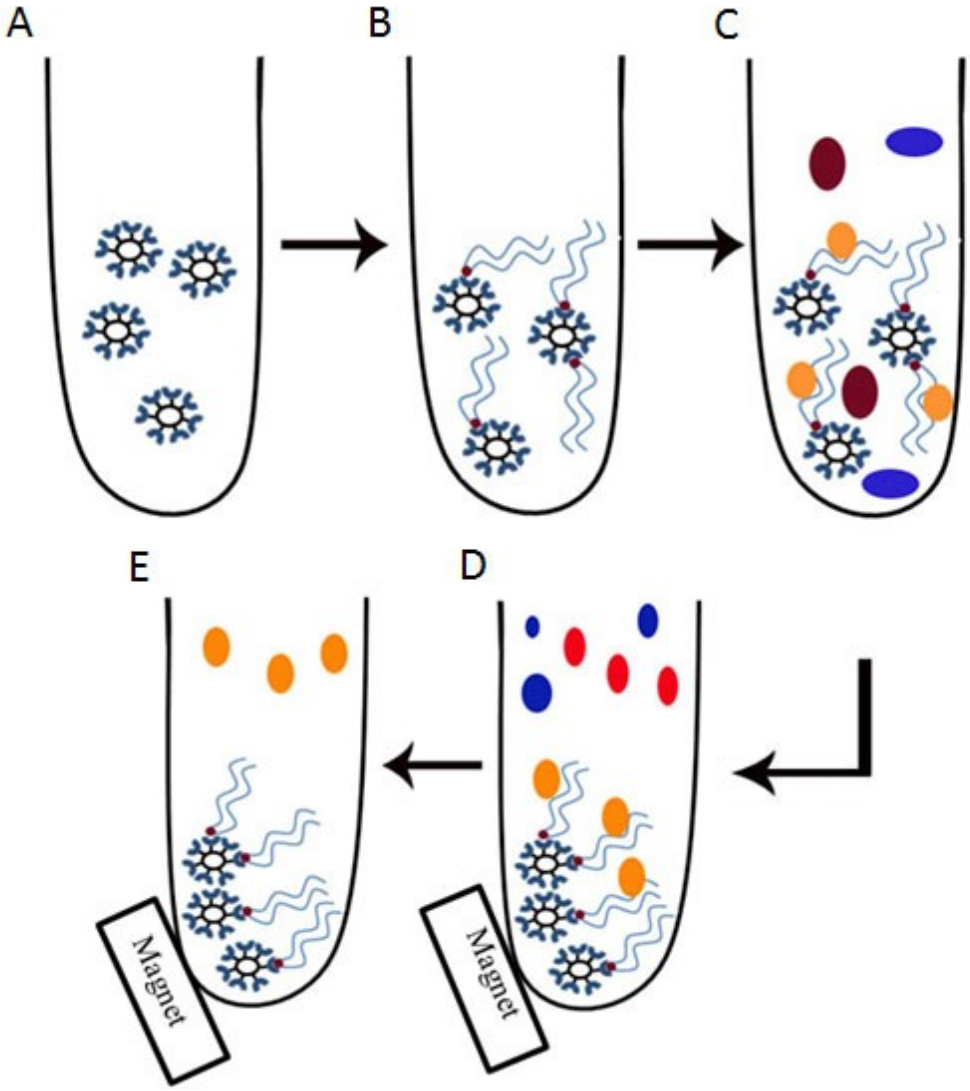


Figure 3.19. Schematic representation of biotin-streptavidin pull-down assay. Streptavidin-coated beads were resuspended (A) and incubated with biotin-tagged DNA fragments (B). Proteins (coloured circles) isolated from EPEC cells were incubated with the DNA-bound beads. Proteins that bound to the DNA were pulled down by a magnet (D), followed by extensive washing to remove unbound proteins. Elution of proteins from the DNA-bound beads was performed by incubation with 1 M NaCl (E). Proteins eluted from the DNA were then separated by SDS-PAGE and visualized by Coomassie stain.

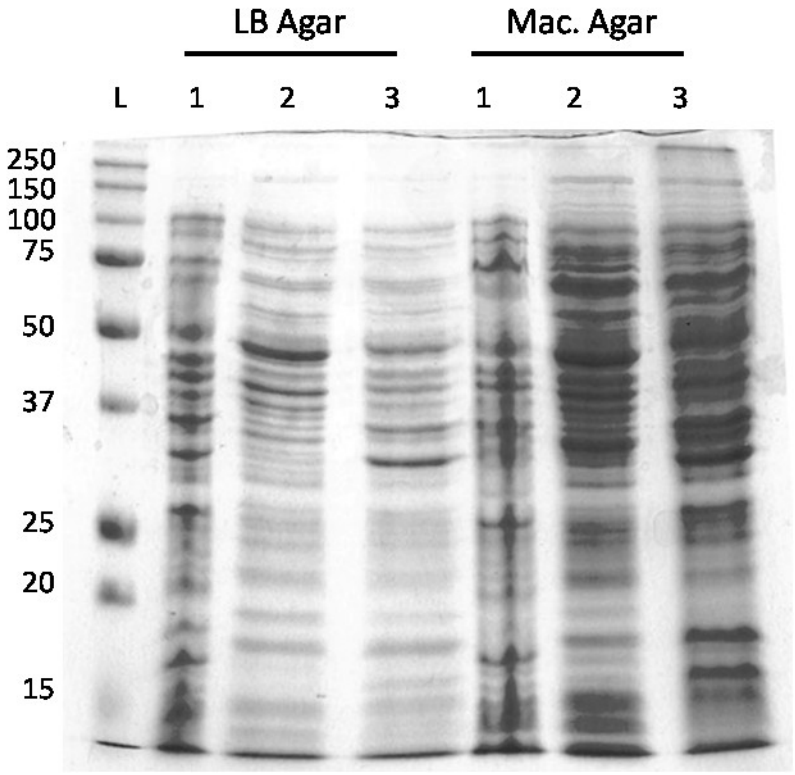


Figure 3.20 EPEC cells grown on LB agar or on MacConkey agar were fractionated using sonication followed by high-speed sonication. Bacteria were grown on LB agar or on MacConkey agar O/N at 37°C. The resulting bacterial emulsion was fractionated by sonication, and soluble (2) and insoluble (3) proteins were isolated by high-speed centrifugation. WCL (1) were also isolated by boiling a sample for 5 min in 2x ESB. Proteins were separated by a 10% SDS-PAGE, followed by coomassie stain. A protein marker (L) was also included with mass represented in kDa and listed to the left.

shown in Figure 3.21. Protein samples that contained nuclease activity displayed an altered band pattern, compared to the uncut 1kb ladder. Reactions that possessed nuclease activity were excluded, and the remaining fractions were pooled and visualized by Coomassie stain (Figure 3.22). Nuclease-free protein fractions were incubated with DNA-bound streptavidin beads. Proteins that bound to the DNA fragment of interest were eluted off using 1 M NaCl solution, separated by SDS-PAGE and visualized by Coomassie stain (Figure 3.23). As shown in Figure 3.23, proteins were successfully eluted off DNA encoding the P_{cesT} promoter, and off DNA encoding the P_{tir} promoter. The Coomassie stain revealed a different band pattern between proteins isolated from EPEC cells grown on LB agar *vs*. MacConkey agar, for both P_{cesT} and P_{tir}. Interestingly, two bands at ~100 kDa and one band at ~50 kDa appear to be present in every sample. These three bands are most prominent in the LB-agar protein sample eluted from the P_{cesT} -encoding DNA, which may implicate a role in activation of this promoter when bacteria are grown on this medium.

Another interesting band, at ~70 kDa, was eluted off of P_{cesT} -encoding DNA from the LB protein sample (Figure 3.23 (black arrow)). A band at the same mass was also eluted from the P_{tir}-encoding DNA, but in lesser abundance. A similar band is absent from proteins collected from EPEC cells grown on MacConkey agar, making this band a potential target for further analysis.

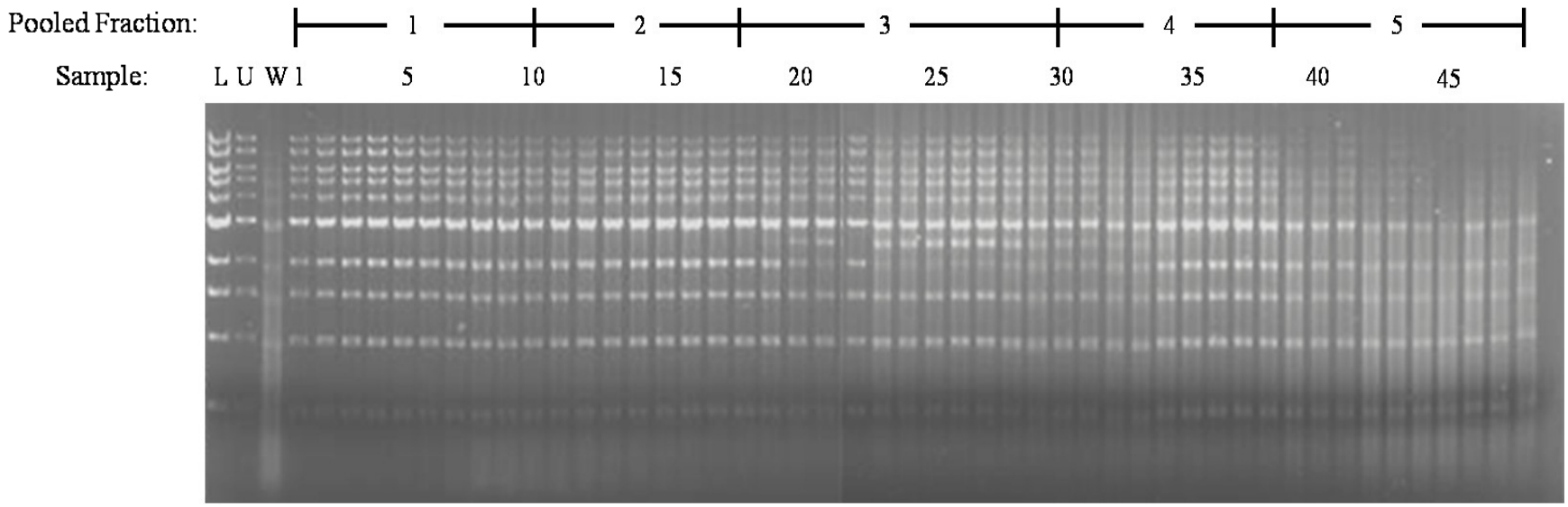
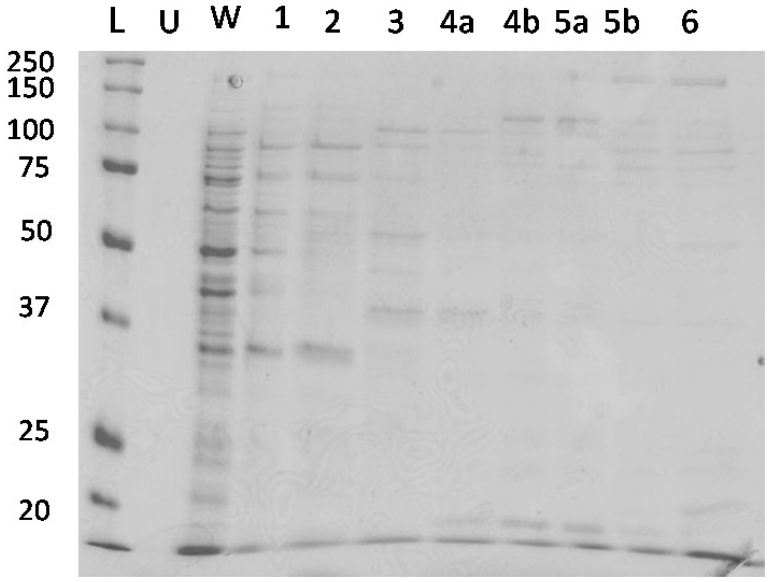


Figure 3.21 Determination of pooled protein fractions by digestion. Proteins isolated from EPEC grown on LB agar were eluted off of a heparin sepharose column and were incubated with 1 kb DNA ladder (NEB) for 1 hr at 37°C. Digestion reactions were visualized on a 1% agarose DNA gel. Protein samples were pooled based on difference of the band pattern, compared to uncut ladder (L). The unbound fraction (U) and the wash fraction (W) were also included. Protein samples 49 and 50 were pooled into a sixth fraction (data not shown).

A



B

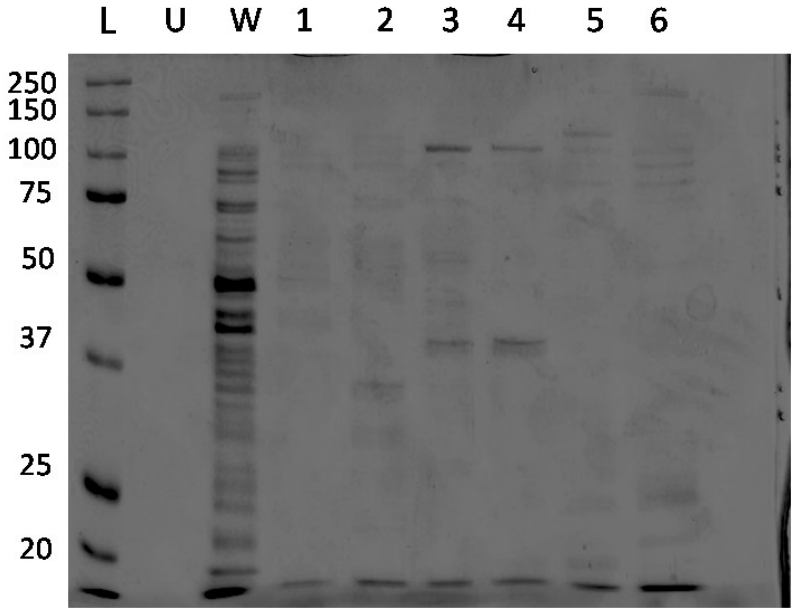


Figure 3.22 Proteins isolated from EPEC cells grown on MacConkey agar or on LB agar were pooled into fractions. Proteins isolated from bacteria grown on MacConkey agar were pooled into eight fractions (**A**), and proteins isolated from bacteria grown on LB agar were pooled into six fractions (**B**). Pooled fractions were determined based on the band pattern seen in the digestion reactions (Figure 3.21) and the presence of DNA nucleases. Protein fractions were separated by SDS-PAGE and visualized by coomassie stain. The sample which was passed over the column (Unbound) and the first wash (Wash) were also visualized.

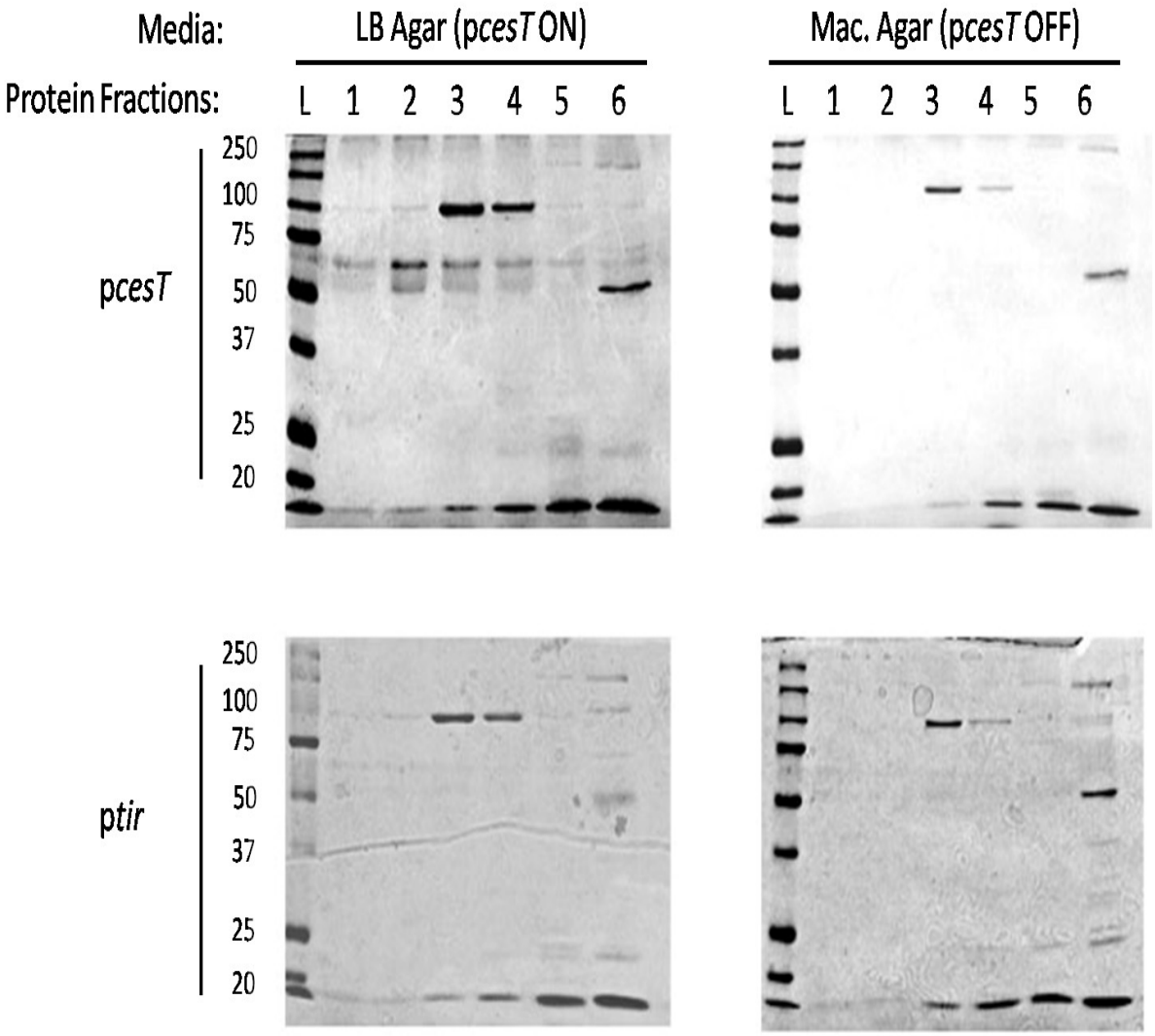


Figure 3.23 Bacterial proteins were eluted from DNA encoding the P_{cesT} and P_{tir} promoters. Bacterial proteins isolated from EPEC cells grown on LB agar or on MacConkey agar were separated into fractions (1-6), as mentioned above. Pooled protein fractions were incubated with streptavidin beads bound to double-stranded DNA encoding P_{cesT} or P_{tir} promoters. Proteins were eluted off the DNA, separated by SDS-PAGE and visualized by coomassie stain. The black arrow indicates a protein of interest at ~70 kDa.

CHAPTER 4. DISCUSSION

4.1 Characterization of the P_{cesT} Promoter

Promoter activity within the *tir-cesT* intergenic region was first observed by Puenté and colleagues (2001). Using primer-extension analysis this research group identified two putative transcriptional start sites, P1 and P2; thus there are two possible promoter regions, each directly upstream of a transcriptional start site. Further analysis of the nucleotide sequence of the *tir-cesT* intergenic region revealed a σ^{70} consensus site encoded upstream of the putative P1 transcriptional start site; no such site was identified upstream of the putative P2 start site (Sanchez-SanMartin *et al*., 2001). My primary objective was to gain insight into the effects of the P1 and P2 putative promoters on *cesT* expression by understanding the temporal activity profiles, the conditions in which they are active, possible transcriptional regulatory mechanisms and elements that are required for promoter activity.

Initial characterization of the putative *cesT* promoters was performed using a luciferase reporter assay, a technique that has been employed to study promoters located within the LEE PAI (Macritchie *et al*., 2008). Transcriptional fusions to a *lux* reporter plasmid were generated to measure the activity from each putative promoter individually, P1*lux* and P2*lux*, and also together (P3*lux*), and were introduced in WT EPEC and grown under conditions known to induce the expression of T3SS virulence genes (Kenny *et al*., 1997). The luciferase reporter assay revealed that the DNA elements directly upstream of the predicted P1 transcriptional start site supported *lux* gene expression (Figure 3.1B), and therefore likely contain a transcriptional promoter. In contrast, DNA elements located directly upstream of the predicted P2 site did not support *lux* gene expression, suggesting

that a P2 promoter region does not exist. From this data I can confirm the initial finding by Puenté and coworkers that there is a promoter region encoded within the *tir-cesT* intergenic region. I can further conclude that this promoter region is located directly upstream of the P1 transcriptional start site, where the predicted σ^{70} consensus sequence is located. Interestingly, the P3*lux* fusion reported higher activity than the sum of the two individual components (P1*lux*, P2*lux*). This observation suggests that there are additional components that may enhance the activity of the P1 promoter which are located in upstream DNA.

Upon initial discovery of the *cesT* promoter, an analysis of the DNA directly upstream of the P1 transcriptional start site revealed sequence elements which constitute a σ^{70} promoter (Sanchez-SanMartin *et al*., 2001). To investigate the conservation of the σ^{70} elements of P_{cesT}, I performed an *in silico* analysis and compared this sequence to an *E. coli* σ^{70} consensus sequence (Mitchell *et al*., 2003). The predicted σ^{70} P_{cesT} promoter shares strong sequence conservation to the *E. coli* consensus sequence with a five-out-of-six match to the TATA box and a three-out-of-six match to the -35 element. A TG motif located at position -14 and -15, found in 20% of *E. coli* σ^{70} promoters, was also identified in the P1 promoter. TG motifs are recognized by the σ^{70}-RNAP holoenzyme, and are essential for maximal activity of promoters (Barne, Bown, Busby, and Minchin, 1997; Mitchell *et al*., 2003). A 'T' encoded at position -18 relative to the transcriptional start site was also present in the P1 promoter. The nucleotide at this position has been identified as an important factor in DNA bending, which can alter intrinsic promoter activity (Singh *et al*., 2011). Both the length and sequence of the spacer region could affect transcription initiation, as the holoenzyme is able to sense conformational changes

of the DNA (Hook-Barnard and Hinton, 2009; Singh *et al*., 2011). The predicted location of the -35 element would yield a spacer region of 16 bp, which differs from the optimal spacer region length of 17 bp that is conserved in 39% of *E. coli* σ^{70} promoters that contain a TG motif (Mitchell *et al*., 2003). This analysis confirmed the hypothesis that a σ^{70} promoter region is encoded directly upstream of the P1 transcriptional start site.

The *in silico* bioinformatics analysis also revealed that the P_{cesT} promoter is well conserved among pathogenic strains of *E. coli* that contain the LEE, and among closely related species such as *Citrobacter*. The sequence corresponding to the -10 and -35 promoter elements do not appear to be strongly conserved; however, nucleotides directly upstream of the -10 element are conserved in all strains examined. It has been described that promoters that lack conservation of the -10 and -35 hexamers have developed 'extended -10' elements which can assist in σ-RNAP holoenzyme-DNA interactions (Barne *et al*., 1997). Given this information, I conclude that the *cesT* promoter is found in a number of human enteric pathogens which encode the LEE PAI, including the highly pathogenic EHEC. Further research is needed to explore this promoter and to examine whether the *cesT* promoter is active in other pathogenic strains.

To determine the role of *cesT*-specific promoter in EPEC pathogenesis, chromosomal mutations were generated with the aim to disrupt promoter activity. The first mutation, EPECΔ10, was generated by base-pair substitution of the TATA box from TATTAT to CTCGAG, the recognition sequence for a restriction endonuclease XhoI. This particular strategy was selected because such a mutation had previously been shown to abolish the activity of the P_{tir} promoter (Sanchez-SanMartin *et al*., 2001). Thus, I had hypothesized that such a mutation to the *cesT* promoter would yield a similar result.

Contrary to what I had hypothesized, the mutated promoter reported higher activity than the WT promoter (Figure 3.3). Previous reports have shown that 'extended -10' elements, such as the 'TG' motif, can compensate for a poor -10 hexamer (Mitchell *et al*., 2003), which may explain why P3Δ10*lux* continued to exhibit activity. It has also been shown that the binding affinity of the RNAP holoenzyme to DNA can be increased when mutations to the -10 element have been made (Niedziela-Majka and Heyduk, 2005), which might also explain how the P3Δ10*lux* fusion reported higher activity.

To determine if the Δ10 chromosomal mutation leads to an increase in CesT protein levels at early time points during an *in vitro* assay, western blot was used coupled with densitometry analysis. Based on the data obtained from luciferase reporter assays which showed that P3Δ10*lux* exhibited higher activity, I had predicted that CesT protein levels would be increased in EPECΔ10. However, it was shown by densitometry analysis that CesT is present in WT cells and EPECΔ10 cells in similar quantities (Figure 3.4). This result may suggest the presence of posttranscriptional control of *cesT* gene expression, as increased promoter activity did not yield more protein.

To further analyze the effect of the Δ10 mutation on the *cesT* promoter, a pedestal assay was performed; this assay can be used to measure the efficiency of Tir translocation. The EPECΔ10 cells maintained their ability to form actin-rich pedestals (Figure 3.5). This result is confirmation that the Δ10 mutation introduced into P_{cesT} does not negatively impact pedestal formation, as CesT is still present and functional in EPECΔ10.

A second mutation was generated, EPECΔintg, in which 83-bp are deleted from the *tir-cesT* intergenic region, with the objective of abolishing P_{cesT} activity by removing elements of the promoter, as well as upstream elements that may influence activity. A P3Δintg*lux* transcriptional fusion reported significantly ($p<0.005$) less activity, compared to a WT P3*lux* fusion (Figure 3.6). However, this result indicated that the mutated promoter still contains elements that allow recognition and transcription initiation by σ^{70}-RNAP, such as elements within the spacer region and the TATA box. It is possible that the P3Δintg*lux* mutant is still able to maintain some promoter activity by recognizing a '-35 like hexamer', as bioinformatics has revealed that promoters that contain a TG motif have greater variation of the -35 element (Mitchell *et al*., 2003).

The reduced activity reported by the P3Δintg*lux* transcriptional fusion could be explained in two ways: firstly, promoter recognition has been altered leading to decreased activity, as mentioned, or secondly, that the DNA sequence upstream of the promoter is necessary for full activation. This second explanation is consistent with my result that a transcriptional fusion containing only the putative P1 promoter region (P1*lux*) reported less activity than a fusion that also contained the upstream DNA (P3*lux*), suggesting that this upstream region contains DNA elements that influence promoter activity. Interestingly, the reduced promoter activity of the P3Δintg*lux* fusion during the luciferase reporter assay led to a decrease in intracellular CesT at early time points during an *in vitro* assay, shown by western blotting coupled with densitometry analysis (Figure 3.7). Further research is needed to investigate if the EPECΔintg mutation effectively alters EPEC pathogenesis.

The LEE PAI is under tight regulation for several reasons; firstly, it would be energetically expensive to express the T3SS apparatus in the 'wrong' environment; and secondly, recent research is providing insightful and convincing evidence that there is a functional hierarchy for protein secretion (Lara-Tejero *et al*., 2011; Thomas *et al*., 2007). Expression of many LEE-encoded factors is subject to repression by the global repressor H-NS (Barba *et al*., 2005; Bustamante *et al*., 2001; Haack *et al*., 2003; Umanski *et al*., 2002). In turn, *ler* expression is tightly regulated by a number of factors that can either activate or repress expression, depending on environmental stimuli. Based on the evidence regarding the transcriptional regulation of the LEE PAI, it can be hypothesized that the *cesT* promoter would also be under tight transcriptional regulation. To investigate the possible mechanisms which might regulate this promoter, the *lux* transcriptional fusions were expressed in different mutant strains.

Before investigating the potential influences of transcriptional regulators on P_{cesT}, a *lux* fusion containing the well-characterized LEE5 operon promoter (P_{tir}*lux*) was generated as a positive control. When introduced into WT, the P_{tir}*lux* fusion reported minimal activity within the first 1.5 hrs during a luciferase reporter assay, while the P3*lux* fusion activity was increasing during this time. These data suggest that P_{cesT} was active early, and that this activity occurred independently of the P_{tir} promoter, as minimal activity was reported by this fusion early during an *in vitro* assay. I speculate that the early activity of P_{cesT} is a mechanism to produce CesT independently of the P_{tir} promoter, and this may prime EPEC cells with CesT to prepare it for an upcoming infection.

To begin investigating the transcriptional regulation of P_{cesT}, *lux* transcriptional fusions were expressed in Δ*escN* and Δ*cesT*. I had suspected that introduction of P3*lux*

into Δ*escN* would result in an increase in activity to satisfy the increased demands of a chaperone, as effectors have been shown to accumulate in an Δ*escN* mutant (Gauthier *et al*., 2003; Thomas *et al*., 2005). However, P_{cesT} maintained similar transcriptional activity profiles in Δ*escN* and WT cells. The activity of P_{tir}*lux* was significantly higher when expressed in Δ*escN* than in WT cells after the first 2 hrs of an *in vitro* assay. These data suggest that the accumulation of effectors in the cytoplasm of Δ*escN* may influence the activity of P_{tir} through a positive regulatory feedback loop (Gauthier *et al*., 2003). Neither the P_{cesT} or P_{tir} promoter showed significantly different activity profiles in the Δ*cesT* background, indicating that neither promoter is influenced by the presence of functional CesT or a regulatory feedback loop involving CesT.

I next investigated the activity of P_{cesT} and P_{tir} in cells deficient for Ler, GrlR or GrlA. As these three proteins have been shown to form a regulatory feedback loop controlling the expression of the T3SS genes from the LEE PAI, I hypothesized that these regulators may also influence the activity of P_{cesT}. When introduced into Δ*ler*, P_{tir}*lux* exhibited 300-fold decrease of activity compared to what is seen in WT cells, showing that this promoter is directly influenced by Ler, a trend consistent with previously reported data (Sanchez-SanMartin *et al*., 2001). In contrast, P3*lux* reported a modest 2-fold decrease in activity. While the decrease of activity of P_{cesT} is statistically significant, these data suggest that the *cesT* promoter may not be directly regulated by the HNS-Ler system. In the absence of Ler, LEE promoters are subject to repression by H-NS; therefore, the reduced activity exhibited by P_{cesT} may be due to general repression of virulence genes and the T3SS apparatus, and not a direct regulatory effect of Ler at this promoter.

Consistent with published data, P_{tir}*lux* exhibited increased activity when introduced into Δ*grlR*, a result of upregulation of *ler* expression through GrlA, and reported less activity when introduced into Δ*grlA*, a result of reduced *ler* expression (Deng *et al*., 2004; Russell *et al*., 2007). I had initially hypothesized that if P_{cesT} was regulated by this feedback loop, changes to the activity profile would occur similar to those observed in P_{tir}*lux*. Unexpectedly, P3*lux* in a GrlR-deficient strain exhibited reduced activity, and exhibited increased activity in a GrlA-deficient strain, compared to WT. These results reflect disruption to the regulatory feedback loop involving Ler and GrlRA. I conclude that P_{cesT} activity is not directly governed by the regulatory feedback loop involving Ler and GrlRA, although it may be indirectly influenced by Ler-dependent events. .

As P_{cesT} activity was not influenced by the presence of CesT or EscN, and expression was not dependent on known transcriptional regulators encoded within the LEE (Ler, GrlR or GrlA), promoter activity was measured in cells of DH5α, a lab strain of *E. coli* that does not encode the LEE PAI. In DH5α cells, P_{cesT} maintained activity, indicating that the LEE PAI is not required for its activity. Interestingly, I observed that P_{cesT} activity was much higher at the time of inoculation (t=0) in an *in vitro* assay in DH5α, compared to expression in other strains discussed thus far. The temporal activity profile of the *lux* fusions in DH5α suggests that P_{cesT} activity may be influenced by mechanisms that are not present in non-pathogenic bacteria.

When P3Δintg*lux*, a mutant with an 83-bp deletion of the *tir-cesT* intergenic regions was introduced into DH5α, this fusion exhibited higher activity than did a WT P3*lux* fusion. As mentioned above, the P3Δintg*lux* transcriptional fusion reported reduced

activity in EPEC cells compared to the P3*lux* fusion. These data support the conclusion that P_{cesT} is differentially regulated in pathogenic and non-pathogenic bacteria.

The expression of P_{cesT} was examined when EPEC cells harbouring the *lux* transcriptional fusions were grown under different conditions, to gain insight on the possible factors that influence activation of this promoter. Two conditions were selected for testing: LB, a rich medium which induces minimal expression of T3SS genes, and M9, a low-calcium minimal medium which has been shown to induce T3SS gene expression (Deng *et al*., 2005). As expression of genes encoding the T3SS is minimal when bacteria are grown in LB, P_{tir}*lux* activity decreased ~20-fold when bacteria were grown in this medium, compared to growth in DMEM. Activity of the P3*lux* transcriptional fusion increased until reaching its peak at 1 hr post inoculation; however, activity was decreased four-fold when bacteria were grown in LB, compared to growth in DMEM. The activity reported by P_{tir}*lux* was consistently lower than the activity reported by P3*lux* at every time point measured during an *in vitro* assay, indicating that P_{cesT} expression is regulated differently than P_{tir}.

The second medium used was M9 medium. Bacteria grown in M9 medium have reduced secretion of translocon components, and increased expression of effector proteins, compared to bacteria grown in DMEM (Deng *et al*., 2005). When WT harbouring *lux* transcriptional fusions were grown in M9 medium, both P_{cesT} and P_{tir} exhibited activity; however, their temporal profiles were altered compared to growth in DMEM. Maximal activity of P_{cesT} was reached at 2.5 hrs, while P_{tir} activity peaked at 7 hrs, which can be explained by a slower growth rate in M9 which was observed

throughout the assay. This shift could also be explained by the reduction in translocon-component secretion, which may disrupt secretion hierarchy.

To summarize what has been explained thus far, the *cesT* promoter, or P_{cesT}, is located directly upstream of the putative P1 transcriptional start site that was initially described by Puenté and colleagues (2001). This promoter region encodes sequence elements of a σ^{70} promoter, which are well conserved among other LEE-containing pathogenic strains of *E. coli*, and closely related species such as *Citrobacter*. The activity of P_{cesT}, as represented by P3*lux*, exhibited early activity during an *in vitro* luciferase reporter assay, and activity occurred independently of the P_{tir} promoter (Δ*ler*). There is supporting evidence to confirm that P_{cesT} transcription is regulated by different mechanisms than P_{tir}. However, it is unclear what mechanisms are responsible for this difference.

4.2 Generation of a β-galactosidase Reporter

I have generated a chromosomally encoded β-galactosidase reporter which could be used to study P_{cesT} activity. There are several benefits to building such a strain. Firstly, this reporter will be present as a single copy, which makes it a better tool for measuring promoter activity than a multi-copy reporter plasmid. Secondly, as transcriptional regulation may be influenced by DNA topology, having P_{cesT} encoded in its native location may make such factors apparent, if they do exist. Thirdly, the β-galactosidase reporter is a commonly used tool in studying gene expression and allows promoter activity to be measured qualitatively or quantitatively by colour change.

A strain of EPEC in which *lacZ* is deleted was generated by homologous recombination, in which the *lacZ* ORF was replaced with a six-basepair restriction enzyme scar. This strain formed white colonies when grown on MacConkey agar and LB agar with X-gal, indicating the loss of lactose metabolism as predicted, due to the loss of LacZ function. The loss-of-function mutation could be complemented by introduction of a plasmid containing *lacZ* in EPECΔ*lacZ*, thus generating EPECΔ*lacZ*/pRK*lacZ*, which formed red and blue colonies when grown on MacConkey agar and on LB agar with X-gal, respectively. Integration of the β-galactosidase reporter plasmid at the *cesT* promoter was performed by homologous recombination, generating EPECΔ*lacZ*::*cesT*VIK. On LB agar with X-gal, the reporter induced a colour change from white to blue, which indicated that integration of the reporter converted a Lac^- strain to a Lac^+ strain. These data supported the previous observation that during the luciferase reporter assay the P_{cesT} promoter was active and can drive the expression of a reporter. Interestingly, a color change was not induced when EPECΔ*lacZ*::*cesT*VIK cells were grown on MacConkey agar. These data suggest that expression of P_{cesT} is altered when bacteria are grown on this medium, possibly through transcriptional regulatory mechanisms. Thus, I set out to identify potential transcriptional regulators that interact with P_{cesT}.

A pull-down assay was used to determine DNA-protein interactions between DNA containing P_{cesT} or P_{tir} and proteins isolated from EPEC cells grown on MacConkey agar or on LB agar. Proteins that bound to the DNA were eluted, separated by SDS-PAGE and visualized by Coomassie stain. Several protein bands of similar molecular weight were present in every sample, two at ~100 kDa and another at ~50 kDa. I hypothesize that the two bands at ~100 kDa are the same protein, although they were

isolated from different fractions. I also predict that, since these proteins were eluted from both P_{cesT} and P_{tir}, and present in bacteria grown on LB and MacConkey agar, they are not the proteins responsible for the observed color phenotype. A distinct band (~70 kDa) was eluted off the DNA containing P_{cesT}, isolated from bacteria grown on LB agar. A band of similar molecular weight was absent from samples prepared from MacConkey medium. Based on the relative size, I predict that the identity of this protein could be σ^{70} or RNA polymerase; both of which are ~70 kDa. Further experimentation, such as mass spectrometry, could determine the identity of this protein.

4.3 Concluding Remarks

My primary objective in this thesis was to characterize the *cesT* promoter, which was initially identified by Puenté and colleagues (2001), for a better understanding of LEE gene expression. Using transcriptional fusions to luciferase reporter genes, I was able to confirm the existence of a promoter located within the *tir-cesT* intergenic region. Furthermore, I was able to specify the location of this promoter, and show by *in silico* analysis that it is well conserved among pathogenic strains of *E. coli*. By examination of the temporal activity profile of P_{cesT}, it was shown that this promoter is active early during an *in vitro* assay and this activity occurs independently of P_{tir}, suggesting that CesT may be important early during host infection. Since CesT interacts with nine effectors in EPEC cells, it is biologically relevant that *cesT* be expressed by a mechanism independent of the P_{tir} promoter, which generates a multigene transcript. Also, given what is known about the role of chaperones in transcriptional regulation in *Salmonella* and *Shigella*, it is possible that CesT has a similar role, which may require *cesT* expression through an alternative mechanism.

As P_{cesT} exhibits activity to varying extent when EPEC cells harbouring *lux* transcriptional fusions are grown in DMEM, M9, and LB medium, I propose that medium components influence promoter expression, likely through transcriptional regulatory mechanisms. The *cesT* promoter was unable to induce a colour change when cells were grown on MacConkey agar, a medium that contains lactose and bile salts, which identifies the only condition tested thus far where P_{cesT} was not active.

Preliminary results from the biotin-pull down assay suggest that there are proteins that bind to the DNA encoding P_{cesT}, but further experimentation is needed to confirm the identity of these proteins. Using a β-galactosidase reporter to study gene expression is an important tool that is commonly used in the field, and can be used to determine the transcriptional regulators that influence the activity of the *cesT* promoter. Furthermore, identification of regulators that influence the activity of P_{cesT} will provide interesting new data on coordinated expression of virulence genes and EPEC pathogenesis.

4.4 Future Research Directions

Further characterization of P_{cesT} is needed to fully understand the relevance of this promoter region to EPEC pathogenesis. My data suggest that a mutant strain with a deficient P_{cesT} region must be generated. From what we have learned about σ^{70} sequence conservation, the 'TG' motif and the 'T' located at position -18 are strongly conserved elements of the *cesT* promoter (Figure 3.2). It is possible that a mutation generated to replace any of these nucleotides could abolish promoter activity, an approach which has had success in the past (Mitchell *et al*., 2003; Singh *et al*., 2011). Once a P_{cesT} -deficient strain has been generated, it may be subject to analysis and the resulting phenotype

established. I would hypothesize that intracellular CesT levels would be severely decreased early during an infection, and decreased EPEC pathogenesis will be observed.

It is clear that the *cesT* promoter is influenced by growth conditions and the presence of additional genes, as transcriptional fusions to the *lux* reporter exhibited altered activity profiles under varying conditions. To confirm the conclusion that P_{cesT} is not active when bacteria are grown on MacConkey agar a luciferase reporter assay could be employed. The luciferase reporter assay could be performed using EPEC cells harbouring *lux* transcriptional fusions grown in MacConkey broth, to quantify P_{cesT} activity under these conditions.

To further clarify the function and purpose of the *cesT* promoter, understanding the regulatory mechanisms responsible for expression is paramount. The β-galactosidase reporter strain is a valuable instrument for future research, and can be used to determine regulatory influences. One technique that could be used is saturated transposon mutagenesis. A transposon mutant library could be created in EPECΔ*lacZ*::*cesT*VIK, and the effect of each mutant on P_{cesT} activity monitored by color change when grown on MacConkey agar, or LB agar with X-gal. Mutations which lead to a color change from white to red colonies on MacConkey agar could indicate a disruption of a repressor protein, by insertion of the transposon. Bacteria grown on LB with X-gal could be used to monitor a color change of colonies from blue to white, indicating a mutation in a protein required for promoter expression, or a color change from blue to a darker blue, which could indicate disruption of a transcriptional repressor. As virulence genes are co-ordinately regulated it will be of great interest to identify genes that are subject to the same modes of regulation as P_{cesT}. This objective could be achieved by microarray

analysis on mutant strains. Identification of such regulatory proteins will clarify how P_{cesT} is regulated, and increase our understanding of factors that influence LEE gene expression in EPEC, and also T3SS gene expression in other human enteric pathogens such as *Salmonella*, *Shigella,* and *Yersinia*.

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