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Shantanu Bhatt Date

Investigation of the Role of the RNA Binding Protein CsrA

in the Virulence of Enteropathogenic *Escherichia coli*

By

Shantanu Bhatt
Doctor of Philosophy

Graduate Division of Biological and Biomedical Sciences
Microbiology and Molecular Genetics

_____
Daniel Kalman
Advisor

_____
Guy M. Benian
Committee Member

_____
Charles P. Moran Jr.
Committee Member

_____
Philip N. Rather
Committee Member

_____
Tony Romeo
Committee Member

_____
Bernard Weiss
Committee Member

Accepted:

_____
Lisa A. Tedesco, Ph.D.
Dean of the James T. Laney School of Graduate Studies

_____
Date

Investigation of the Role of the RNA Binding Protein CsrA

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By

Shantanu Bhatt
B.S., Denison University, 2005

Advisor: Daniel Kalman, Ph.D.

An abstract of
A dissertation submitted to the Faculty of the
James T. Laney School of Graduate Studies of Emory University
in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

in Graduate Division of Biological and Biomedical Sciences
Microbiology and Molecular Genetics

2011

Abstract

Investigation of the Role of the RNA Binding Protein CsrA

in the Virulence of Enteropathogenic *Escherichia coli*

By **Shantanu Bhatt**

The CsrA holoprotein is an RNA-binding protein that affects the stability and/or translation of transcripts. The gene was originally isolated in *E. coli* as a repressor of glycogen production. CsrA orthologs have since been discovered in numerous bacteria. For examples, in *Erwinia carotovora* subsp. carotovora, *Pseudomonas aeruginosa*, and *Salmonella* Typhimurium, besides regulating conserved ancestral processes, CsrA controls diverse virulence-associated traits. Despite its global regulatory role in innocuous and pathogenic bacteria, CsrA remains uncharacterized in any pathovar of *E. coli*.

We investigated the role of CsrA in the virulence of enteropathogenic *Escherichia coli* (EPEC). Inactivation of *csrA* profoundly diminished the infectivity of EPEC as evident from reduced pedestal formation on tissue culture cells. Molecular analysis revealed that the observed defect resulted from reduced transcript levels of the *LEE4* operon, which primarily encodes for the regulatory and structural components of a type 3 secretion system (T3SS). Purified CsrA protein specifically bound to the *LEE4* transcript, suggesting that CsrA presumably stabilizes the transcript and promotes pedestal formation. Intriguingly, modest overexpression of *csrA*, like inactivation, also repressed the *LEE4* operon. However, unlike inactivation, overexpression of *csrA* also silenced the other LEE-encoded transcription units. Overexpression of *csrA* exerted its effect by repressing the global activator of LEE, GrlA, as evident from reduced transcript levels. Furthermore, CsrA appeared to exert a direct effect on GrlA as the purified protein specifically bound to the *grlRA* transcript. Thus, CsrA appears to modulate the LEE in a dose-dependent manner. Besides virulence, CsrA also controlled flagellar-based motility and glycogen production in EPEC.

Additionally, *csrA* mutation abolished the ability of EPEC to synthesize diffusible exotoxins that paralyze and kill the worm *Caenorhabditis elegans*. Overexpression of *tnaA*, which encodes for the toxin-synthesizing enzyme tryptophanase, in the *csrA* mutant, but not vice-versa, rescued the ability of the mutant to kill worms. Moreover, tryptophanase activity was abolished in the *csrA* mutant. Collectively, these results suggest that CsrA is upstream of *tnaA* in a regulatory circuit that is essential for exotoxin synthesis and the killing of worms by EPEC.

In summary, our results suggest that *csrA* is a pleiotropic broad-host range virulence determinant of EPEC.

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Acknowledgements

"*The journey is the reward*"*....* An ancient Chinese adage that has assisted me in dueling arduous and challenging quests in life. Indeed, my journey through graduate school has been one of the most rewarding experiences of my life. Granted, there were occasions when I detested it and other times when I enjoyed it immensely. However, if I were given a choice to relive these years....I would...in an instant for I learnt the joys and frustrations of research and developed lifelong friendships through the experimental experience – something that is difficult to fathom through the eyes of another. This quest would not have been attainable had it not been for all my friends, family, and mentors who shared my joys and my sorrows, my failures and my successes, my trials and tribulations, and who uplifted me when I needed it most. I sincerely **THANK YOU** for all that you have done for me and I hope to be able to do the same for you when you need me.

First and foremost, I'd like to dedicate this dissertation to the memory of my maternal grandparents, Mrs. Leela Sharma and Mr. Bishan Dutt Sharma, who raised me since I was less than a month old and because of who, I am, who I am. Not a day goes by that I don't think of you two for you have left indelible marks on my life and I hope I can always make you proud. I will forever cherish your memories that are a source of immense support, unconditional love, and inspiration to me when I most need them. I know you will be watching me at my defense just as you have always watched over me since you departed. Amma and Papaji, I love you both and miss you sorely.

I'd like to sincerely thank my mentor, Dan Kalman, who has always supported me in my endeavors and given me immense freedom to pursue the projects that I have wanted

to even if they were beyond his area of expertise. Dan, your telescopic vision provides the perfect compliment to my microscopic vision and I would not have been able to accomplish what I have without your guidance and support, and for that I will forever be indebted to you. I'd also like to thank my committee members – Bernie Weiss, Guy Benian, Charlie Moran, Tony Romeo, and Phil Rather – who have always been there to field my questions and suggest outlets to extend my research even just when it seemed to have reached a dead end. I'd like to specially acknowledge Bernie Weiss who on occasions stopped midway of his own experiments to address my scientific dilemmas. Dr. Weiss, Thank you.

I'd also like to thank the Kalman lab members for putting up with me during some of my worst moods as a graduate student and for providing helpful suggestions concerning my project.

I'm grateful to my undergraduate mentor, Dr. Christine (Chris) Weingart who played an important role in directing me towards bacteriology by providing me the earliest opportunity to conduct research. Moreover, I'm exceedingly grateful to you for hearing my graduate school predicaments and providing meaningful advice to tackle those problems. Your guidance has helped shape my career goals in wanting to eventually teach at a small liberal arts college.

I'm grateful to my mother, Dr. Pushpa Bhatt, and my father, Ashok Dev Bhatta, who have always believed in me and helped me is so many ways that recounting all of them would take up more pages than my dissertation. I'd like to acknowledge my aunts – Dr. Mamta Sharma, Geeta Kandpal, Priyam Sharma – my uncles – Dr. Sanjay Sharma,

Naveen Chandra Sharma, and Dr. Rakesh Sharma (deceased) – who provided me the much needed familial love and support when I most needed it. I love you all.

I'd also like to extend my eternal gratitude and gratefulness to my friends both in Atlanta and elsewhere who patiently listened to my rants when all hell had broken loose. In particular, I'm grateful to have friends like Pancham Gajjar, Larry Jacobs, Jane Makepeace and I cherish their omnipresent love and support.

I'd also like to thank two of my closest, recently married, friends who played invaluable roles in my quest for my PhD – Luke Saba Uebelhoer and Ruth Jean Napier. Luke, (aka my brother from a different mother), I'm going to miss the bike rides immensely. They were a much needed source of de-stressing and mutual commiserating when we were completely engulfed by festering rage. And finally...Ruth... If there is a God then I'm grateful to him that you joined the lab; despite my failed attempts to get you to do otherwise. For once I'm extremely happy that I was unsuccessful. We've gone through a lot together as labmates and over time our friendship has evolved, strengthened, and matured. I'm grateful to you for your genuine concern and critiques about my personal and professional life. Lukie and Ruthie, you guys have made lasting impressions on my life and I want you to remember that despite any distances I'm always going to be there for you and I intend to keep our friendship alive beyond the grave. I LOVE you two.

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CHAPTER 1

Background and Significance

Escherichia coli – From Saprotropism to Parasitism

Escherichia coli is a Gram-negative, nonsporulating, and facultative anaerobe that inhabits the intestine of warm-blooded animals and reptiles (Tenaillon *et al.*, 2010, Berg, 1996, Gordon & Cowling, 2003). The bacterium is one of the most comprehensively characterized organisms that has served as a paradigm for groundbreaking research in fields as diverse as genetics, biochemistry, physiology, and molecular biology (Shuman & Silhavy, 2003, Tenaillon et al., 2010). *E. coli* was originally isolated, in 1885, from the feces of a healthy infant by the German paediatrician, Theobald Escherich, who christened it "*Bacterium coli commune*", and described them as short rod-shaped cells (Escherich, 1885). For over half a century since its discovery, *E. coli* was regarded a benign saprotroph – an organism that fulfills its nutritional requirements by exclusively foraging on decayed organic matter (Chen & Frankel, 2005). However, this overtly simplistic view has undergone a metamorphosis with the identification and characterization of numerous pathovars of *E. coli* that contribute to significant morbidity and mortality worldwide (Kosek *et al.*, 2003, Croxen & Finlay, 2010, Kaper *et al.*, 1997, Kaper *et al.*, 2004, Russo & Johnson, 2003).

Virulent *E. coli* isolates can be classified into two broad categories – intestinal pathogenic *E. coli* (InPEC) or extraintestinal pathogenic *E. coli* (ExPEC). Currently, there are eleven pathovars of *E. coli*, of which eight – enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), diffusely adherent *E. coli* (DAEC), cell-

detaching *E. coli* (CDEC), and adherent invasive *E. coli* (AIEC) – are intestinal pathogens, and three – uropathogenic *E. coli* (UPEC), neonatal meningitis *E. coli* (NMEC), and necrotoxigenic *E. coli* (NTEC) – are extraintestinal pathogens. Recent estimates suggest that collectively, pathogenic *E. coli* result in the death of over 2 million individuals annually (Kosek et al., 2003, Russo & Johnson, 2003, Tenaillon et al., 2010), several hundred thousand of which are attributed to infections by EPEC and EHEC (Chen & Frankel, 2005, Kaper et al., 2004, Mellies *et al.*, 2007a). Consequently, EPEC and EHEC are major public health concerns and have emerged as the most exhaustively characterized of all the *E. coli* pathotypes (Bhatt *et al.*, 2011, Chen & Frankel, 2005, Kaper et al., 1997, Kaper et al., 2004, Mellies et al., 2007a, Tenaillon et al., 2010).

EPEC and EHEC: Discovery, pathogenesis, epidemiology, and drug development

EPEC was the first pathogenic strain of *E. coli* to be implicated in outbreaks of watery diarrhea amongst infants in the 1940s (Bray, 1945, Bray & Beavan, 1948). The bacterium was first reported by John Bray, who referred to it as "*Bacillus coli neopolitanum*" (Bray, 1945). The term EPEC was coined in 1954 by Neter to describe pathogenic strains of *E. coli* that were observed in the intestines of infected children, but rarely observed in healthy individuals or patients afflicted with non-diarrheal illnesses (Neter *et al.*, 1955). These outbreaks, referred to as "summer diarrhea", were very frequent in industrialized nations and were associated with high mortality rates (Rowe, 1979, Levine & Edelman, 1984, Robins-Browne, 1987). Volunteer studies that were conducted in the early 1950s with *E. coli* isolates harvested from infants with gastroenteritis confirmed the virulence of

these strains (Neter & Shumway, 1950, June *et al.*, 1953, Ferguson & June, 1952, Koya *et al.*, 1954a, Koya *et al.*, 1954b). Young adults who were infected with EPEC strains of the serotype O111 and/or O55 developed diarrheal disease whereas individuals who were infected with *E. coli* strains obtained from healthy babies did not develop diarrhea (Neter & Shumway, 1950, Ferguson & June, 1952, June et al., 1953). However, nowadays, other than individual cases, EPEC is rarely associated with pediatric diarrhea in developed countries (Nataro & Kaper, 1998). By contrast, EPEC remains a primary cause of infantile diarrhea in developing countries (Gomes *et al.*, 1991, Cravioto *et al.*, 1996, Cravioto *et al.*, 1990, Cravioto *et al.*, 1988, Senerwa *et al.*, 1989b, Senerwa *et al.*, 1989a). It is estimated that approximately 30-40% of infantile diarrhea in developing countries results from EPEC infections (Chen & Frankel, 2005), with some outbreaks reporting mortality rates as high as 30% (Senerwa et al., 1989b).

On the other hand, it was only in the early 1980s that pathogenic strains of *E. coli* that synthesized a toxin with cytolytic activity toward African green monkey kidney cells (Vero cells) were first identified (Karmali *et al.*, 1983a, Karmali *et al.*, 1983b). These strains were also isolated from individuals with bloody diarrhea and hemolytic uremic syndrome (HUS) (Karmali et al., 1983b)– a condition that arises due to kidney damage induced by Shiga toxins (Karch *et al.*, 2005), which are encoded within cryptic prophages of EHEC that are absent in EPEC (Mellies et al., 2007a). The term "enterohemorrhagic" was coined by Levine *et al.* in 1987 to describe serotypes of *E. coli* that were hemorrhagic colitis (Levine *et al.*, 1987). Furthermore, these strains also hybridized to the CVD 419 probe that complements the sequence of the hemolysin gene, which is encoded within the EHEC-specific plasmid, pO157 (Levine et al., 1987). Since then, numerous

studies have confirmed the role of EHEC as the etiologic agent of nonbloody or bloody diarrhea and HUS (Karch et al., 2005), fulfilling Koch's postulates of linking a pathogen to a disease.

In contrast to EPEC, EHEC infections occur in individuals of all age groups and appear to be endemic to developed nations (Mellies et al., 2007a). Furthermore, the infectious dose of EHEC is far lesser than that of EPEC. It has been estimated that approximately 100 colony-forming units (cfu) of EHEC are sufficient to induce disease in humans whereas the infectious dose of EPEC is far more at approximately $10^8 – 10^{10}$ cfu (Bieber *et al.*, 1998, Donnenberg *et al.*, 1993a, Griffin & Miner, 1995, Mellies et al., 2007a). Finally, the two pathogens also exhibit differences in tissue tropism with EHEC colonizing the large intestine whereas EPEC infections occur in the small intestine (Reece *et al.*, 2001, Mellies et al., 2007a). It has been suggested that different isoforms of the outer membrane protein intimin contribute, in part, to the observed niche specialization of EPEC and EHEC (Reece et al., 2001, Mellies et al., 2007a).

Despite apparent differences in the epidemiology and certain aspects of pathogenesis, these pathotypes also possess vast stretches of conserved genomic segments that confer similar pathophysiological traits on these two enterovirulent bacteria (Bhatt et al., 2011, Mellies et al., 2007a, McDaniel *et al.*, 1995, McDaniel & Kaper, 1997, Kaper et al., 2004, Nataro & Kaper, 1998). One such defining trait, essential for the virulence of EPEC and EHEC, is their capacity to form attaching and effacing (A/E) lesions on the surface of infected cells – a signature pathomorphological trait that often serves as a diagnostic test for EPEC and EHEC infections (Mellies et al., 2007a, Bhatt et al., 2011, Knutton *et al.*, 1989). In fact, the ability to form A/E lesions is restricted to a few closely related Gram-

negative bacteria, and as such is the primary criteria to define the A/E family of gastrointestinal pathogens, the other members of which include the murine pathogen *Citrobacter rodentium* (Luperchio *et al.*, 2000, Luperchio & Schauer, 2001, Schauer & Falkow, 1993a, Schauer & Falkow, 1993b), rabbit diarrheagenic *E. coli* (RDEC) (Karaolis *et al.*, 1997), and *Escherichia albertii* (Huys *et al.*, 2003, Hyma *et al.*, 2005, Oaks *et al.*, 2010).

A/E lesions are characterized by intimate adherence of the bacterium to the host cell (**attachment**) and the concomitant destruction of the cellular microvilli in the vicinity of the infecting bacterium (**effacement**) (Mellies et al., 2007a, Bhatt et al., 2011, Knutton et al., 1989, Knutton *et al.*, 1987, Chen & Frankel, 2005). The disintegration of the microvilli involves the depolymerization of filamentous actin and other cytoskeletal elements, which profoundly reduces the surface area of the microvilli ablates the capacity of intestinal cells to absorb nutrients (Bhatt et al., 2011, Chen & Frankel, 2005, Mellies et al., 2007a). As a consequence, ingested nutrients and fluids are unabsorbed into the intestinal cells and remain confined to the intestinal lumen, leading to liquefied feces (diarrhea) – the classical symptomology associated with infections by EPEC and EHEC (Bhatt et al., 2011, Chen & Frankel, 2005, Kaper et al., 2004, Mellies et al., 2007a, Nataro & Kaper, 1998). Dissociated actin monomers are subsequently conscripted beneath the adherent bacterium, remodeled, and repolymerized to form actin-dense pseudopod-like protrusions, termed “pedestals”, that extend outward from the surface of the infected cell and are crowned by the infecting bacterium (Figure 1.1) (Mellies et al., 2007a, Bhatt et al., 2011, Knutton et al., 1989, Knutton et al., 1987, Chen & Frankel,

2005, Kaper et al., 2004, Nataro & Kaper, 1998, Campellone *et al.*, 2002, Campellone & Leong, 2003).

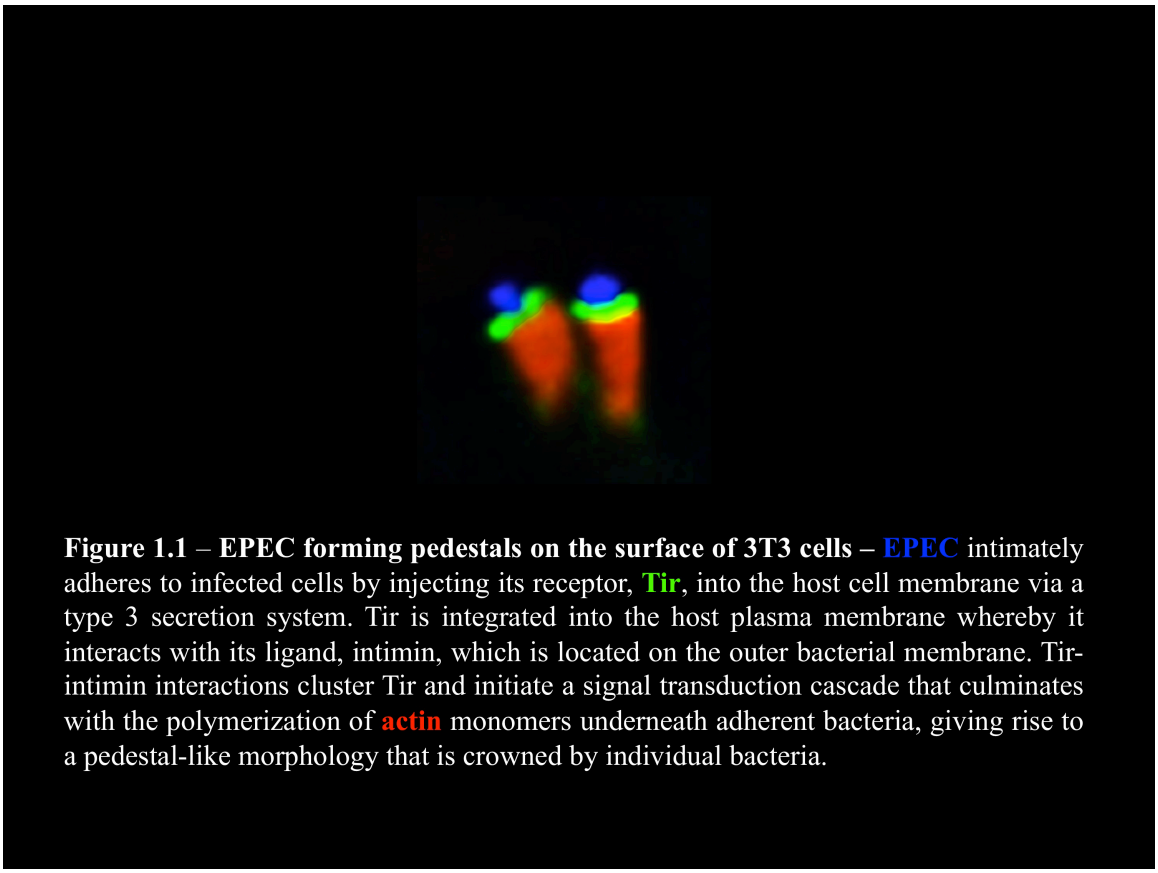


Figure 1.1 – EPEC forming pedestals on the surface of 3T3 cells – **EPEC** intimately adheres to infected cells by injecting its receptor, **Tir**, into the host cell membrane via a type 3 secretion system. Tir is integrated into the host plasma membrane whereby it interacts with its ligand, intimin, which is located on the outer bacterial membrane. Tir-intimin interactions cluster Tir and initiate a signal transduction cascade that culminates with the polymerization of **actin** monomers underneath adherent bacteria, giving rise to a pedestal-like morphology that is crowned by individual bacteria.

Experimental challenge studies in human volunteers suggest that EPEC mutants that are unable to form pedestals *in vitro* display profoundly reduced virulence *in vivo* including diminished or complete absence of diarrhea, fever, malaise, as well as reduced stool volume (Donnenberg et al., 1993a, Donnenberg *et al.*, 1993b). Furthermore, similar findings were obtained in studies in which non-human mammalian hosts that were

infected with mutants of EPEC and EHEC that lacked the regulatory genes (*ler*), structural components (*escN*, *escV*, *sepQ*, *escC*), and effectors of pedestal formation (*tir*, *eae*, *espD*) were attenuated *in vivo* (Donnenberg et al., 1993a, Deng *et al.*, 2004, Deng *et al.*, 2003, Dziva *et al.*, 2004). Collectively, these results suggest that pedestal formation is an important correlate of the diarrhea and the accompanying disease associated with infections by EPEC and EHEC. Therefore, a comprehensive understanding of the environmental cues and the regulatory factors that govern pedestal formation is not only essential for understanding the cellular and molecular pathogenesis of EPEC and EHEC infections but also for designing any effective preventative or therapeutic measures to combat these pathogens. The latter is of grave significance as recent reports have identified EPEC and EHEC isolates with resistance to multiple antibiotics (Scaletsky *et al.*, 2010, Teel *et al.*, 2002, Zhang *et al.*, 2000). Paradoxically, certain DNA-damaging drugs, which are routinely used against other pathogens, actually potentiate the virulence of EPEC and EHEC (Scaletsky et al., 2010, Teel et al., 2002, Zhang et al., 2000, Mellies *et al.*, 2007b). Consequently, the arsenal of drugs used to treat EPEC and EHEC infections is severely depleted and there is dire urgency for discovering or synthesizing inhibitors that can curb the morbidity and mortality associated with these pathogens.

Genetic organization of the LEE in EPEC and EHEC

The ability of EPEC and EHEC to form pedestals resides within a 35-45 kb pathogenicity island termed the locus of enterocyte effacement (LEE) (Figure 1.2) (Bhatt et al., 2011, Kaper et al., 2004, McDaniel et al., 1995, McDaniel & Kaper, 1997, Mellies et al., 2007a, Croxen & Finlay, 2010, Deng et al., 2004, Mellies *et al.*, 1999, Elliott *et al.*,

2000, Hansen & Kaper, 2009). In the prototypical EPEC strain belonging to the serotype O127:H6 as well as its EHEC counterpart belonging to the serotype O157:H7, the LEE is inserted into the *selC* tRNA encoding gene (Perna *et al.*, 1998, Bertin *et al.*, 2004, Sperandio *et al.*, 1998, Zhu *et al.*, 2001). However, in certain non-conventional and traditionally understudied serotypes of EPEC and EHEC, the LEE is inserted in the *pheV* and *pheU* phenylalanine tRNA genes (Muller *et al.*, 2009, Sperandio et al., 1998). Collectively, these molecular observations suggest that EPEC and EHEC have acquired the LEE, via horizontal gene transfer, on multiple occasions during the course of their evolutionary history.

The LEE is genetically organized into five major polycistronic operons, *LEE1*, *LEE2*, *LEE3*, *LEE4*, and *LEE5*, a dimorphic transcription unit, *grlRA* and *grlA*, and numerous monocistronic genes including *escD*, *map*, *espG*, *cesF*, and *rorf3* (Bhatt et al., 2011, Elliott et al., 2000, Mellies et al., 2007a, Mellies et al., 1999, Sanchez-SanMartin *et al.*, 2001, Tauschek *et al.*, 2010, Sperandio *et al.*, 1999, Sperandio *et al.*, 2001, Deng *et al.*, 2001, Deng et al., 2004) (Figure 1.2). The transcription units housed within the LEE encode for proteins that constitute the regulatory factors and the structural components of a specialized type three secretion system (T3SS) that connects the bacterial cytoplasm to that of the host (Mellies *et al.*, 1997, Elliott et al., 2000, Deng et al., 2004, Sperandio *et al.*, 2000, Bhatt et al., 2011). This macromolecular complex functions as a nanosyringe for the injection of effector molecules directly into the infected host cell, some of which are also encoded within the LEE (Mellies et al., 1997, Elliott et al., 2000, Deng et al., 2004, Sperandio et al., 2000, Bhatt et al., 2011, Deng *et al.*, 2005, Deng et al., 2003,

Elliott *et al.*, 1998, Croxen & Finlay, 2010, Deng *et al.*, 2010, Sekiya *et al.*, 2001) (Figure 1.3).

The oligomeric T3SS consists of approximately 25 architectural proteins, with the majority of them being conserved in phylogenetically distant bacterial pathogens of animals, plants, and insects (Ghosh, 2004). Remarkably, most of the conserved structural components of the T3SS are homologous to the bacterial flagellum, suggesting a common evolutionary origin of the two morphological organelles (Aizawa, 2001, Galan *et al.*, 1992, Hueck, 1998). Based on molecular phylogenetic analysis there are currently two schools of thought that trace the evolution of the flagella and the T3SS (Gophna *et al.*, 2003, Nguyen *et al.*, 2000, Saier, 2004). The first model contends that the T3SS arose by the duplication of a subset of flagellar genes and the two morphogenetic systems evolved independently of each other, with minimal genetic interchange (Nguyen et al., 2000, Saier, 2004). However, a competing model argues that the T3SS is as ancient as the flagella and the two share a common ancestor, rather than the former originating from the latter (Gophna et al., 2003). The physiological roles associated with the flagella are motility and chemotaxis (Macnab, 1996, Stock & Surette, 1996), whereas the T3SS functions to anchor the bacterium on a biotic substratum, such as a cell (Mellies et al., 2007a, Ghosh, 2004). Motility and adherence are two seemingly opposing traits. Not surprisingly, environmental stimuli and the cognate signal transduction and regulatory pathways that regulate flagella often coregulate genomic islands, such as the LEE, that house T3SSs (Hansen & Kaper, 2009, Iyoda *et al.*, 2006, Iyoda & Watanabe, 2005, Altier *et al.*, 2000, Lawhon *et al.*, 2003).

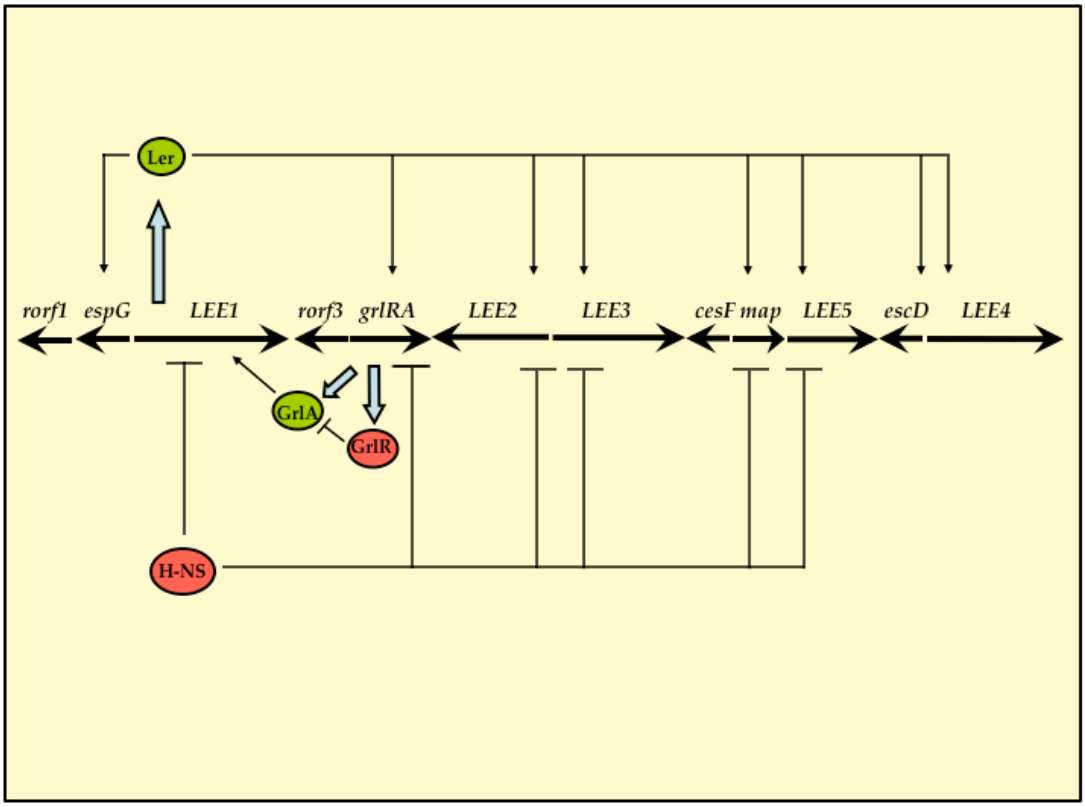


Figure 1.2 – Genetic and regulatory architecture of the LEE – The LEE is xenogenically silenced by the ancestral regulatory protein H-NS, which recognizes and binds to AT-rich segments within the LEE by forming extended nucleoprotein filaments. In response to diverse environmental cues, the H-NS-dependent repression of *LEE1* is relieved and Ler, an H-NS homolog, is induced. Ler functions as an antirepressor of H-NS and activates transcription from the other *LEE*-encoded transcription units including *grlRA*, *grlA*, *LEE2*, *LEE3*, *LEE4*, and *LEE5* by preventing the formation of H-NS-DNA nucleoprotein complexes. GrlA and GrlR encode for an activator and its cognate antiactivator respectively. GrlA recognizes sites upstream of *LEE1* and promotes the transcription of *ler* in a positive feedback loop, whereas GrlR binds to GrlA and sequesters it preventing the GrlA-mediated activation of *ler* thereby constituting a negative feedback loop. The synchronized expression from the LEE leads to the assembly of a T3SS that connects the bacterial cytosol to that of the host and culminates with pedestal formation and the trafficking of other effectors into the host that contribute to disease.

Supramolecular structure of the LEE-encoded T3SS

The *LEE1*, *LEE2*, *LEE3* operons and the *escD* transcription unit code for the conserved architectural components of the T3SS that traverses the inner membrane, peptidoglycan layer, and the outer membrane of the bacterium (Mellies et al., 2007a, Bhatt et al., 2011, Mellies et al., 1999, Deng et al., 2004, Beltrametti *et al.*, 2000) (Figure 1.3). Assembly of this morphogenetic organelle gives rise to a hollow nanofilamentous pipeline that connects the bacterial cytosol to the extracellular environment (Mellies et al., 2007a, Deng et al., 2004, Daniell *et al.*, 2001, Sekiya et al., 2001) (Figure 1.3). The *LEE4* operon codes for the regulator SepL (Kresse *et al.*, 2000, Beltrametti *et al.*, 1999, Deng et al., 2005, O'Connell *et al.*, 2004), the extracellular structural elements, EscF (Knutton *et al.*, 1998, Wilson *et al.*, 2001, Sekiya et al., 2001), EspA (Kenny *et al.*, 1996), EspB (Ide *et al.*, 2001, Foubister *et al.*, 1994), EspD (Lai *et al.*, 1997), the chaperones – CesD2 (Neves *et al.*, 2003) and L0017 (Su *et al.*, 2008)– and the effector EspF (McNamara & Donnenberg, 1998, McNamara *et al.*, 2001). SepL and SepD (encoded within the *LEE2* operon) constitute a "gatekeeper switch" (Deng et al., 2005, Wang *et al.*, 2008), which in response to high extracellular concentrations of calcium ions (Cornelis *et al.*, 1998), promotes the selective export of EscF, EspA, EspB, and EspD while actively repressing the transport of effectors by sequestering them (Deng et al., 2005, Wang et al., 2008, O'Connell et al., 2004). EscF comprises the extracellular needle tip of the T3SS that protrudes out of the outer bacterial membrane (Knutton et al., 1998, Wilson et al., 2001, Sekiya et al., 2001). EscF serves as a docking site for the recruitment and nucleation of EspA monomers, which polymerize to form a helical filamentous tube that forms the biophysical bridge between the EscF needle tip and the membrane of the

host cell (Sekiya et al., 2001, Wilson et al., 2001, Daniell et al., 2001, Knutton et al., 1998) (Figure 1.3). Subsequently, EspB and EspD are trafficked through the mature T3SS and integrated into the plasma membrane of the host, where they form a portal that permits the entry of effector molecules into the host (Figure 1.3) (Shaw *et al.*, 2001, Wachter *et al.*, 1999, Wolff *et al.*, 1998, Ide et al., 2001). Insertion of EspB and EspD into the plasma membrane of the host completes the morphogenesis of the mature type three secreton that forms a contiguous unobstructed channel linking the bacterial cytosol to that of the host for directly injecting effectors into the host cytosol.

Assembly of the T3SS provides the bacterium access to the host cytosol, where the calcium concentrations are extremely low (~100-300 nM) (Barrero *et al.*, 1997, Dominguez, 2004). The reduction in calcium levels serves as a physiological stimulus to inactivate the SepL/SepD gatekeeper (Deng et al., 2005, Wang et al., 2008); consequently, secretion of translocators is inhibited and the sequestered effectors are constitutively trafficked into the host via the T3SS (Bhatt et al., 2011, Mellies et al., 2007a, Croxen & Finlay, 2010, Deng et al., 2010, Deng et al., 2005, O'Connell et al., 2004, Wang et al., 2008). Consistent with this model, the calcium-dependent effect occurs exclusively in a SepL/SepD-dependent manner as *sepL* and *sepD* mutants are insensitive to the presence of calcium and exhibit profoundly reduced secretion of translocators and enhanced constitutive export of effectors (Deng et al., 2005, Wang et al., 2008).

At least 40 structurally and functionally diverse effectors are injected directly into the host via the T3SS of A/E pathogens (Croxen & Finlay, 2010, Kaper et al., 2004, Deng et al., 2010). The translocated intimin receptor, Tir, and its ligand, the adhesin intimin, are

two effectors that are encoded within the *LEE5* operon and are essential for pedestal formation (Kenny *et al.*, 1997b, Batchelor *et al.*, 2000, Luo *et al.*, 2000, Liu *et al.*, 2002). Whereas intimin localizes, likely in a T3S-independent manner, to the outer membrane of the bacterium (Wentzel *et al.*, 2001), Tir is trafficked into the host via the T3SS and subsequently integrated into the host plasma membrane where it isomerizes to attain a hairpin-loop conformation (Deibel *et al.*, 1998, DeVinney *et al.*, 1999, Kenny et al., 1997b, Batchelor et al., 2000, Liu *et al.*, 1999, Liu et al., 2002, Luo et al., 2000) (Figure 1.3). Tir contains a central extracellular domain that recognizes intimin whereas its amino and carboxy terminal domains are localized to the host cytosol (Hartland *et al.*, 1999, de Grado *et al.*, 1999, Kenny, 1999). Intimin-Tir interactions promote the clustering of Tir and initiate a signal transduction cascade that recruits several cytoskeletal and regulatory factors which cooperate to recruit and polymerize actin monomers that culminate with pedestal formation underneath the adherent bacterium (Touze *et al.*, 2004, Campellone & Leong, 2003, Campellone & Leong, 2005, Campellone *et al.*, 2004, Kalman *et al.*, 1999, Bommarius *et al.*, 2007, Swimm *et al.*, 2004). Other effectors hijack additional host cell signaling pathways and lead to membrane depolarization, ionic imbalance, reduced nutrient and water uptake, tight-junction disruption, dysregulated paracellular trafficking, and pedestal formation that collectively contribute to the observed morbidity and mortality associated with EPEC and EHEC infections (Croxen & Finlay, 2010, Kaper et al., 2004, Deng et al., 2010).

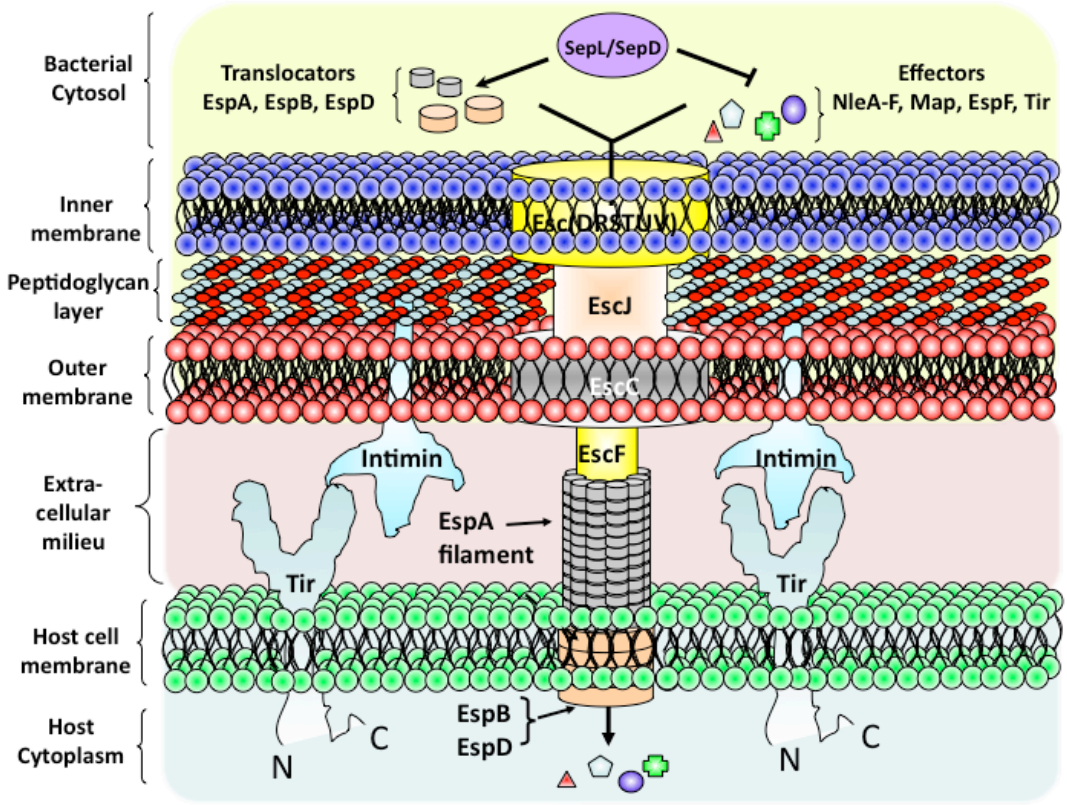


Figure 1.3 – Supramolecular structure of the type III secretion system (T3SS) – *LEE1*, *LEE2*, *LEE3* operons and *escD* encode for the structural components of the type three-secretion system (T3SS) that span the inner and outer membrane of the bacterium as well as the peptidoglycan layer. The *LEE4* operon specifies the extracellular translocators of the T3SS including EscF, EspA, EspB and EspD. The gatekeeper SepL/SepD facilitates the export of translocators, while repressing the effectors, in response to high calcium concentrations present in the extracellular milieu. EscF forms the protruding needle tip of the T3SS that serves as a nucleating site for EspA. EspA monomers polymerize to form a hollow filamentous tube that connects the EscF needle tip to the host cell membrane. Subsequently, EspB and EspD are translocated through this conduit and integrated into the host cell membrane to form a pore thereby conjugating the bacterial cytosol to that of the host. After assembly and anchorage, the bacterium perceives the reduced calcium deplete cytoplasm of the host cell and SepL/SepD is inactivated. Consequently, translocator export is inhibited and effectors (such as Tir, EspF, Map and other non-LEE encoded [Nle A-F] effectors) are trafficked into the host cell. Tir is integrated into the host cell membrane and interacts with its ligand, the adhesin intimin, located on the outer membrane of the bacterium to mediate pedestal formation.

Core transcriptional architecture of the LEE

Like most horizontally acquired genomic islands, the LEE of EPEC and EHEC possesses a low GC (or high AT) content of ~38.36 % and ~39.59 % respectively (Elliott *et al.*, 1999), which is significantly lower than the average GC content of ~50.8 % observed for the ancestral *E. coli* chromosomal backbone (Blattner *et al.*, 1997). Due to the high AT content, the LEE is subject to xenogenic silencing by global regulatory factors that recognize and bind AT rich genetic elements such as the heat stable nucleoid structuring protein, H-NS (Mellies et al., 2007a, Bhatt et al., 2011, Umanski *et al.*, 2002) (Figure 1.2). In its functional form H-NS exists as a homo- or heteromeric protein, each subunit of which folds into distinct N-terminal and C-terminal domains that are connected by a flexible linker region (Dillon & Dorman, 2010, Dorman, 2004). The N-terminal domain is involved in oligomerization whereas the C-terminal domain possesses the capacity to recognize and bind to nucleic acids (Dorman, 2004, Dillon & Dorman, 2010). H-NS does not have an obvious consensus binding motif; rather, it exhibits promiscuous sequence specificity with a preference for AT rich sequences that possess an inherent curvature (Dorman, 2004, Dillon & Dorman, 2010). H-NS represses transcription by looping and bridging of DNA, preventing isomerization of the RNA Polymerase (RNAP)-promoter closed complex to an open complex conformation, and by trapping the RNAP in the open complex (Fang & Rimsky, 2008, Dorman, 2004). With regards to the LEE, H-NS binds to *cis*-regulatory sequences of multiple LEE-encoded transcription units and represses transcription (Mellies et al., 2007a, Haack *et al.*, 2003, Bustamante *et al.*, 2001, Barba *et al.*, 2005) (Figure 1.2). Thus, the benefit conferred onto EPEC and EHEC, by the LEE, is only evident when the H-NS-mediated silencing is

alleviated. This is accomplished in response to a plethora of environmental cues such as physiological pH, temperature, osmolarity, pheromones (such as AI-3 and indole that are synthesized by gastrointestinal bacteria), growth phase, ferric nitrate ($Fe(NO_3)_3$), calcium (Ca^{2+}), magnesium (Mg^{2+}), manganese (Mn^{2+}), bicarbonate (HCO_3^-), and ammonium chloride (NH_4Cl), amongst others (Mellies et al., 1999, Abe *et al.*, 2002, Kenny *et al.*, 1997a, Sircili *et al.*, 2004, Sperandio et al., 1999, Sperandio, 2002, Sperandio et al., 1998, Sperandio *et al.*, 2002, Umanski et al., 2002, Elliott et al., 2000). These molecular cues are perceived by an elaborate network of sensory, signal transduction, and regulatory factors that integrate them to gene expression from the LEE (Elliott et al., 2000, Gomez-Duarte, 1995, Hansen & Kaper, 2009, Kaper et al., 2004, Kaper & Sperandio, 2005, Lodato & Kaper, 2009, Mellies et al., 1999, O'Connell et al., 2004, Sperandio, 2002, Sperandio et al., 1999, Sperandio et al., 2002, Shin *et al.*, 2001, Clarke *et al.*, 2006, Edrington *et al.*, 2009, Hughes *et al.*, 2009, Reading *et al.*, 2007, Russell *et al.*, 2007, Sharp & Sperandio, 2007, Sperandio et al., 2001).

Currently, over 40 regulators of the LEE have been identified, with the majority of them affecting transcription (Mellies et al., 2007a, Bhatt et al., 2011). Most of the extracellular and intracellular factors exert their effects by selectively counteracting the H-NS-mediated repression from the *LEE1* operon (Kenny et al., 1997a, Bhatt et al., 2011, Mellies et al., 2007a, Friedberg *et al.*, 1999, Umanski et al., 2002). As an example, when the ambient temperature is ~27˚C, H-NS represses transcription from the *LEE1*, *LEE2*, *LEE3*, *LEE4*, *LEE5*, *grlRA*, *grlA*, and the *espG* transcription units by forming extended nucleoprotein filaments (Mellies et al., 2007a, Friedberg et al., 1999, Umanski et al., 2002). The H-NS protomers in the ternary complex interact with each other to generate

loops in the DNA that give rise to a heterochromatin-like, condensed, and transcriptionally inactive protein-DNA ternary complex (Dame *et al.*, 2005, Dame *et al.*, 2002, Dame *et al.*, 2000, Dorman, 2004, Dillon & Dorman, 2010). As the temperature is elevated to 37˚C – the physiological body temperature of mammals – the contact between the H-NS subunits is disrupted at the *LEE1* operon and this destabilizes the nucleoprotein complex culminating with derepression of *LEE1* (Dorman, 2004, Mellies et al., 2007a, Friedberg et al., 1999, Umanski et al., 2002). Interestingly, H-NS continues to repress the other LEE operons (Umanski et al., 2002). The first gene of the *LEE1* operon encodes for the LEE-encoded master regulator Ler, which, in turn, activates transcription from the *grlRA*, *grlA*, *LEE2*, *LEE3*, *LEE4*, *LEE5*, and the *espG* transcription units in a synchronized temporal manner to promote biogenesis of pedestals (Elliott et al., 2000, Mellies et al., 1999, Mellies et al., 2007a, Deng et al., 2004, Barba et al., 2005, Deng et al., 2005, Sperandio et al., 2000) (Figure 1.2). The *grlRA* operon encodes for a transcriptional repressor, GrlR (global regulator of LEE repressor), and an associated transcriptional activator, GrlA (global regulator of LEE activator) of the LEE (Deng et al., 2004). GrlA is a DNA-binding protein that further amplifies the transcription of *ler* by directly binding upstream of the *LEE1* operon (Huang & Syu, 2008, Barba et al., 2005) (Figure 2A). By contrast, GrlR functions as an antiactivator as it interacts with GrlA to form inactive heterodimers thereby limiting the unwarranted transcription of Ler (Jobichen *et al.*, 2007, Jimenez *et al.*, 2010) (Figure 1.2). Thus, Ler and GrlA are components of a positive feedback loop whereas Ler and GrlR constitute a negative feedback loop that control the kinetics and expressivity of the genes governing pedestal formation (Deng et al., 2004, Elliott et al., 2000). Transcription from the other LEE-

encoded operons results in the synthesis and the ensuing biogenesis of a T3SS that connects the bacterium to the host cytosol (Mellies et al., 2007a, Bhatt et al., 2011). Subsequently, effectors are injected into the host cytoplasm that lead to pedestal formation (Kenny et al., 1997b, Croxen & Finlay, 2010).

Mechanism of action of Ler

Ler is an H-NS paralogue that exhibits a high degree of homology to the C-terminal DNA binding domain of H-NS (Sperandio et al., 2000). Genetic, bioinformatic, and biochemical evidence suggest that Ler functions as an antirepressor by competing with H-NS for overlapping binding sites and disrupting the H-NS-LEE nucleoprotein complex to derepress transcription from the other LEE-encoded operons (Mellies et al., 2007a, Sperandio et al., 2000, Haack et al., 2003, Bustamante et al., 2001, Barba et al., 2005, Sanchez-SanMartin et al., 2001). Remarkably, Ler also activates the LEE in an *hns*-independent manner as inactivation of *ler* leads to a further reduction in gene expression from the LEE in an *hns* mutant (Laaberki *et al.*, 2006). StpA, another paralogue of H-NS, has recently been shown to repress the LEE (Tauschek et al., 2010). Moreover, in an *hns stpA* double mutant, the LEE is constitutively expressed (Tauschek et al., 2010). Therefore, besides H-NS, Ler might derepress the LEE by counteracting the StpA-dependent silencing of the LEE.

Ler-mediated activation of the other LEE operons is a classic example of a single input regulatory module (SIM) whereby a single regulator synchronizes and coregulates the expression of a defined set of genes, which are involved in the same physiological process (Alon, 2006, Alon, 2007). In such circuits the ordered pattern of activation from

the different promoters correlates with the functional order in which the gene products are required (Alon, 2006, Alon, 2007). For instance, it has been demonstrated that Ler first activates transcription of the structural components of the T3SS and only after the assembly of the translocon are the LEE-encoded effectors, which are trafficked through the assembled T3SS, synthesized (Wang et al., 2008). Such a regulatory architecture ensures that gene products are synthesized at appropriate times and minimizes the unnecessary accumulation of a product at a time point when it is not necessary (Alon, 2006, Alon, 2007). Such a regulatory motif also explains the observation that increasing the temperature does not alleviate the H-NS mediated repression from all the LEE-encoded operons, as such an effect would lead to discordant expression from all the transcription units of the LEE and likely be deleterious to bacterial fitness.

Investigations into the regulation of the LEE have primarily focused on transcription factors, and regulators that target transcripts or proteins have remained largely ignored for the better part of the last two decades (Bhatt et al., 2011, Mellies et al., 2007a). However, increasing evidence suggests that posttranscriptional and posttranslational regulatory systems are key elements that refine transcriptional output, and mediate a more rapid adaptive response than is possible by transcriptional regulation alone (see below and refer to chapter 4). Consequently, elucidating the posttranscriptional and posttranslational motifs that converge on the LEE is critical for developing a complete model of the regulatory plasticity of the LEE.

Posttranscriptional and posttranslational regulation of the LEE

There are several reasons why posttranscriptional and posttranslational controls may

have evolved to complement transcriptional control. First, the operon organization of bacterial genes limits the capacity of transcription factors to differentially modulate genes within the same transcription unit. For instance, Ler activates the transcription of all the genes encoded within *LEE2, LEE3*, *LEE5* and *LEE4* operons without selectively affecting the expression of genes within individual transcription units (Elliott et al., 2000, Mellies et al., 2007a). Moreover, such a genetic organization is particularly constraining when gene products required in different stoichiometric ratios are encoded on the same transcript. For instance, the *LEE4*-encoded polycistronic transcript encodes a regulator (SepL), the structural components of a mature T3SS (EspA, EspD, EspB, and EscF), chaperones (CesD2 and L0017), and an effector (EspF), (Lodato & Kaper, 2009). While most of the genes in the transcript contribute to T3SS in general, they are required in different concentrations; the translocators EspA, EspB and EspD are made in excess relative to the regulator SepL (Lodato & Kaper, 2009). In EHEC, this is accomplished by posttranscriptional processing within the intracistronic segment of *sepL*, followed by the selective degradation of *sepL* and the concomitant stabilization of *espA*, *espD*, *espB* (Lodato & Kaper, 2009, Roe *et al.*, 2003).

Second, posttranscriptional and posttranslational mechanisms allow bacteria to control gene expression over a wide dynamic range, degrading some transcripts or proteins when they are no longer needed, or retaining others in abeyance for rapid mobilization at a later time (Waters & Storz, 2009). For instance, the RNA-binding protein CsrA represses translation from some transcripts by promoting their degradation (e.g. *pgaABCD*) (Wang *et al.*, 2005) or without affecting transcript stability (e.g. *hfq*) (Baker *et al.*, 2007). Likewise, the adaptor protein RssB can target the alternative

stationary phase sigma factor, RpoS, for degradation by the ClpXP protease or simply bind to and sequester it, consequently repressing the expression of RpoS-activated genes (Hengge, 2009). Thus, by affecting substrate activity, stability and/or abundance (Waters & Storz, 2009, Nogueira & Springer, 2000, Hengge, 2009, Hengge & Turgay, 2009, Deng et al., 2005, Wang et al., 2008), posttranscriptional and posttranslational mechanisms fine-tune gene expression in a way not easily accomplished by transcriptional controls alone.

Third, posttranscriptional and posttranslational controls of gene regulation provide a means to rapidly and globally adapt to diverse environmental stimuli. The use of global regulators of ancestral processes for this purpose allows bacterial pathogens to coordinate virulence with other physiological processes. Examples of such global regulatory factors include CsrA, Hfq, DsrA, and ClpXP, whose activities are described in detail below.

Lastly, posttranscriptional regulation is energetically efficient because small RNAs (sRNAs) governing this process, such as DsrA and CsrB, or small proteins such as CsrA and Hfq, which exert much of the posttranscriptional control, can be synthesized quickly and with less energy compared to the relatively larger transcription factors (Waters & Storz, 2009). In summary, posttranscriptional and posttranslational mechanisms provide a complement to transcriptional control for highly plastic regulatory responses to diverse environmental stimuli (Waters & Storz, 2009).

Carbon Storage Regulatory (Csr) system in Eubacteria

One such posttranscriptional regulatory system that coordinates a plethora of cellular responses in eubacteria is the carbon storage regulator, CsrA (Romeo *et al.*, 1993). *csrA*

(carbon storage regulator A) was originally isolated by Tony Romeo and colleagues in a transposon mutagenesis screen of *E. coli*, which was designed to identify *trans*-acting regulatory factors involved in glycogen biosynthesis (Romeo et al., 1993). One of the transposon insertion mutants, TR1-5, hyperproduced glycogen. Consistent with this observation, the expression of key genes involved in glycogen biosynthesis was upregulated. Strikingly, the mutant also displayed altered cell size and increased adherence to abiotic surfaces suggesting that *csrA* exerts pleiotropic effects (Romeo et al., 1993). In subsequent years, orthologs of CsrA were discovered in numerous pathogenic bacteria (Timmermans & Van Melderen, 2010, Lucchetti-Miganeh *et al.*, 2008). The first report to implicate *csrA* in pathogenesis was documented in the phytopathogen *Erwinia carotovora* where the *csrA* ortholog, *rsmA* (repressor of secondary metabolites), was isolated as a repressor of extracellular enzymes, acyl homoserine lactone-mediated quorum sensing, and virulence in the phytopathogen (Cui *et al.*, 1995).

The Csr/Rsm regulatory architecture has been best characterized in *E. coli* where the activity of CsrA is very stringently regulated. The CsrA/RsmA family of proteins function as homodimeric RNA-binding proteins in which each monomer is composed of 61 amino acids and is approximately 7 kDa in size (Rife *et al.*, 2005, Schubert *et al.*, 2007, Gutierrez *et al.*, 2005, Heeb *et al.*, 2006, Dubey *et al.*, 2003, Mercante *et al.*, 2006, Romeo et al., 1993). CsrA regulates gene expression posttranscriptionally by binding to AGGA/ANGGA motifs in leader segment or cistrons of transcripts and influencing their stability and/or translation (Baker et al., 2007, Baker *et al.*, 2002, Dubey et al., 2003, Liu & Romeo, 1997, Romeo, 1998, Sabnis *et al.*, 1995, Wang et al., 2005, Wei *et al.*, 2001, Yang *et al.*, 2010, Yakhnin *et al.*, 2011). Besides CsrA, this regulatory circuit also

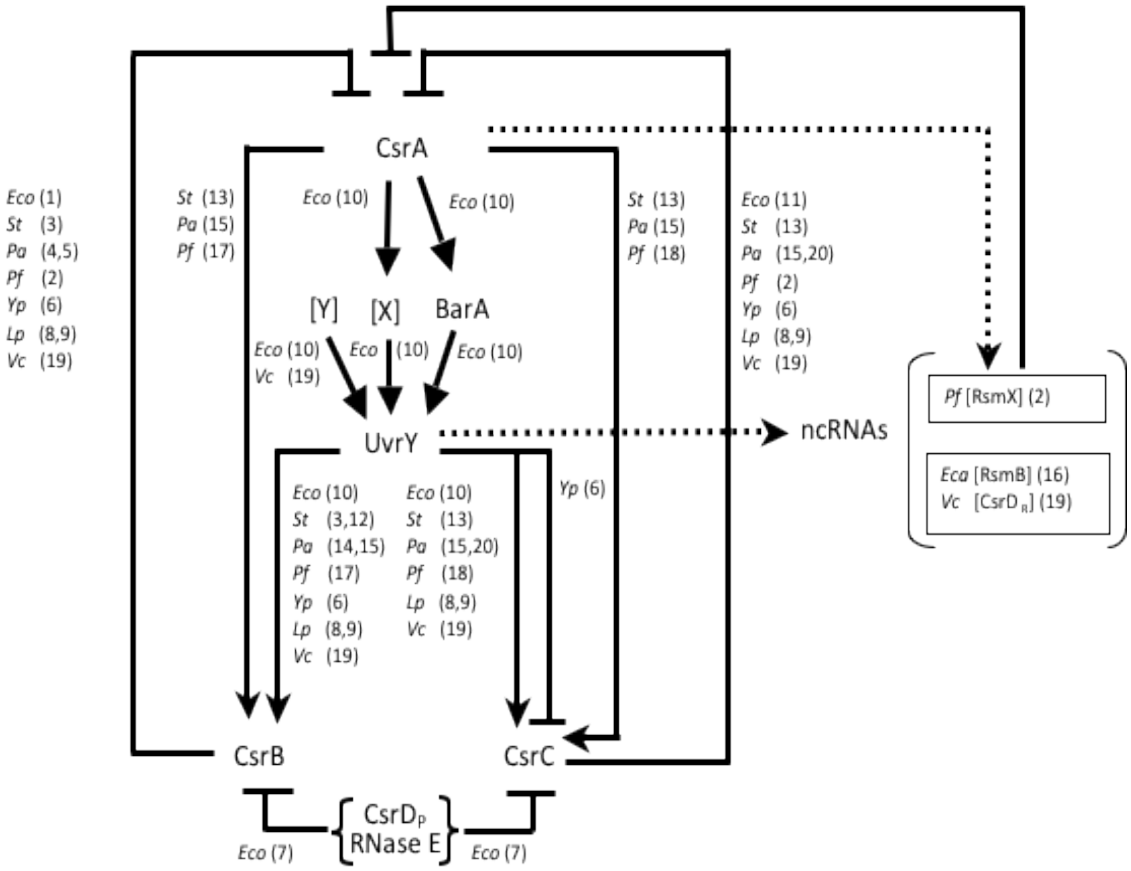
includes the two-component system (TCS) BarA-UvrY (Suzuki *et al.*, 2002), and the untranslated regulatory sRNAs, CsrB (Liu *et al.*, 1997), and CsrC (Weilbacher *et al.*, 2003). BarA is the membrane-associated histidine sensor kinase. It belongs to the family of unorthodox tripartite sensor kinases whereby multiple phosphorelay events occur within the sensor kinase prior to the transfer of the phosphoryl moiety to the cognate response regulator (Ishige *et al.*, 1994). UvrY, the cognate response regulator of BarA, belongs to the LuxR/UhpA family of transcriptional factors and is localized to the bacterial cytoplasm (Pernestig *et al.*, 2001). Formate, acetate and propionate activate the BarA-UvrY signal transduction system; however, the direct biochemical interaction between these ligands and BarA remains to be established (Chavez *et al.*, 2010). *csrB* encodes for a 369 nucleotide transcript (Romeo, 1998), whereas the length of the CsrC sRNA is 245 nucleotides (Wassarman *et al.*, 2001, Weilbacher et al., 2003). CsrB and CsrC possess multiple imperfect CsrA binding sites, characterized by the signature sequence ANGGA/AGGA in single-stranded segments, that bind to and titrate out multiple CsrA molecules (Weilbacher et al., 2003, Babitzke & Romeo, 2007, Liu et al., 1997, Dubey *et al.*, 2005).

In response to unidentified environmental cues, CsrA activates the transcription of the sRNA CsrB and CsrC, indirectly, through the response regulator UvrY (Suzuki et al., 2002); consequently, CsrA participates in a negative feedback loop to modulate its own activity. *csrA* does not influence the expression, but rather appears to affect the activity of UvrY (Suzuki et al., 2002). This effect is mediated through two mutually exclusive pathways with one dependent on the expression of the UvrY cognate sensor kinase, BarA, and the other independent of BarA (Suzuki et al., 2002) (Figure 1.4).

Phosphorylation of UvrY activates the protein and it binds to sites in the promoter regions of CsrB and CsrC to activate their transcription (Weilbacher et al., 2003). CsrB possesses approximately 18-20 putative CsrA binding sites and sequesters ~9-10 CsrA dimers (Babitzke & Romeo, 2007, Liu et al., 1997), whereas CsrC contains approximately 9-10 recognizable sites for CsrA and titrates out ~4-5 CsrA dimers (Weilbacher et al., 2003, Babitzke & Romeo, 2007). Consequently, CsrB and CsrC bind to and sequester CsrA into globular ribonucleoprotein complexes, thereby preventing CsrA from regulating the transcripts in its regulon (Weilbacher et al., 2003, Liu et al., 1997, Babitzke & Romeo, 2007) (Babitzke & Romeo, 2007). This core Csr/Rsm regulatory architecture incorporating *csrA-barA-uvrY-csrB-csrC* appears to be broadly conserved in other gammaproteobacteria including *Salmonella enterica* serovar Typhimurium (Altier et al., 2000, Fortune *et al.*, 2006, Teplitski *et al.*, 2003), *Pseudomonas aeruginosa* (Kay *et al.*, 2006, Brencic & Lory, 2009), *Erwinia carotovora* (Liu *et al.*, 1998, Heeb & Haas, 2001, Cui *et al.*, 2001), *Vibrio cholerae* (Lenz et al., 2005) and *Legionella pneumophila* (Edwards *et al.*, 2010, Sahr *et al.*, 2009) (Table 1.1 and Figure 1.4).

An additional component of the Csr system that has so far been exclusively characterized in non-pathogenic *E. coli* includes the specificity factor protein CsrD (Suzuki *et al.*, 2006). CsrD targets CsrB and CsrC for endonucleolytic cleavage by the single-strand specific endoribonuclease, RNase E (Suzuki et al., 2006); consequently promoting the activity of CsrA and influencing the expression of genes in the CsrA regulon. In striking contrast to CsrA, which displays a broad phylogenetic distribution with orthologues conserved in the families Enterobacteriaceae, Vibrionaceae, and

Pseudomonadaceae, conserved CsrD orthologues appear to be phylogenetically restricted to *E. coli* and closely related bacterial species (Suzuki et al., 2006). Thus, it is likely that CsrD functions as a local regulator in species and genera closely related to *E. coli*, whereas in distantly related bacteria the CsrB/RsmY and CsrC/RsmZ decay occurs through heterologous regulatory systems employing similar or distinct mechanisms.


CsrA
[Y]
[X]
BarA
UvrY
ncRNAs
CsrB
CsrC
CsrD
RNase E
Eco (1)
St (3)
Pa (4,5)
Pf (2)
Yp (6)
Lp (8,9)
Vc (19)
St (13)
Pa (15)
Pf (17)
Eco (10)
Eco (10)
Eco (10)
Vc (19)
Eco (10)
Eco (10)
Eco (10)
St (3,12)
Pa (14,15)
Pf (17)
Yp (6)
Lp (8,9)
Vc (19)
Eco (10)
St (13)
Pa (15,20)
Pf (18)
Lp (8,9)
Vc (19)
Yp (6)
St (13)
Pa (15)
Pf (18)
Eco (11)
St (13)
Pa (15,20)
Pf (2)
Yp (6)
Lp (8,9)
Vc (19)
Pf [RsmX] (2)
Eca [RsmB] (16)
Vc [CsrD R] (19)
Eco (7)
Eco (7)

Bacterium	Csr family	TCS SK/RR*	ncRNA	Reference(s)
Escherichia coli	CsrA	BarA/UvrY	CsrB, CsrC	(Suzuki et al., 2002, Liu et al., 1997, Weilbacher et al., 2003, Romeo et al., 1993)
EPEC	CsrA	BarA/UvrY	CsrB, CsrC	(Bhatt *et al.*, 2009) unpub data
EHEC	CsrA	BarA/UvrY	CsrB, CsrC	(Bhatt et al., 2009) unpub data
Salmonella Typhimurium	CsrA	BarA/SirA	CsrB, CsrC	(Fortune et al., 2006, Altier et al., 2000, Teplitski et al., 2003)
Pseudomonas aeruginosa	RsmA	GacS/GacA	RsmY, RsmZ	(Brencic & Lory, 2009, Brencic *et al.*, 2009, Heurlier *et al.*, 2004, Kay et al., 2006, Sonnleitner *et al.*, 2006, Sorger-Domenigg *et al.*, 2007, Pessi *et al.*, 2001)
Pseudomonas fluorescens	RsmA RsmE**	GacS/GacA	RsmX, RsmY, RsmZ	(Heeb & Haas, 2001, Reimmann *et al.*, 2005, Valverde *et al.*, 2003, Blumer *et al.*, 1999)
Erwinia carotovora subsp. carotovora	RsmA	ExpS/ExpA	RsmB	(Cui et al., 1995, Liu et al., 1998)
Vibrio cholerae	CsrA	VarS/VarA	CsrB, CsrC, CsrD	(Lenz *et al.*, 2005)
Legionella pneumophila	CsrA**	LetS/LetA	RsmY, RsmZ	(Sahr et al., 2009, Rasis & Segal, 2009, Cazalet *et al.*, 2004)
Yersinia pseudotuberculosis	CsrA	BarA/UvrY	CsrB, CsrC	(Heroven *et al.*, 2008)

* - SK – Sensor histidine kinase; RR – Response regulator; ** - In *Pseudomonas fluorescens* RsmE is the RsmA/CsrA paralog (Reimmann et al., 2005) whereas in *Legionella pneumophila* multiple CsrA homologs have been identified (Cazalet et al., 2004).

Figure 1.4 & Table 1.1 –

The Csr regulatory architecture in gammaproteobacteria – In *E. coli* the RNA-binding protein CsrA activates the transcription of the non-coding RNAs, CsrB and CsrC, by affecting the activity, but not the expression, of UvrY. This effect is both dependent and independent [**X**] of the UvrY-cognate sensor kinase, BarA. The CsrA and BarA-UvrY mediated transcriptional activation of *csrB* and *csrC* has been observed in other bacteria [(**Figure 4)** and refer to **Table 1** for a list of the orthologues of the Csr regulatory system in other eubacteria]. However, in other gammaproteobacteria it remains to be determined whether CsrA and BarA/UvrY-dependent activation of *csrB* and *csrC* is part of the same regulatory pathway. In both *E. coli*, and other gammaproteobacteria, there is evidence that BarA-independent factors also influence the expression and/or activity the UvrY family of response regulators and consequently affect the transcription of the ncRNAs CsrB and CsrC. These ncRNAs contain multiple CsrA binding sites and act by sequestering multiple CsrA homodimers into globular ribonulceoprotein complexes thereby inhibiting CsrA activity, thus antagonizing the CsrA-mediated effect on the genes in its regulon. The role of CsrD in the Csr/Rsm circuitry has only been addressed in *E. coli*, where it destabilizes CsrB and CsrC by targeting it for degradation by the single-strand specific endoribonuclease RNase E. In summary, the Csr (with or without $CsrD_P$) circuitry is a "buffering" or "homeostatic" circuit where the activity of CsrA is stringently regulated by periodic fluctuations in the concentrations of the sRNAs (CsrB, CsrC and other ncRNAs) and the specificity factor CsrD. Abbreviations are as such - *Eco – Escherichia coli*, *St - Salmonella typhimurium*, *Pa - Pseudomonas aeruginosa*, *Pf - Pseudomonas fluorescens*, *Yp - Yersinia pseudotuberculosis*, *Lp - Legionella pneumophila*, *Vc - Vibrio cholerae*. Lines with arrowheads indicate activating pathways whereas blunt-ended lines indicate repressive pathways.

Structural analysis of CsrA

A major breakthrough in understanding the molecular basis of CsrA/RsmA-mediated RNA recognition arose from the elucidation of the three-dimensional structure of the functional form of the protein (Rife et al., 2005, Schubert et al., 2007, Gutierrez et al., 2005, Heeb et al., 2006, Dubey et al., 2003, Mercante et al., 2006) (Figure 1.5). The bioactive form of CsrA is composed of two identical, interdigitated monomers that form an entropically favorable hydrophobic core that consists of 10 β-strands and two winglike α-helices (Mercante et al., 2006). The CsrA dimer exhibits a barrel-like topology that is also enthalpically stabilized by an elaborate arrangement of hydrogen bonds between the backbone amino and carbonyl groups (Heeb et al., 2006, Mercante et al., 2006). Thus, the

typical thermodynamic parameters, increased entropy and reduced enthalpy that facilitate protein folding in general, also contribute to the conformational topology of CsrA.

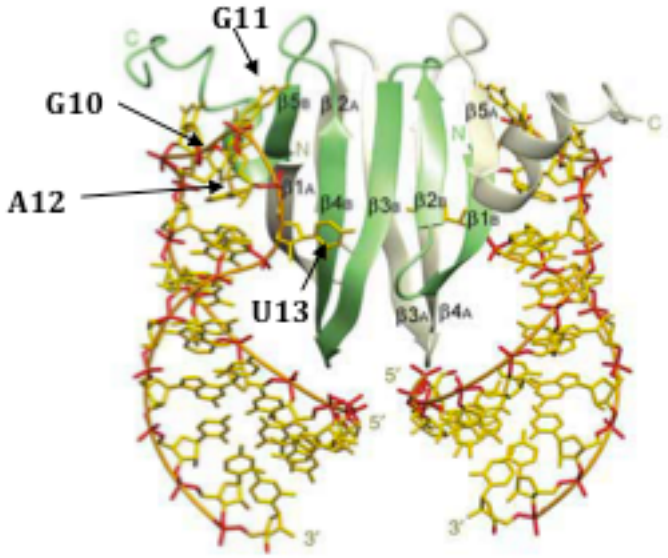


Figure 1.5 – NMR solution structure of CsrA/RsmA/RsmE in complex with two *hcnA* molecules – The functional form of CsrA consists of interdigitated homodimers. Two RNA recognition sites are located on the opposite faces of the molecule. The β5 strand and the α helix located downstream of it, from one subunit, align parallel to the β1 strand, from the other subunit, to form each RNA recognition site. Transcripts that bind to CsrA contain the invariant trinucleotide sequence GGA, which is usually located in the single stranded segments such as the loops of hairpin structures. Picture adapted and modified with permission (Schubert et al., 2007).

The secondary structure of each CsrA subunit consists of 5 β-strands and a C-terminal α-helix (Mercante et al., 2006). Comprehensive alanine-scanning mutagenesis identified two sub-domains, region 1 and region 2, that are critical for RNA-binding and the ensuing activity of CsrA (Mercante et al., 2006). Region 1 corresponds to the β1 strand (residues 2-7) that is located at the extreme N-terminus of CsrA, whereas region 2

includes the β5 strand as well as the first two residues of the downstream α-helix (residues 40-47) and is positioned closer to the C-terminus of the polypeptide (Mercante et al., 2006). The higher order quaternary structure reveals that region 1 and region 2 of opposite monomers localize and are aligned parallel and contiguous with respect to each other to form an RNA-binding domain (Mercante et al., 2006, Schubert et al., 2007). Thus, a CsrA homodimer presents two RNA-contact domains with each of them located on the opposite surface of the functional molecule (Mercante et al., 2006). This topological organization addresses the previous experimental observation that the sRNA, CsrB, which possesses ~ 18 CsrA binding sites, titrates out ~ 9 CsrA molecules (Liu et al., 1997).

Physiological roles of CsrA amongst eubacteria

CsrA orthologs have been discovered in both Gram-negative and Gram-positive bacteria in which CsrA sits atop regulatory hierarchies and modulates an array of physiological processes (Wei et al., 2001, Bhatt et al., 2009, Altier et al., 2000, Brencic & Lory, 2009, Baker et al., 2007, Jackson *et al.*, 2002, Wang et al., 2005, Baker et al., 2002, Dubey et al., 2003, Romeo et al., 1993, Heeb & Haas, 2001, Heeb et al., 2006, Irie *et al.*, 2010, Pessi et al., 2001, Romeo, 1998, Yakhnin *et al.*, 2007, Lucchetti-Miganeh et al., 2008, Timmermans & Van Melderen, 2010, Cui et al., 1995, Goodman *et al.*, 2004, Lenz et al., 2005, Forsbach-Birk *et al.*, 2004, Kerrinnes *et al.*, 2009). For instance, in *E. coli* the role of CsrA has expanded to include, besides carbon homeostasis and biofilm formation (Romeo et al., 1993, Romeo, 1998, Sabnis et al., 1995, Baker et al., 2002, Dubey et al., 2003, Jackson et al., 2002, Wang et al., 2005), peptide uptake (Dubey et al.,

2003), motility (Wei et al., 2001, Yakhnin et al., 2007), synthesis of secondary metabolites such as cyclic di-GMP (Jonas *et al.*, 2008), colicin biosynthesis (Yang et al., 2010), and acetate metabolism (Wei *et al.*, 2000) amongst others.

Besides controlling ubiquitous ancestral processes, CsrA/RsmA also controls numerous, biologically unlinked, virulence-associated traits. For example, the opportunistic pathogen, *P. aeruginosa*, infects injured, burned, or immunocompromised patients (Hauser, 2009). The bacterium almost invariably colonizes the lungs of cystic fibrosis patients, where it exacerbates disease by resulting in acute or chronic respiratory infections (Hauser, 2009). The BarA and UvrY orthologs, GacS and GacA respectively, along with RsmA, play an indispensible role in reciprocally regulating the expression of virulence determinants involved in the chronic and acute phases of infection (Hauser, 2009, Laskowski *et al.*, 2004, Goodman et al., 2004, Yahr & Wolfgang, 2006). As is the case in *E. coli*, GacS activates its cognate response regulator, GacA, which in turn binds to the promoter element upstream of the sRNA genes *rsmY* and *rsmZ* and activates transcription (Brencic et al., 2009, Goodman et al., 2004, Heurlier et al., 2004). RsmY and RsmZ bind to and sequester multiple RsmA molecules, thereby inhibiting its activity (Kay et al., 2006). Environmental cues that favor entry into the acute phase of infection do so by modulating the sensor kinase, RetS, which when activated forms inactive heterodimers with GacS and disrupts the GacS-GacA signal transduction pathway, leading to the activation of RsmA (Goodman *et al.*, 2009). RsmA activates the T3SS, swarming motility, synthesis of rhamnolipds, lipases, *toxA*, and *lipA* – determinants that play important roles in bacterial colonization (Heurlier et al., 2004). Reciprocally, RsmA represses the expression of pyocyanin, hydrogen cyanide, and PA-IL lectin (Hauser,

2009, Pessi et al., 2001). RsmA also silences *psl*, involved in the biosynthesis of the exopolysacchride necessary for the formation of the biofilm matrix, and the type VI secretion system HSl-l (Irie et al., 2010, Brencic & Lory, 2009). The negatively regulated genes are critical for entry and persistence in the chronic phase of infection (Hauser, 2009). *toxA*, pyocyanin, and hydrogen cyanide are involved in the killing of *Caernorhabditis elegans*, whereas the T3SS plays an important role in the acute infectious phase of *P. aeruginosa* (Mahajan-Miklos, 1999, Tan, 1999b, Tan, 1999a, Gallagher L.A., 2001). Recent evidence suggest that chronic infections may actually select for isolates that exhibit reduced or abolished expression of the T3SS, implicating the selective role of this specialized secretion system in the acute but not chronic infectious phase. The observed RsmA-dependent effect likely occurs via the cAMP-dependent homologue of CRP, Vfr (Goodman et al., 2004), as *vfr* activates the T3SS and its transcript levels are dramatically reduced in the *rsmA* mutant (Burrowes *et al.*, 2006). The switch from the acute to chronic phase is promoted by the alternative sensor kinase, LadS, which activates the GacS-GacA pathway to promote transcription of the RsmA antagonists RsmY and RsmZ (Goodman et al., 2004, Ventre *et al.*, 2006). As a consequence, expression of the T3SS is abolished and determinants, which promote entry into the chronic phase, such as HSl-1 and exopolysaccharides are induced (Hauser, 2009). Therefore, RsmA is a critical homeostatic switch that enables *P. aeruginosa* to alternate between the acute and chronic phases of infections.

Similarly, CsrA regulates the Salmonella pathogenicity island 1 (SPI-1) in *Salmonella enterica* serovar Typhimurium (Altier et al., 2000, Lawhon et al., 2003). SPI-1, a 40 kb genomic segment located at centisome 63 (Behlau & Miller, 1993, Altier et al., 2000,

Lawhon et al., 2003), encodes for a T3SS that is required for the ability of *S.* Typhimurium to invade epithelial cells *in vitro* and for disease *in vivo* experimental models of infection (Altier et al., 2000, Lawhon et al., 2003). Inactivation of the *csrA* profoundly diminished the invasiveness of *S.* Typhimurium (Altier et al., 2000, Lawhon et al., 2003). Remarkably, overexpression of *csrA*, from a heterologous inducible promoter, genocopies the phenotype observed with the *csrA* mutan, suggesting that CsrA is capable of functioning as an activator or repressor of SPI-1 in a dose-dependent manner (Altier et al., 2000). Thus CsrA-mediated regulation of SPI-1 constitutes an incoherent feedforward regulatory loop whereby a regulatory factor activates a target gene through one pathway but represses it via another (Alon, 2006, Alon, 2007). The *csrA* mutant and overexpressor exhibit reduced expression of *invF*, *prgH*, and *sipC* that encode for the regulatory, structural, and secreted components of a T3SS respectively, phenotypically mimicking a mutation in the SPI-1 encoded master regulator *hilA* (Altier et al., 2000). Consistent with this observation, *hilA* expression is diminished in the *csrA* mutant and overexpressor (Altier et al., 2000). However, the effect of *csrA* on *hilA* is indirect and mediated through the transcriptional factors *hilC* and *hilD* (Altier et al., 2000). CsrA also coregulates accessory traits that exacerbate the virulence of S. Typhimurium (Lawhon et al., 2003). For instance, the *csrA* mutant exhibits reduced flagellar synthesis in *S.* Typhimurium and flagella is an important virulence factor, as aflagellate mutants of *S.* Typhimurium exhibit reduced virulence in an *in vivo* model of infection (Lawhon et al., 2003). Additionally, CsrA also regulates metabolic traits in *Salmonella* that may play crucial roles in acclimating the pathogen to the gastrointestinal tract (Lawhon et al., 2003). For example, CsrA regulates the *mal*, *pdu*, and *eut* operons

that are involved in metabolizing maltodextrins, propanediol, and ethanolamine respectively – nutrients that are ubiquitously present in the mammalian gastrointestinal tract (Lawhon et al., 2003). Thus, CsrA might confer an evolutionary advantage on *Salmonella* by coordinately controlling the T3SS, flagella and enhancing the potential of the bacterium to metabolize diverse and prevalent carbon sources within its pathoecological niche. CsrA also regulates virulence in *Shigella flexneri* (Gore & Payne, 2010), *Dickeya dadantii* (Yang *et al.*, 2008), *Erwinia carotovora* (Cui et al., 2001, Cui et al., 1995) and *Vibrio cholerae* (Lenz et al., 2005), amongst other pathogens (Barnard *et al.*, 2004, Forsbach-Birk et al., 2004, Heroven et al., 2008).

Rationale for the putative role of CsrA in the virulence of EPEC and EHEC

Multiple lines of evidence suggest a role for CsrA in the virulence of EPEC and EHEC –

1. The observation that CsrA/RsmA modulates the T3S-dependent virulence in an array of eubacterial pathogens related to EPEC and EHEC raises the possibility that CsrA might exert similar effects on the LEE-encoded T3SS of EPEC and EHEC.
2. The flagella and T3SS are architecturally conserved polymeric organelles that often perform opposing functions; consequently regulatory factors that govern one of these morphogenetic pathways often regulate the other. For instance, in A/E pathogens transcriptional (GrlR, GrlA, IHF) (Iyoda et al., 2006, Yona-Nadler *et al.*, 2003), posttranscriptional (Hfq) (Hansen & Kaper, 2009), and

posttranslational (ClpXP) (Iyoda & Watanabe, 2005) factors synchronize the expression of the flagella with that of the LEE-encoded T3SS. A similar role has been demonstrated for CsrA in *S.* Typhimurium (Altier et al., 2000, Lawhon et al., 2003) and *E. carotovora* subsp. carotovora (Chatterjee *et al.*, 2010, Cui et al., 1995). In benign *E. coli*, CsrA binds to the leader segment of the *flhDC* transcript, which encodes for the master transcriptional activator of the flagella, and stabilizes the transcript (Wei et al., 2001). The primary sequence of CsrA as well as the putative binding site in the untranslated leader segment of *flhDC* are conserved in EPEC and EHEC suggesting that the CsrA-mediated regulation of flagella is likely conserved in EPEC and EHEC (unpub. observations). Additionally, bioinformatic analysis reveal recognizable CsrA-binding sites in the leader segment of the LEE-encoded *grlRA* and *LEE4* operons that encode for the regulatory and structural components of the T3SS respectively (unpub. observations). Collectively, these observations suggest that CsrA might coregulate the T3SS and flagella in EPEC and EHEC.

3. Finally, colonization studies with EHEC suggest that the ability of the bacterium to switch between glycolytic and gluconeogenic carbon sources is critical for the *in vivo* pathogenicity of the bacterium (Miranda *et al.*, 2004). Consequently, regulatory or structural factors that regulate either or both processes might contribute to bacterial virulence. Consistent with this observation, glycolytic genes or media promote the expression of the T3SS, whereas gluconeogenic genes or media repress it (Gore & Payne, 2010, Kenny et al., 1997a). This effect is likely because the former process is an exergonic process that can be coupled to

the expression and assembly of the metabolically expensive T3SS whereas the latter is an endergonic process and depletes much of the energy currency of the cell. CsrA promotes glycolysis and inactivation of *csrA* inhibits the secretion of effectors in *Shigella flexneri* – an effect that is dependent on the glycolytic gene *pfkA* (Gore & Payne, 2010). Similarly, in benign *E. coli*, CsrA binds to the leader segment of the *glgCAP* transcript and destabilizes it (Baker et al., 2002), consequently inhibiting gluconeogenesis and glycogen biosynthesis (Romeo et al., 1993). These binding sites are conserved in EPEC and EHEC (unpub. observations). Cumulatively, these observations suggest that in EPEC and EHEC, CsrA likely regulates glycolysis and gluconeogenesis and consequently exerts an indirect effect on the T3S-dependent virulence of these pathogens.

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CHAPTER 2

The RNA Binding Protein CsrA is a Pleiotropic Regulator of the Locus of Enterocyte Effacement Pathogenicity Island of Enteropathogenic *Escherichia coli* †

Shantanu Bhatt[1,2], Adrianne Nehrling Edwards[2], Hang Thi Thu Nguyen[3], Didier Merlin[3], Tony Romeo[4], and Daniel Kalman[1,2]

[1] Department of Pathology and Laboratory Medicine, [2] Microbiology and Molecular Genetics Program, [3]Department of Medicine, Division of Digestive Diseases, Emory University School of Medicine, Atlanta, GA, USA 30322

[4] Department of Microbiology and Cell Science, P.O. Box 110700. University of Florida, Gainesville, FL 32611-0700 USA

† - This chapter consists of a manuscript that was published as –

Bhatt, S., A. N. Edwards, H. T. Nguyen, D. Merlin, T. Romeo & D. Kalman, (2009) The RNA binding protein CsrA is a pleiotropic regulator of the locus of enterocyte effacement pathogenicity island of enteropathogenic *Escherichia coli*. ***Infect Immun*** **77: 3552-3568.**

Adrianne Nehrling Edwards conducted the competitive RNA-Electrophoretic mobility shift assay in Figure 5D and Hang Thi Thu Nguyen performed the transepithelial resistance assay depicted in Figure 2.2. Shantanu Bhatt performed all other experiments. Shantanu Bhatt, Tony Romeo, and Daniel Kalman wrote the manuscript.

Abstract

The attaching and effacing (A/E) pathogen enteropathogenic *Escherichia coli* (EPEC) forms characteristic actin-filled membraneous protrusions upon infection of host cells termed pedestals. Here we examine the role of the RNA-binding protein, CsrA, in the expression of virulence genes and proteins that are necessary for pedestal formation. The *csrA* mutant was defective in forming actin pedestals on epithelial cells and in disrupting Transepithelial Resistance (TER) across polarized epithelial cells. Consistent with reduced pedestal formation, secretion of the translocators EspA, EspB, EspD and the effector Tir were substantially reduced in the *csrA* mutant. Purified CsrA specifically bound to the *sepLespADB* mRNA leader, and the corresponding transcript levels were reduced in the *csrA* mutant. In contrast, Tir synthesis was unaffected in the *csrA* mutant. Reduced secretion of Tir appeared to be in part due to decreased synthesis of EscD, an inner membrane architectural protein of the type three secretion system (TTSS) and EscF, a protein that forms the protruding needle complex of the TTSS. These effects were not mediated through the locus of enterocyte effacement (LEE) transcriptional regulators GrlA or Ler. In contrast to the *csrA* mutant, multicopy expression of *csrA* repressed transcription from *LEE1*, *grlRA*, *LEE2*, *LEE5*, *escD* and *LEE4,* an effect mediated by GrlA and Ler. Consistent with its role in other organisms, CsrA also regulated flagellar motility and glycogen levels. Our findings suggest that CsrA governs virulence factor expression in an A/E pathogen by regulating mRNAs encoding translocators, effectors or transcription factors.

Introduction

Enteropathogenic *Escherichia coli* (EPEC) is a major etiologic agent of infantile diarrhea in developing countries, causing the death of several hundred thousand children per year (Nataro & Kaper, 1998, Chen & Frankel, 2005). EPEC and the related enterohemorrhagic *E. coli* (EHEC) cause attaching and effacing (A/E) lesions that are characterized by the disruption of the intestinal microvilli and reorganization of the cytoskeleton in infected cells to form actin-filled membrane protrusions, termed "pedestals," emanating beneath bacteria attached to the cell surface (Moon *et al.*, 1983, Knutton *et al.*, 1987a, Knutton *et al.*, 1987b).

The LEE of EPEC is a pathogenicity island that is necessary and sufficient for the formation of pedestals (McDaniel & Kaper, 1997). It consists of five major polycistronic operons including *LEE1*, *LEE2*, *LEE3*, *LEE5* and *LEE4*, a bicistronic operon *grlRA* and several monocistronic genes (Elliott *et al.*, 2000, Mellies *et al.*, 1999, Deng *et al.*, 2004). The *LEE1*, *LEE2* and *LEE3* operons encode for the architectural components of a TTSS whereas *LEE4* encodes for the translocator exporter SepL, the secreted translocators EspA, EspB, EspD, the chaperones CesD2, and L0017, the needle complex forming component of the TTSS, EscF and the effector protein EspF. SepL, along with SepD, forms a molecular switch that coordinates the hierarchical secretion of EspA, EspB and EspD over effectors in response to calcium and other environmental signals (Deng *et al.*, 2005, Donnenberg *et al.*, 1993). EspA is secreted to form a hollow filamentous organelle that connects the protruding EscF needle of the bacterial TTSS to the host cell membrane (Knutton *et al.*, 1998, Daniell *et al.*, 2001, Sekiya *et al.*, 2001). EspB and EspD are translocated through this filamentous TTSS and integrated into the host cell membrane

where they form a pore that allows effector molecules to be injected directly into the host cytosol (Wachter *et al.*, 1999, Ide *et al.*, 2001). The *LEE5* operon encodes for the effector Tir, its ligand, the adhesin intimin and the Tir chaperone CesT (Mellies et al., 1999). Tir is translocated into the host cytosol via the TTSS and subsequently integrated into the host cell membrane, where it serves as a receptor for intimin, which is present on the outer bacterial membrane (Kenny, 1999). The interaction of Tir and Intimin results in firm attachment of the bacterium to the infected cell. Tir recruits cellular factors such as tyrosine kinases, Nck, and N-WASP, that activate the Arp2/3 complex and initiate actin polymerization beneath the attached bacteria (Kalman *et al.*, 1999, Gruenheid *et al.*, 2001, Swimm *et al.*, 2004, Bommarius *et al.*, 2007) culminating with the formation of pedestals.

Coordinated spatiotemporal expression from the LEE is critical for pedestal formation by EPEC. Such a mode of regulation is achieved by the presence of a plethora of transcription factors such as Ler, PerC, GrlA, GrlR, QseA, IHF, Fis, and H-NS (Mellies et al., 1999, Deng et al., 2004, Sharp & Sperandio, 2007, Friedberg *et al.*, 1999, Goldberg *et al.*, 2001, Umanski *et al.*, 2002) in response to diverse environmental conditions including pH, osmolarity, $Fe(NO_3)_3$, Ca^{2+}, temperature, quorum sensing, and HCO^-_3 (Sperandio *et al.*, 2001, Abe *et al.*, 2002, Kenny *et al.*, 1997, Umanski et al., 2002, Mellies et al., 1999, Sperandio, 2002, Sperandio *et al.*, 2002, Sperandio *et al.*, 2003, Sircili *et al.*, 2004). Most of these transcription factors affect the expression from the LEE by activating the transcription of *ler*, which in turn activates transcription from *grlRA*, *LEE2*, *LEE3*, *LEE5*, *escD* and *LEE4* (Figure 2.9) (Mellies et al., 1999, Bustamante

et al., 2001, Elliott et al., 2000, Haack *et al.*, 2003, Deng et al., 2004, Barba *et al.*, 2005, Mellies *et al.*, 2007).

Whereas our understanding of the mechanisms of transcriptional regulation of the LEE is extensive, information about posttranscriptional and posttranslational regulation is more limited. Posttranscriptional control has been suggested in the negative regulation of *espADB* mRNA in EHEC strains that secrete high levels of EspA. However, the mechanistic basis for this phenomenon has not been established (Roe *et al.*, 2003). In terms of posttranscriptional and posttranslational regulation, the detailed molecular mechanisms for only the endoribonuclease RNase E (Lodato & Kaper, 2009) and the protease, ClpXP, have been elucidated (Iyoda & Watanabe, 2005). Whereas in EHEC RNase E generates the *sepL* and *espADB* transcripts by splicing at the C-terminal end of *sepL* in the precursor *sepLespADB* transcript (Lodato & Kaper, 2009), the protease ClpXP positively regulates the LEE posttranslationally by affecting the expression of RpoS and GrlR (Iyoda & Watanabe, 2005). The ribosome binding GTPase, BipA, and the non-coding RNA, DsrA, have also been shown to upregulate the expression of the LEE in EPEC and EHEC respectively, although the detailed molecular mechanisms involved in the regulation have yet to be elucidated (Grant *et al.*, 2003, Laaberki *et al.*, 2006).

We have identified *csrA* as a posttranscriptional regulator of EPEC necessary for paralyzing and killing the nematode *Caenorhabditis elegans* (S. Bhatt and D. Kalman, manuscript in preparation). Previous studies have demonstrated that genes involved in nematode pathogenesis may also facilitate pedestal formation on mammalian cells (Anyanful *et al.*, 2005, Mellies *et al.*, 2006). This latter observation prompted us to investigate the possible role of *csrA* in mammalian pathogenesis.

CsrA and its orthologue RsmA are homodimeric RNA binding proteins (Liu & Romeo, 1997, Gutierrez *et al.*, 2005, Rife *et al.*, 2005, Heeb *et al.*, 2006, Babitzke & Romeo, 2007) that regulate gene expression posttranscriptionally by binding to sites containing the AGGA/ANGGA motif in the leader segment of transcripts and altering their stability and/or translation (Liu & Romeo, 1997, Baker *et al.*, 2002, Wang *et al.*, 2005, Wei *et al.*, 2001, Baker *et al.*, 2007, Dubey *et al.*, 2005, Lucchetti-Miganeh *et al.*, 2008). Transcripts such as *flhDC*, whose expression is activated by CsrA, have putative CsrA binding site(s) located distantly from the Shine-Dalgarno sequence. Binding of CsrA enhances their stability and leads to an increase in the steady-state levels of such transcripts (Romeo, 1998, Wei et al., 2001). For transcripts that are negatively regulated, CsrA binds to sequences that overlap or lie in close proximity to the Shine-Dalgarno sequence, thereby preventing the 30S ribosomal subunit from binding to the transcript and inhibiting translation and/or facilitating mRNA decay (Baker et al., 2007, Dubey *et al.*, 2003, Baker et al., 2002, Wang et al., 2005).

The Carbon storage regulation (Csr) system also includes the non-coding sRNAs, CsrB and CsrC which bind tightly to and sequester 9-10 and 4-5 CsrA dimers respectively, thereby preventing CsrA from exerting its effect on the mRNAs in its regulon (Weilbacher *et al.*, 2003, Liu *et al.*, 1997). CsrA activates the expression of CsrB and CsrC through the BarA-UvrY two-component regulatory system (Suzuki *et al.*, 2002, Weilbacher et al., 2003, Pernestig *et al.*, 2003). The last component of the Csr system is CsrD, a protein containing vestigial GGDEF and EAL domains, that targets CsrB and CsrC for degradation by the endoribonuclease RNase E thereby increasing intracellular CsrA activity (Suzuki *et al.*, 2006). These negative feedback loops of the Csr system

demonstrate that CsrA activity is finely tuned and suggest that the Csr system functions as a homeostatic circuit (Suzuki et al., 2002, Suzuki et al., 2006). The role of CsrA and RsmA in regulating virulence factors and host interactions of mammalian and plant pathogens is well established in *Salmonella enterica* serovar Typhimurium, *Erwinia carotovora*, *Pseudomonas aeruginosa*, *Helicobacter pylori*, *Legionella pneumophila* and *Yersinia pseudotuberculosis* (Heroven *et al.*, 2008, Altier *et al.*, 2000, Lawhon *et al.*, 2003, Barnard *et al.*, 2004, Kerrinnes *et al.*, 2009, Cui *et al.*, 1995, Forsbach-Birk *et al.*, 2004, Pessi *et al.*, 2001, Burrowes *et al.*, 2006).

In this report, we provide evidence that in EPEC, a functional *csrA* allele is necessary for the formation of pedestals, and for membrane depolarization of epithelial cells. CsrA exerts its effect by binding to the leader segment of the *sepLespADB* mRNA and enhancing the steady-state transcript and protein levels. In contrast to the *csrA* mutant phenotype, modest overexpression of *csrA* globally repressed transcription from the LEE operons by binding to and repressing the expression of global regulator of the LEE-activator, GrlA. Furthermore, we also provide evidence that CsrA-mediated effects appear through at least one other regulatory factor. Lastly, we show that *csrA* also regulates other virulence-associated traits such as motility and glycogen biosynthesis. Our results extend the role for *csrA* as a regulator of virulence of the LEE genes of EPEC and likely other A/E pathogens, which also possess highly conserved *csrA* orthologs and putative CsrA binding sites in the leader segments of LEE genes.

Materials and Methods

Bacterial Strains, Plasmids, Primers & Media

Bacteria were grown in Luria-Bertani (LB) broth or DMEM containing phenol red (Immunofluorescence microscopy) or lacking glutamine and phenol red (Western blotting and quantitative real-time PCR) with appropriate antibiotic supplements when needed. Antibiotics used were streptomycin (100 μg/ml), chloramphenicol (25 or 50 μg/ml), kanamycin (50 μg/ml), tetracycline (15 μg/ml), and ampicillin (50 or 100 μg/ml).

For determining growth rates, bacterial cultures were grown overnight at 37˚C/250 rpm in LB or LB supplemented with the appropriate antibiotic. Overnight cultures were diluted to a starting optical density of approximately 0.01 in DMEM containing glucose (4.5 g/L) but lacking phenol red and glutamine. The cultures were grown standing at 37˚C in a 5% CO_2 incubator and growth was measured every hour and intermittently at half hours over a period of 24 hours. The growth curve for each strain was determined at least twice from different experimental samples with each sample being assayed in duplicate. Growth curves are representative of one such experiment. Strains, plasmids and oligonucleotides used are listed in Tables 1 & 2.

csrA was disrupted by the one step gene inactivation method described by Datsenko and Wanner and modified for EPEC by Murphy and Campellone (Datsenko & Wanner, 2000, Murphy & Campellone, 2003). Briefly, the plasmid pKD3 was used as a template to amplify a chloramphenicol (*cat*) resistance cassette flanked on its 5'side with 51 nucleotides (101-151 nucleotides downstream of the translation initiation codon of the *csrA* ORF) and on its 3' side with 49 nucleotides (152-186 nucleotides downstream of the translation initiation codon of the *csrA* ORF and 14 nucleotides downstream of the

translation termination codon) using the primers 5'csrAP2(Wanner) and 3'csrAP1(Wanner). The PCR product was gel purified and 250-500 ng of the product was electroporated into EPEC expressing the lambda red recombinase proteins Gam, Exo and Bet from the plasmid pTP223 under an IPTG-inducible promoter (Poteete & Fenton, 1984). Expression of the lambda red recombinase system promotes the site-specific substitution of the wild type *csrA* allele with the mutant *csrA::cat* PCR product because of the presence of identical flanking sequences in the PCR product. Recombinants were selected on LB plates containing chloramphenicol. *grlA* was tagged in the chromosome with a 3X-FLAG tag in a similar manner using pKD4 as template (Uzzau *et al.*, 2001, Iyoda & Watanabe, 2005, Murphy & Campellone, 2003). The *csrA* disruption was verified by locus specific PCR using the primer pairs 5'csrA and c2 or 3'csrA(EPEC) and c1 whereas *grlA* tagging was verified by using the primer pair 5'grlA(qRT-PCR) and k1 (Datsenko & Wanner, 2000). PCR products of the expected size were generated and verified by sequencing.

csrA-(pBR322) and *csrA*-(pCRA16) were constructed by tranformation of the *csrA* disruptant by the empty vector pBR322 (Sambrook *et al.*, 1989) or pCRA16. Construction of pCRA16 has been described previously (Suzuki et al., 2006). Briefly, a BamHI-EcoRI fragment of approximately 500 bp containing the K12 *csrA* gene under its putative promoter(s) was restricted from pCSR10 (Romeo *et al.*, 1993), end-filled with the Klenow fragment of DNA Polymerase I and cloned into the blunt *VspI* site of the *bla* gene of pBR322 to generate pCRA16. The *csrA* ORF is oriented in the same direction as the *bla* gene (Suzuki et al., 2006).

EPEC*csrA-lacZ-csrA+* is the *csrA* disruptant expressing the wild type *csrA* allele under its putative promoter(s). *csrA* was amplified from EPEC by colony PCR using the primers 5'csrA-EcoRV and 3'csrA-EcoRV. The 685 bp PCR product contains the intergenic sequence spanning from the last 11 nucleotides of the *csrA* upstream gene, *alaS*, to the -35 region of the *csrA* downstream gene, *serV*, and includes *csrA* under its native *cis* regulatory elements (as determined by qRT-PCR and Western blot for LEE-encoded genes regulated by CsrA). The PCR product was gel purified, treated with EcoRV and cloned into the EcoRV site of the *lacZ* gene in the plasmid pJRlacZins (Kraiss *et al.*, 1998) to generate pJRcsrA6. The cloned *csrA* gene is oriented opposite to the direction of transcription of the *lacZ* gene. The *csrA* sequence in pJRcsrA6 was verified by sequencing using the same set of primers. pJRcsrA6 contains the R6Kγ origin of replication and can only replicate in π-protein expressing strains such as S17-1λpir (Simon *et al.*, 1983). This plasmid was transferred into the *csrA* mutant of EPEC by conjugation as described previously (Kraiss et al., 1998). Integrants were selected by plating dilutions onto LB plates supplemented with chloramphenicol, ampicillin, X-gal (40 μg/ml) and IPTG (5 mM) and incubated overnight at 37˚C. Transconjugants that arose on these plates were confirmed to be EPEC derivatives and contained both the wild type and the mutant *csrA*- alleles. One such colony was selectively purified and serial dilutions were plated onto LB plates devoid of NaCl but supplemented with 5% sucrose to counterselect for the loss of *sacB* gene encoded on the plasmid backbone. One such colony that arose was confirmed to be ampicillin sensitive, chloramphenicol resistant, sucrose resistant (*sacB*-), white when plated onto LB plates containing X-gal and IPTG

(*lacZ*-) and contained both the *csrA* wild type and the *csrA*- mutant allele as determined by PCR and was used for all subsequent experiments.

Table 2.1 – Bacterial strains and plasmids used in this study

Strain	Relevant genotype	Reference
EPEC	Wild-type EPEC 2348/69 serotype O127:H6, Str^r	Jim Kaper
EPEC*csrA*-	EPEC 2348/69 *csrA::cat*, Str^r cat^r	This study
EPEC*csrA-lacZ-csrA*+	EPEC 2348/69 *csrA::cat lacZ::csrA*, Str^r cat^r	This study
EPEC *grlA*3XFLAG	EPEC 2348/69 Φ(*grlA*-3X FLAG), Kan^r Str^r	This study
EPEC*csrA- grlA*3XFLAG	EPEC 2348/69 *csrA::cat* Φ(*grlA*-3X FLAG), cat^r Kan^r Str^r	This study
EPEC*csrA-lacZ-csrA*+ *grlA*3XFLAG	EPEC 2348/69 *csrA::cat lacZ::csrA* Φ(*grlA*-3X FLAG), cat^r Kan^r Str^r	This study
EPEC(pBR322)	EPEC 2348/69 transformed with the empty vector pBR322, Tet^r Amp^r Str^r	This study
EPEC(pCRA16)	EPEC 2348/69 transformed with pCRA16, Tet^r Str^r	This study
EPEC*grlA* 3XFLAG(pBR322)	EPEC 2348/69 Φ(*grlA*-3X FLAG) containing pBR322, Kan^r Tet^r Amp^r Str^r	This study
EPEC*grlA* 3XFLAG(pCRA16)	EPEC 2348/69 Φ(*grlA*-3X FLAG) containing pCRA16, Kan^r Tet^r Str^r	This study
EPECΔ*ler*	EPEC 2348/69 *ler* deletion	Jay Mellies
EPECΔ*tir*	EPEC 2348/69 *tir* deletion	B. Brett Finlay
EPECΔ*espA*	EPEC 2348/69 *espA* deletion	James B. Kaper
EPECΔ*espB*	EPEC 2348/69 *espB* deletion	James B. Kaper
EPECΔ*espD*	EPEC 2348/69 *espD* deletion	James B. Kaper
S17-1λpir	*E.coli pir* lysogen of S17-1 (*thi pro hsdR hsdM*$^+$ *recA* RP4-Tc::Mu-Km::Tn7) Tp^r Str^r	Phil Rather

DH5α	*supE44* Δ*lacU169* (Φ80 *lacZ*ΔM15) *hsdR17 recA1 endA1 gryA96 thi-1 relA1*	Bettina Bommarius

Plasmids

pTP223	*gam, bet,* and *exo* genes of the λ phage cloned under the control of the *lac* promoter, Tet^r	Kenan Murphy
pBR322	Cloning vector, Tet^r Amp^r	85
pCRA16	*csrA* gene from K-12 cloned into the blunt-ended VspI site of *bla* of pBR322, Tet^r	97
pJRlacZIns	Suicide vector, Amp^r	Vanessa Sperandio
pJRcsrA6	*csrA* gene from EPEC under its regulatory sequences cloned into the EcoRV site of *lacZ* in pJRLacZIns, Amp^r	This study

Tet^r – tetracycline resistance, cat^r – chloramphenicol resistance, Str^r – streptomycin resistance, Amp^r – ampicillin resistance, Tp^r – trimethoprim resistance

Table 2.2 – Oligonucleotides used in this study

Primers	Sequence
5'csrAP2(Wanner)	TAAATGCCCCGAAGGAAGTTTCTGTTCACCGTGAAGAGATCT ACCAGCGTACATATGAATATCCTCCTTA
3'csrAP1(Wanner)	GAGACGCGGAAAGATTAGTAACTGGACTGCTGGGATTTTTC AGCCTGGAGTGTAGGCTGGAGCTGCTTC
c1*	TTATACGCAAGGCGACAAGG
c2*	GATCTTCCGTCACAGGTAGG
k1*	CAGCCGATTGTCTGTTGTGCCC
5'csrA	GGATAATGCCGGGATACAGAGAGAC
3'csrA[EPEC]	ATTTTGAGGGTGCGTCTCACCG
5'csrA-EcoRV	GCGGCC**GATATC**AATTGCAATAATATAAGCGTCAGGCAATG
3'csrA-EcoRV	GCGGCC**GATATC**GTCAAACAATTTTTCCCACACTTTTATCG
5'grlA-3X-FLAG	TCTAATATCTGGAACGAAATGATCTTGAGGCGGAAAAAGGA GAGTGACTACAAAGACCATGACGG
3'grlA-3X-FLAG	GAGAAAAAGGCTTACCCTGGAAAACAAAACCCTTAAATATA GCTTCATATGAATATCCTCCTTAG
RT-qRT-PCR primers	
5'rrsB	CTTACGACCAGGGCTACACAC
3'rrsB	CGGACTACGACGCACTTTATG
5'ler	GCAGTTCTACAGCAGGAAGCA
3'ler	CGAGCGAGTCCATCATCAG
5'flhD	TGCATACCTCCGAGTTGCTG
3'flhD	GCGTGTTGAGAGCATGATGC
5'tir**	GCAGAAGACGCTTCTCTGAATA
3'tir**	CCCAACTTCAGCATATGGATTA
5'espA**	GCTGCAATTCTCATGTTTGC
3'espA**	GGGCAGTGGTTGACTCCTTA
5'grlR	TTAGCAATGAAGACTCCTGTGG
3'grlR	AGAGAGAACCCCCTGATACAC
5'grlA	AGGCGGTTCCGATAGAAAGT
3'grlA	GCCTCAAGATCATTTCGTTCC
5'escJ	CCAAAGAAATGGACAAAAGTGG
3'escJ	GCTGGGTGGGAAAATAACCT
5'sepL	GAAAGAAGAGGAAGGCACGAC
3'sepL	CAAACATCGCCAAAGTAGGA
5'escD	CACGCCCTATGAAGCAGATAA
3'escD	CAACGCAAAAGTAGCACCAA
5'espD	GCTGCTACGGCTACTTCAGG
3'espD	GCTGTGGTTCTGTTCCCTCT
5'espB	TAGGCTCTTTTGCTGCCATT
3'espB	TTCGCCAGTGCTTTAGTTGA
5'escF	ACAAATGGGTGAAGTAGGTAAAACG
3'escF	GAACCGCAAACTGCAACTCTAAC

***In vitro* transcription primers**	
5'-T7p-grlR-EPEC	TAATACGACTCACTATAGGGCATTGCAATCTGGAGAAAAAG
3'-T7p-grlR-EPEC	CAGTGATCATATTTCCATTTT
5'-T7p-escD-EPEC	TAATACGACTCACTATAGGGGATGTAAGTTCACCATATTTT
3'-T7p-escD-EPEC	CGGAAGTTGTAATTCCCGATT
5'-T7p-sepL-EPEC	TAATACGACTCACTATAGGGGTCTAAGAATAGAGTAGAAAG
3'-T7p-sepL-EPEC	CATTAGCCATTGGAAACTCAC
phoB-T7 (*E.coli* K-12)	TAATACGACTCACTATAGGGGCATTAATGATCGCAACCTATT TATTACAACAGGGCAAATCATG
GCphoB-T7 (*E.coli* K-12)	CATGATTTGCCCTGTTGTAATAAATAGGTTGCGATCATTAAT GCCCCTATAGTGAGTCGTATTA

* - Reference for c1, c2 and c3 primers (Datsenko & Wanner, 2000); ** - Reference for *tir* and *espA* specific primers (Leverton & Kaper, 2005).

Cell culture and Immunofluorescence microscopy

3T3 cells were maintained and passaged under standard culture conditions in DMEM supplemented with 10% fetal bovine serum and penicillin and streptomycin (cDMEM). Pedestal formation by EPEC and its isogenic derivatives on 3T3 cells was performed as described previously (Anyanful et al., 2005).

Preparation of cell lysates, TCA precipitation and Western Blotting

Synthesis of EspA, and Tir and secretion of EspA, EspB, EspD and Tir was analyzed by Western blotting. Bacterial cultures were grown under standing conditions at 37˚C in a 5% CO_2 incubator to an optical density of 0.2-0.3, 0.5 or 1.0 and pelleted by centrifugation at 3000 rpm. The proteins in the supernatant were precipitated at -20˚C by the addition of Tricholoroacetic acid to a final concentration of 10%. The precipitated proteins were pelleted and washed with acetone and centrifuged. The pellets were air-dried to ensure complete evaporation of the acetone prior to suspending them in 100 µl of a 1:1 volumetric ratio of 2X Laemmli buffer containing ß-mercaptoethanol (Laemmli,

1970) and 1M Tris-HCl (pH 6.8). The suspensions were boiled for 10 minutes prior to loading onto a 12% SDS-PAGE gel. To ensure equal loading the volume of the sample loaded was normalized to the optical density of the cultures when harvested.

For determining protein levels in the cell lysate the cell pellets were suspended in 200 µl of 50 mM Tris-HCl (pH~7.5) and sonicated to disrupt the cell membrane. Cell debris was pelleted by centrifugation and the supernatant transferred to a fresh epindorf tube. Protein concentrations were determined using the Lowry Assay (Lowry *et al.*, 1951) as recommended by the manufacturer (Bio-rad) after which the lysates were mixed with an equal volume of 2X Laemmli buffer containing ß-mercaptoethanol. The lysates were boiled at 95˚C for 10 minutes. Equal amount of protein was loaded onto a 12% SDS-PAGE gel. Proteins were electroblotted onto a PVDF membrane and probed with one of the following antibodies: mouse monoclonal anti-FLAG M2 (F-3165) (10 µg/ml), rabbit α-Ler (1:2500), rabbit α-EspA (1:50,000), rabbit α-EspB (1:10,000), rat α-EspD (1:500), or rabbit α-Tir (1:50,000). α-FLAG was purchased from SIGMA. Each experiment was repeated atleast 3 times with similar results in each experiment.

RNA Isolation

RNA isolation was performed by using the MasterPure^{TM} RNA Purification Kit from *EPICENTRE* as recommended by the manufacturer with the exception that the DNase I treatment was performed for 45 minutes and then an additional 5 µl of DNase I was added to the samples and the reaction continued for another 45 minutes prior to inactivation of the DNase I. The DNase I treated RNA was subjected to phenol-chloroform extraction followed by isopropanol precipitation and the procedure repeated.

Precipitated RNA was resuspended in 50 µl of TE buffer (10 mM Tris-HCl [pH 7.8],1 mM EDTA) and 1 µl of ScriptGuard RNase inhibitor was added to the RNA samples.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

The iScript™ One-Step reverse transcription quantitative real-time PCR (RT-qRT-PCR) kit with SYBR® Green (Bio-rad) was used to determine the transcript levels of genes as recommended by the manufacturer with some modifications. Each reaction was done in a total volume of 25 µl. The reaction ingredients consisted of the following- 1X SYBR Green RT-PCR Reaction Mix [12.5 µl of 2X reaction buffer containing 0.4 mM of dATP, dCTP, dGTP and dTTP, magnesium ions, iTaq DNA Polymerase, 20 nM fluorescein, SYBR Green I dye and stabilizers], Primer pair (0.75 µl at a final concentration of 300 nM), nuclease free water, iScript Reverse Transcriptase (0.5 µl) and DNase I treated RNA (50 ng). Reaction conditions were as follows- (i) 53˚C/20 mins; (ii) 95˚C/10 mins; (iii) ([95˚C/0.5 min; 60˚C/1 min] x40); (iv) 4˚C/∞. Melt curve analysis were done to ensure the specificity of the generated product. Melting curve conditions were the following- (i) 95˚C/1 min; (ii) 55˚C/1min; (iii) ([55˚C/10 sec with an increment of 0.5˚C] x 40 cycles). Control reactions were conducted to ensure the absence of genomic DNA contamination in purified RNA and the absence of primer dimer formation. RT-qRT-PCR was performed in duplicate or triplicate from multiple experimental samples. The cycle threshold method $2^{-\Delta\Delta Ct}$ was used for quantifying the transcript levels after normalizing them to the housekeeping gene *rrsB*. The relative transcript levels for EPEC or EPEC(pBR322) in the first sample of the first experiment was defined as 1 and all other values were determined relative to this. The unpaired

student's *t*-test was used to assay for statistical significance. A *p*-value <0.01 was considered statistically significant for RT-qRT-PCR based analysis.

***In vitro* transcription and RNA Electrophoretic Mobility Shift Assay**

The MEGAshortscript™ Kit from Ambion was used for *in vitro* transcription as recommended by the manufacturer. Briefly, a PCR product containing the T7 promoter at its 5' end and the entire leader segment encompassing the putative CsrA binding sites of *grlR*, *sepL* and *escD* were generated. Transcripts were run out on a 5% polyacrylamide gel and the band corresponding to the correct size transcript was excised and the gel fragment suspended in 250 µl of the elution buffer (0.5 M Ammonium Acetate, 1 mM EDTA, 0.2% SDS) along with 2 µl of Superase-in™ (Ambion) and eluted overnight at 4˚C and then at room temperature for 30 minutes. The eluate was subjected to phenol-chloroform extraction and ethanol precipitation and suspended in TE [10 mM Tris-HCl [pH 8.0], 1 mM EDTA] buffer. 500 ng of the RNA was run out on a gel to verify the size and integrity of the RNA. 30-40 pmoles of the RNA was dephosphorylated and the RNA purified by another round of phenol-chloroform extraction and ethanol precipitation. The purified RNA was suspended in 10 µl of nuclease-free water. RNA was radioactively labeled and suspended in 10 µl of TE buffer. Because the *csrA* ORF and the CsrA protein are identical between EPEC and K-12, C-terminally His-tagged CsrA, purified from *E.coli* K-12, was used for the binding reactions. Purification of recombinant His-tagged CsrA has been described elsewhere (Mercante *et al.*, 2006). His-tagged CsrA was suspended in protein dilution buffer [10 mM Tris-HCl (pH 7.5), 2 mM Dithiothreitol (DTT), 10% glycerol]. RNA was denatured at 85˚C and slowly cooled to 25˚C over 25

minutes in the Bio-rad ICycler prior to addition in the binding reaction. The components of the binding reaction included 50 pM RNA in TE, 1X binding buffer [10 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 100 mM KCl], 3.25 ng/µl of total yeast RNA, 20 mM DTT, 7.5% glycerol, 0.01 µl of Superase-in^{TM} (Ambion) and 2 µl of different concentrations of His-tagged CsrA in a total volume of 10 µl. The reactions were incubated at 37˚C for 30 minutes after which the samples were loaded onto a 10% native polyacrylamide gel. The gel was exposed to a phosphorimager plate overnight and the bands detected using a phosphorimager. ImageQuaNT (Molecular Dynamics) was used to perform densitometric analysis. The apparent equilibrium dissociation constant (K_d) for CsrA was determined from two independent experiments using the equation –

$$Y = (Ymax*((x/ K_d)^n)) /(1+((x/ K_d)^n))$$

For competition based gel shift experiments unlabeled specific and non-specific (*phoB*) transcripts were added at a final concentration of 500 pM, 5 nM and 50 nM which represents 10, 100 and 1000 fold excess relative to the labeled transcript.

Transepithelial Resistance Assay

Resistance of cells in response to EPEC and its mutant strains was monitored in real time using the electric cell-substrate impedance sensing (ECIS) 1600R device (Applied BioPhysics, Troy, NY). Caco2-BBE cells were seeded in ECIS 8W1E electrodes (5×10^5 cells/400 ml/electrode) and kept at 37°C in 5% CO_2 and 90% humidity. When cells were confluent, cells were washed and changed to serum-free, antibiotic-free DMEM. Resistance of cells was measured at a frequency of 500 Hz and a voltage of 1 V. Details on the operation, equivalent resistance-capacitance circuit, and modification of the ECIS

system can be found in the manufacturer's instructions. Bacterial strains were grown overnight in Luria-Bertani (LB) broth and transferred to serum-free and antibiotic-free DMEM, and confluent Caco2-BBE monolayers were infected at a multiplicity of infection of 10. Transepithelial resistance was measured continuously post-infection. TER assays were conducted atleast three times from separate experimental samples with each sample assayed in triplicate per experiment. The depicted results are representative of one such experiment.

Motility Assay

Cultures were grown to an OD_{600} of 1.0 and 1 µl of the culture was stabbed onto 0.3% ½ X DMEM [containing glucose (2.25 g/L) and sodium pyruvate (0.5 mM) but lacking phenol red and glutamine]. The stabbed agar plates were incubated upright overnight at 37˚C and the diameter of the "diffusion halo" was measured 30 hours later to determine the extent of motility of the bacterium. Motility assays were performed on 3 separate occasions.

Glycogen biosynthesis assay

A loopful of bacterial cultures grown to an OD_{600} of 1.0 was streaked onto Kornberg media (1.1% K_2HPO_4, 0.85% KH_2PO_4, 0.6% yeast extract, 2% glucose, 1.5% agar) (Romeo et al., 1993) after which the plates were incubated overnight at 37˚C. The plates were then exposed to iodine crystals to determine the extent of glycogen biosynthesis in the different bacterial strains. Iodine forms complexes with glycogen as a result of which strains producing glycogen appear blackish-brown whereas strains that do not synthesize

glycogen do not exhibit any color change after exposure to iodine. Glycogen biosynthesis assays were performed on 3 separate occasions. The pictures are representative of one such experiment.

Results

csrA regulates adherence and pedestal formation on mammalian cells

Disruption of *csrA* does not affect the growth of EPEC as evident from similar doubling times for the wild type (48.04 ± 0.8 mins) and the *csrA* mutant (41.15 ± 9.2 mins) (Figure 2.1A, B and C). Because *csrA* was necessary for virulence against *C. elegans* (data not shown; manuscript in preparation), we next assessed whether disruption of *csrA* affected virulence in mammalian cells. To do this, we assessed the capacity of EPEC to adhere to and form pedestals on mammalian 3T3 cells (Knutton *et al.*, 1989), and to disrupt the resistance across polarized epithelial cells (Canil *et al.*, 1993, Guttman *et al.*, 2006). Although cells were infected with the same number of bacteria, the *csrA* mutant exhibited reduced adherence compared to EPEC or the single copy complemented strain (n=4; Figure 2.1D; compare DAPI staining in column 2 row 3 (*csrA*-) with column 1 row 3 (EPEC) or column 3 row 3 (*csrA-lacZ-csrA*+)). Furthermore, whereas clustering of the bacteria was evident for the wild type strain and the complemented strain (yellow arrows), the *csrA* mutant primarily appeared as solitary isolated cells.

Using fluorescence microscopy in conjunction with antibodies against phosphotyrosine and Tir, and FITC-phalloidin to visualize polymerized actin, we next assessed formation of actin pedestals. In cells infected with EPEC, >80% of cells displayed >10 pedestals per cell (Figure 2.1E), and Tir and phosphotyrosine (pY) staining were evident directly beneath attached bacteria and at the tip of actin pedestals (Figure 2.1D, Column 1). By contrast, with the *csrA* mutant, the percentage of cells displaying pedestals was reduced by ~35 fold (Figure 2.1E), as was the number of pedestals per cell (Figure 2.1D). Accordingly, phosphotyrosine and Tir staining was diminished in cells

infected with the *csrA* mutant compared to wild type EPEC (Figure 2.1D, Column 2). Complementation of the mutant with *csrA* in a single copy restored adherence, pedestal formation, and localization of phosphotyrosine and Tir to levels seen with wild type EPEC (Figure 2.1D, Column 3 and Figure 2.1E).

Disruption of Transepithelial resistance across polarized Caco-2-BBE cells depends on *csrA*

Previous reports have shown that adherence, pedestal formation and the effectors EspF and Map mediate the capacity of EPEC to disrupt transepithelial resistance (TER) across polarized Caco-2-BBE cells (Canil et al., 1993, McNamara *et al.*, 2001, Dean & Kenny, 2004). Because mutation of *csrA* diminishes the ability of EPEC to adhere and form pedestals, we next examined its effects on TER. For EPEC, the time required to disrupt the TER to 50% of the value at the point of infection ($t_{1/2}$) was ~5.5 hours, whereas the *csrA* mutant required approximately 2.5 times as long ($t_{1/2}$ of ~14.3 hours; Figure 2.2). By 9.5 hours post-infection (p.i.), EPEC decreased the resistance across the cells by over 76% (>4 fold), whereas the *csrA* mutant reduced the TER by less than 15% during the same period. The complemented strain regained the ability to disrupt the TER with significantly faster kinetics compared to the *csrA* mutant ($t_{1/2}$ of ~ 7.9 hours), and by 9.5 hours it had decreased the TER by over 63% (Figure 2.2). Interestingly, even after prolonged infection by the *csrA* mutant there was still some residual end-point resistance (~12000 Ω) that was reproducibly higher than that observed in cells infected with EPEC (7000 Ω) or with the complemented strain (5000 Ω) (Figure 2.2). Taken together, these

data suggest that *csrA* not only regulates the time of onset, but also the kinetics and extent of membrane depolarization of mammalian cells *in vitro*.

csrA is necessary for synthesis and secretion of translocators, but only affects the secretion of the effector Tir

Mutations in *espA*, *espB*, *espD* or *tir* negate the ability of EPEC and *Citrobacter rodentium* to form pedestals on tissue culture cells *in vitro* or to colonize mice, respectively (Kenny & Finlay, 1995, Kenny *et al.*, 1996, Knutton et al., 1998, Deng et al., 2004, Cleary *et al.*, 2004, Lai *et al.*, 1997, Kenny, 1999, Foubister *et al.*, 1994). Disruption of *csrA* reduced the secreted (Figure 2.3A) as well as the bacterium-associated (Figure 2.3B) levels of EspA, EspB and EspD. Complementation of the *csrA* mutant with *csrA* expressed in a single copy under its putative promoter(s) restored both the secreted (Figure 2.3A) and the bacterium-associated (Figure 2.3B) amount of EspA, EspB and EspD to near wild type levels.

Two prominent bands corresponding to Tir were visible in the secreted fraction of the wild type and the complemented strain (Figure 2.3A; lanes 1 & 3), but absent in the *tir* mutant (data not shown). Whereas the slower migrating band displayed a modest reduction, the faster migrating band was absent in the *csrA* mutant (Figure 2.3A; compare lane 1 with 2). Our results are consistent with previous observations identifying at least two variants of Tir in EPEC, one of which migrates at 80 kDa (upper band) and the other, likely representing a truncated form, at 70 kDa (lower band) (Elliott et al., 2000, Elliott *et al.*, 1999). Both bands were routinely observed in the secreted fraction (Figure 2.3A), but only the 80 kDa band was consistently observed in cell lysates (Figure 2.3B). In contrast

to the secretion of Tir, the bacterium-associated levels of Tir was not substantially affected by the disruption of *csrA* (Figure 2.3B). Overall, these data suggest that disruption of *csrA* reduces the secretion of Tir without appreciably affecting the amount of Tir protein associated with the bacterium or its synthesis (see below).

csrA regulates the transcript levels of the architectural components of the TTSS

Because the bacterium-associated levels of EspA, EspB and EspD were reduced in the *csrA* mutant, we next explored the possibility that reduced levels of protein were due to reduced transcript levels. RT-qRT-PCR for *espA*, *espD* and *espB* demonstrated that transcript levels for these genes were reduced by approximately 16, 28, and 14 fold, respectively, in the *csrA* mutant (Figure 2.4A). Complementation with *csrA* in a single copy restored the transcript levels to those seen in the parental strain (Figure 2.4A). Thus, reduced steady-state protein levels of the translocators in the mutant can be accounted for by reduced transcript levels. Interestingly, whereas *espA* and *espB* showed similar fold reductions in transcript levels, *espD* exhibited a 1.75 fold greater reduction (Figure 2.4A).

A σ^{70} promoter for the *LEE4* operon is located upstream of *sepL*, which is the first gene in the operon (Mellies et al., 1999). In EPEC and EHEC *sepL*, *espA*, *espD* and *espB* are transcribed as a single polycistronic transcript (Lodato & Kaper, 2009, Mellies et al., 1999). In EHEC this polycistronic transcript is processed at the C-terminus of *sepL* to yield two transcripts containing *sepL* and *espADB* respectively (Lodato & Kaper, 2009), whereas for EPEC this phenomenon has yet to be demonstrated. SepL facilitates the secretion of translocators (Kresse *et al.*, 2000, O'Connell *et al.*, 2004, Deng et al., 2005) without affecting their synthesis. Because CsrA binds to the 5' leader segment of

transcripts and affects gene expression we scanned the RNA sequence in the leader segment of the *sepL* transcript for putative CsrA binding sites. Two such sites were identified (Figure 2.5A & B), suggesting that CsrA might bind to and affect the *sepLespADB* transcript levels. Consistent with this prediction, *sepL* transcript levels were reduced by ~17.2 fold in the *csrA* mutant (Figure 2.4A). Together, these data suggest that *csrA* not only regulates the synthesis of EspA, EspB, and EspD, but may directly affect their export by regulating the levels of SepL. Consistent with data showing similar levels of bacterium-associated Tir in the wild type and *csrA* mutant, no significant differences in the *tir* transcript levels were observed in these strains (Figure 2.4B). Thus, reduced secretion of Tir in the mutant is likely due to an effect of *csrA* on a regulator or a component of the TTSS.

EscF forms the needle complex of the TTSS that connects the EscC outer membrane protein of the TTSS to the EspA filament. Inactivation of *escF* diminishes secretion of EspA, EspB, EspD and pedestal formation by EPEC (Wilson *et al.*, 2001). *escF* is present on the same polycistronic transcript as *sepLespADB* (Mellies et al., 1999) suggesting that its trancript levels might also be reduced in the *csrA* mutant. However, the transcript levels of *escF* did not show the same extent of reduction as *sepLespADB* and were reduced by only ~5.5 fold in the *csrA* mutant (Figure 2.4B). It is possible that besides being present on the longer transcript, *escF* is also transcribed from additional promoter(s) in the intergenic region between *espB* and *escF* and this transcript is not subject to CsrA-mediated regulation.

Besides *escF,* we also assayed for the transcript levels of *escD*. EscD is homologous to the *Yersinia* YscD protein, both of which are predicted to be inner membrane proteins

and components of the TTSS necessary for the secretion of translocators and effectors (Ogino *et al.*, 2006). *escD* is located between the *eae* gene of the *LEE5* operon and the *sepL* gene of the *LEE4* operon (Mellies et al., 1999). *escD* and *LEE4* have overlapping divergent promoters and their transcriptional initiation nucleotides are adjacent to one another albeit on opposite strands (Mellies et al., 1999). We found that *escD* transcript levels were reduced ~2.5 fold in the *csrA* mutant (Figure 2.4B) and restored when the mutant was complemented with *csrA* in single copy. Overall, these data suggest that reduced synthesis of EscF and EscD in the *csrA* mutant may in part be responsible for reduced secretion of Tir and the translocators by affecting assembly of the TTSS. Expression from the *LEE1* and *LEE2* operons, which encode for other components of the TTSS, was not significantly affected in the *csrA* mutant (data not shown).

Purified CsrA binds to the leader segment of the *sepLespADB* but not *escD* transcript

Two putative CsrA binding sites were identified in the 5' leader segment of *sepL* (Figure 2.5A & B) suggesting that CsrA might regulate the *sepLespADB* mRNA levels by directly binding to the transcript. Binding of CsrA to the leader segment of the *sepL* transcript was detectable at a protein concentration of 10 nM (Figure 2.5C), with an apparent equilibrium binding contant of 23 ± 1.7 nM (Figure 2.5E). No unbound transcript was evident at a CsrA concentration of 80 nM (Figure 2.5C), and multiple shifted ribonucleoprotein complexes were evident at concentrations ≥160 nM (Figure 2.5C). The binding of CsrA to the *sepL* transcript was specific, as shown by competition assays with unlabeled *sepL* and the non-specific RNA *phoB,* to which CsrA does not bind

(Figure 2.5D). The binding data and RT-qRT-PCR results suggest that increased steady-state levels of *sepL*, *espA*, *espD*, *espB* and *escF* in the wild type strain is the result of direct interaction between CsrA and the polycistronic transcript. CsrA failed to bind to the *escD* transcript (data not shown), suggesting that *csrA*-mediated activation of *escD* occurred through an intermediate regulator.

The transcription factor GrlA activates the transcription of *ler* (Huang & Syu, 2008, Deng et al., 2004, Barba et al., 2005), which in turn upregulates the steady-state transcript levels of *escD* (Deng et al., 2004, Elliott et al., 2000, Mellies et al., 1999). To determine whether the effect of *csrA* on *escD* and *LEE4* were also occurring through *grlA* and *ler,* we assayed for the expression of these genes. Protein levels of Ler and FLAG-tagged GrlA were unchanged in the *csrA* mutant (Figure 2.5F & G), suggesting that the effects of *csrA* on *escD* and the *LEE4* occurred independently of Ler and GrlA.

Overexpression of *csrA* globally represses the expression from the LEE

Previous studies with *csrA* suggest that fine tuning of the activity of CsrA is critical for the virulence of *S.* Typhimurium (Altier et al., 2000). This observation prompted us to assess the effect of increasing the activity of CsrA in EPEC. The non-coding sRNAs CsrB and CsrC bind to and sequester CsrA, thereby inhibiting its activity. These RNAs are strongly regulated *in vivo*, and deletion of both *csrB* and *csrC* increases CsrA activity in the cell, a condition that would be more relevant for *in vivo* interpretation (Suzuki et al., 2002, Weilbacher et al., 2003). Because CsrB and CsrC exhibit compensatory regulation, loss of either RNA leads to an increase in the remaining RNA (Weilbacher et al., 2003). Thus, single deletions have weak or negligible effects on CsrA-regulated genes

and processes. In this regard, isogenic single mutants of *csrB* or *csrC* show no difference in the expression of Tir (data not shown), and we were unable to construct a *csrBcsrC* double mutant of EPEC, for unknown reasons. Thus high activity of CsrA was achieved by increasing the levels of CsrA via expression of *csrA* from a plasmid. Multicopy expression of *csrA* resulted in a modest ~1.5 ± 0.1 fold increase in the CsrA protein levels above the empty vector containing strain (Figure 2.6A). Overexpression of CsrA reduced transcript levels of *escJ, tir* and *espA* encoded on the *LEE2*, *LEE5* and *LEE4* operons and the monocistronic gene *escD* by 76-, 43-, 13- and 45- fold respectively (Figure 2.6B). Consistent with the observed reduction in transcript levels of *tir* and *espA,* the overall steady-state protein levels were also reduced (data not shown). Reduced *tir* transcript levels in the overexpressor, but not the *csrA* mutant (Compare Figure 2.6B with 4B), suggest that at some level of regulation disruption and overexpression of *csrA* affect Tir levels via distinct pathways.

In EPEC, *grlR* and *grlA* are encoded on the same bicistronic transcript because a PCR product was generated in an RT-qRT-PCR reaction using a primer specific for *grlR* and another for *grlA* (data not shown). The *grlRA* operon encodes for a repressor, GrlR, and an activator, GrlA (Deng et al., 2004). GrlA activates the expression from the LEE by upregulating the transcription of *ler* (Deng et al., 2004, Barba et al., 2005) whereas binding of GrlR to GrlA has been proposed to prevent such activation (Huang & Syu, 2008, Jobichen *et al.*, 2007, Creasey *et al.*, 2003). Ler in turn upregulates the transcription from *LEE2*, *LEE3*, *LEE5*, *LEE4* and *escD* (Elliott et al., 2000, Mellies et al., 1999). Because we observed reduced transcript levels of genes under the *ler* regulon, we assessed the expression of *ler*, *grlR* and *grlA* in the *csrA* overexpressing strain. The

transcript levels of the three genes were reduced by 7-, 14- and 29- fold respectively in the overexpressor as determined by RT-qRT-PCR (Figure 2.6C). The corresponding protein levels of Ler and 3X-FLAG tagged GrlA were also substantially reduced (Figure 2.6D), suggesting that overexpression of *csrA* globally shuts off the transcription from the LEE by repressing the expression of *grlA*. We were unable to assay for GrlR because no antibody was available, and we were unable to epitope tag *grlR* on the chromosome. Notably, no Ler protein was detectible in the *csrA* overexpressor even when *ler* was expressed from a multicopy plasmid despite the fact that complementation of EPECΔ*ler* with the same plasmid resulted in Ler protein levels that were ~17-fold higher than EPEC containing a single chromosomal copy of *ler* (data not shown).

We could not attribute the observed differences in gene expression from the LEE to the subtle alteration in growth exhibited by the *csrA*-overexpressing strain. While barely observable on the logarithmic scale (Figure 2.1A), in a linear plot of growth the *csrA*-overexpressing strain exhibited a biphasic growth (black arrows in Figure 2.1B), which resulted in a mild but detectible growth defect (Figure 2.1B). This growth defect was evident only after the strain had grown past the early log phase beyond an OD_{600} of ~ 0.25-0.3 as both the strains grew at similar rates in the early log phase with doubling times of ~51.61±8.3 and ~54.70±6.4 minutes for EPEC(pBR322) and EPEC(pCRA16), respectively (Figure 2.1C). Steady-state levels of Ler protein were reduced in the *csrA* overexpressor (Figure 2.6E) even when both strains were growing at the same rate (OD_{600} ~ 0.2), thereby ruling out the possibility that the observed difference in the expression of virulence factors was due to a difference in the growth rate.

CsrA binds to the 5' leader segment of the *grlRA* transcript

Three predicted CsrA binding sites were identified in the leader segment of the *grlRA* transcript (Figure 2.7A & B). Transcripts that are repressed by CsrA often have binding sites that overlap the Shine-Dalgarno sequence or are located nearby (Baker et al., 2007, Wang et al., 2005). One of the predicted CsrA binding sites in *grlRA* is located one codon downstream of the translation initiation codon (Figure 2.7B). Bioinformatic analysis together with the observation that transcript levels for *grlR* and *grlA* were reduced in the *csrA*-overexpressing strain (Figure 2.6C & D), suggested that CsrA, when present at high levels, might repress these genes by binding to the leader segment of the *grlRA* transcript. Binding of CsrA to the *grlRA* leader segment was evident at a CsrA concentration of 2.5 nM; at 80 nM, all of the transcript was present in the bound form (Figure 2.7C). Multiple shifted ribonucleoprotein species were evident at CsrA concentrations as low as 20 nM (Figure 2.7C, S1 & S2). On further increasing the CsrA concentration (≥320 nM), the transcript was evident in supershifted CsrA-RNA complexes S3 and S4 (Figure 2.7C). Binding to the *grlRA* leader was specific because unlabeled *grlRA* but not *phoB* RNA competed with the labeled transcript (Figure 2.7D). The apparent equilibrium-binding constant of CsrA for *grlRA* was 6 ± 0.8 nM (Figure 2.7E). Taken together, these data suggest that overexpression of CsrA globally represses the transcription from the LEE in part by binding to the *grlRA* transcript and repressing the expression of GrlA.

***csrA* regulates motility and glycogen biosynthesis in EPEC**

In non-pathogenic *E. coli* K-12 CsrA activates flagellar biogenesis by directly binding to the untranslated leader segment of the *flhDC* transcript and increasing its half-life (Wei

et al., 2001). Similarly, inactivation of *csrA* rendered EPEC non-motile, whereas complementation of the mutant with *csrA* restored motility (Figure 2.8A). High levels of CsrA appeared to have a dose-dependent effect on motility, as the overexpressor was hypermotile compared to the empty vector containing strain (Figure 2.8A). Consistent with the observed reduction in motility, *flhD* transcript levels exhibited a ~6-fold reduction in the *csrA* mutant and the transcript levels were restored in the complemented strain (Figure 2.8B). Besides motility, *csrA* also regulated glycogen biosynthesis in EPEC with a *csrA* mutation resulting in elevated glycogen levels (Figure 2.8C). Glycogen biosynthesis was repressed in a dose-dependent manner as multicopy presence of *csrA* in EPEC (EPEC(pCRA16)) did not elicit any color change on exposure to iodine in comparison to EPEC containing the empty vector (EPEC(pBR322)) (Figure 2.8C).

Discussion

We have shown that *csrA* regulates gene expression from the LEE pathogenicity island of EPEC and is necessary for pedestal formation and disuption of transepithelial resistance; two *in vitro* correlates of pathogenesis. Whereas disruption and multicopy expression of *csrA* lead to a reduction in the levels of the *LEE4* encoded transcripts, the mechanistic basis differs. The two predicted CsrA binding sites observed in the leader segment of the *sepL* transcript do not overlap the Shine-Dalgarno sequence of *sepL* and are located 17 and 45 nucleotides upstream. Our results suggest that binding of CsrA to the leader segment of the *sepLespADB* transcript results in increased steady-state transcript levels in EPEC. In contrast, multicopy expression of *csrA* decreases transcription from the LEE operons, including *LEE4*, by repressing the expression of the transcriptional activator GrlA (Figure 2.9).

csrA disruption and overexpression also differentially regulate expression of the virulence factor Tir. Whereas *tir* transcript and the bacterium-associated Tir protein levels are unaffected in the *csrA* mutant, reduced secretion of Tir in the *csrA* mutant is partly due to reduced synthesis of EscD and EscF. In the multicopy expressor, by contrast, *tir* transcript levels are greatly diminished resulting in reduced Tir synthesis.

It is noteworthy that *csrA,* when expressed in a single copy, upregulates the expression of genes that form the architectural components of the TTSS (e.g. *escD* and *escF*), translocators that form a filamentous conduit between the EscF needle of the Type III Secretion System and the host cytosol (e.g. *espA*, *espB*, and *espD*), and the "gatekeeper switch" *sepL*. SepL and SepD interact (Deng et al., 2005, O'Connell et al., 2004) to facilitate the hierarchical secretion of translocators over the effectors in response to

changes in calcium and possibly other environmental signals (Deng et al., 2005), perhaps as a means of ensuring the formation of a functional secretion apparatus and subsequent delivery of effectors directly into the host cytosol rather than the media. EPEC with a mutation in *sepL* is insensitive to the presence or absence of calcium and displays dysregulated secretion of effectors and translocators (Deng et al., 2005). Thus, SepL may be a component of a sensor apparatus that detects reduced intracellular calcium concentration in the host cell, and then promotes the release of effectors. Our results suggest that *csrA* is immediately proximal to *sepL* in a regulatory cascade and thus it might play a role in coordinating this hierarchical secretion of translocators over effectors by posttranscriptionally regulating the expression of *sepL* in response to different environmental stimuli. Future experiments aimed at addressing whether *csrA* transcript or CsrA protein levels are affected by environmental factors known to regulate the expression and/or secretion of the *LEE4* encoded genes will shed light on the possible involvement of CsrA in such a hierarchical secretion mechanism.

We have demonstrated that multicopy expression of *csrA* globally represses the expression from the *LEE*, likely via GrlA. A *grlA* mutation might be expected to act epistatically with respect to a *grlR* mutation, because GrlR affects the expression from the LEE locus by binding to and inhibiting GrlA activity (Creasey et al., 2003, Huang & Syu, 2008). Hence a reduction in GrlR and GrlA should lead to an overall transcriptional repression from *lee1,* resulting in reduced levels of the master regulator Ler. Consistent with this idea, *ler* transcript and Ler protein levels, as well as transcription from all LEE operons tested, were reduced in the CsrA overexpressor.

It is intriguing that the affinity of CsrA is higher for the *grlRA* transcript than for the *sepL* transcript, especially as the repression of *grlRA* by CsrA appears to occur only at high CsrA concentrations. A likely explanation is that although CsrA initially binds to the *grlRA* transcript at low concentrations (K_d ~6 nM), it does not produce physiologically relevant changes in gene expression, as evidenced by unchanged GrlA and Ler protein levels in the *csrA* mutant and the wild type strain. However, at higher CsrA concentrations, higher order complexes that affect gene expression may form. Consistent with this idea, multiple CsrA-*grlRA* ribonucleoprotein species are observed in gel shifts at higher CsrA concentrations (40 nM) (Figure 2.7C, band S2). It is unlikely that S2 represents an artifact resulting from nonspecific aggregates of CsrA because whereas almost all of the *grlRA* leader transcript is evident in the supershifted species S2 at 80 nM, *sepL* transcript remained in the S1 form. Notably, a concentration of 40 nM is within the range at which CsrA binds to other *E. coli* mRNAs (Baker et al., 2007). Our data also raises the possibility of at least one additional CsrA-regulated factor that affects gene expression from the LEE in lieu of the fact that *escD* transcript levels are reduced in the *csrA* mutant, but purified CsrA does not bind to its leader segment.

CsrA is a pleiotropic regulator in EPEC as it also regulates motility and glycogen metabolism. The *flhDC* mRNA leader segment contains binding sites for CsrA that mediate its effects on motility in *E. coli* K-12 (Wei et al., 2001). These binding sites are conserved in EPEC. Thus it is likely that reduced *flhDC* transcript levels in the *csrA* mutant of EPEC lead to reduced motility. However, the regulation between EPEC and *E.coli* K-12 does not appear to be identical. *E.coli* K-12 is motile under diverse environmental conditions including LB, LB supplemented with exogenous carbon

sources such as acetate, and succinate, whereas glucose concentrations of 10 mM represses motility (Wei et al., 2001). By contrast, EPEC remains motile on 0.5X DMEM, which contains 12.5 mM glucose, whereas an isogenic *csrA* mutant is non-motile. Thus, unlike K12, glucose does not appear to repress motility in EPEC, and *csrA* is required for maximal motility on DMEM.

Thus, CsrA represents a novel posttranscriptional regulatory molecule in A/E pathogens that activates and represses the expression of the LEE-encoded genes in a concentration-dependent manner. Based on putatively identified CsrA binding sites in the leader segment of *sepL* and *grlRA* of EHEC and *Citrobacter rodentium* (Figure 2.5B & 7B), and the conserved mode of regulation of *ler* by GrlR and GrlA (Deng et al., 2004, Barba et al., 2005), we predict that CsrA functions similarly in these A/E pathogens.

An outstanding question is how EPEC and other A/E pathogens integrate transcriptional and posttranscriptional control of the LEE, with motility and metabolism as they transit through the digestive tract. Our results raise the possibility that an important component of this coordinate regulatory system may be CsrA. Thus, regulation of the concentration or activity of CsrA, through transcriptional control or via its binding partners CsrB/C, in response to extracellular signals from other bacteria, or even signals from the host, may have pronounced effects on pathogenesis *in vivo*. We speculate that it would be advantageous for EPEC to mimic commensal strains, perhaps in a manner akin to the *csrA* overexpressor, during passage through the upper gastrointestinal tract. Thus, increased expression or activity of CsrA would be expected to repress the expression of virulence factors encoded on the LEE (Figure 2.9), thereby minimizing energy expenditures on unneeded transcripts and reducing the possibility of detection of

pathogen specific antigens by the innate immune system. Moreover, increased flagellar motility might facilitate transit to the sites of colonization. As EPEC approaches the small intestine decreasing CsrA protein levels or activity may prove advantageous. Thus, reducing flagellar motility in coordination with increased transcription and translation of translocators, followed by effectors would permit the bacteria to efficiently attach and colonize. Using the mutants we have developed, we are currently testing how coordinate regulation of flagellar motility, metabolism, and LEE expression by CsrA contributes to pathogenesis and immune evasion *in vivo*.

Acknowledgements

We thank Bernie Weiss and Phil Rather for critical evaluation of the manuscript and Guy Benian, Charlie Moran and the members of the Kalman, Romeo, and Weiss labs for helpful discussions and suggestions. We are grateful to Dr. June Scott (Emory University) for providing the wild type EPEC strain EPEC 2348/69, Dr. Jim Kaper and Dr. Michael Donnenberg (University of Maryland) for strains (EPECΔ*espA*, EPECΔ*espB*, EPEC Δ*espD*), and antibodies (α-Tir, α-EspA, and α-EspB), Dr. Jay Mellies (Reed College) for EPECΔ*ler*, Dr. Vanessa Sperandio (UT-Southwestern - Dallas) for pJRLacZIns, Dr. Rebekah DeVinney (University of Calgary) for the α-EspD antibody, Dr. Phil Rather (Emory University) for S17-1λpir, Dr. Bettina Bommarius (Emory University) for DH5α, and Dr.Ilan Rosenshine (The Hebrew University, Jerusalem) for α-Ler. This work was supported by NIH grant R01DK074731-01 and R01-A1056067-01 (to D.K.). S.B. is a recipient of the National Science Foundation Award # 0450303 Subaward # I-66-606-63 to Emory University.

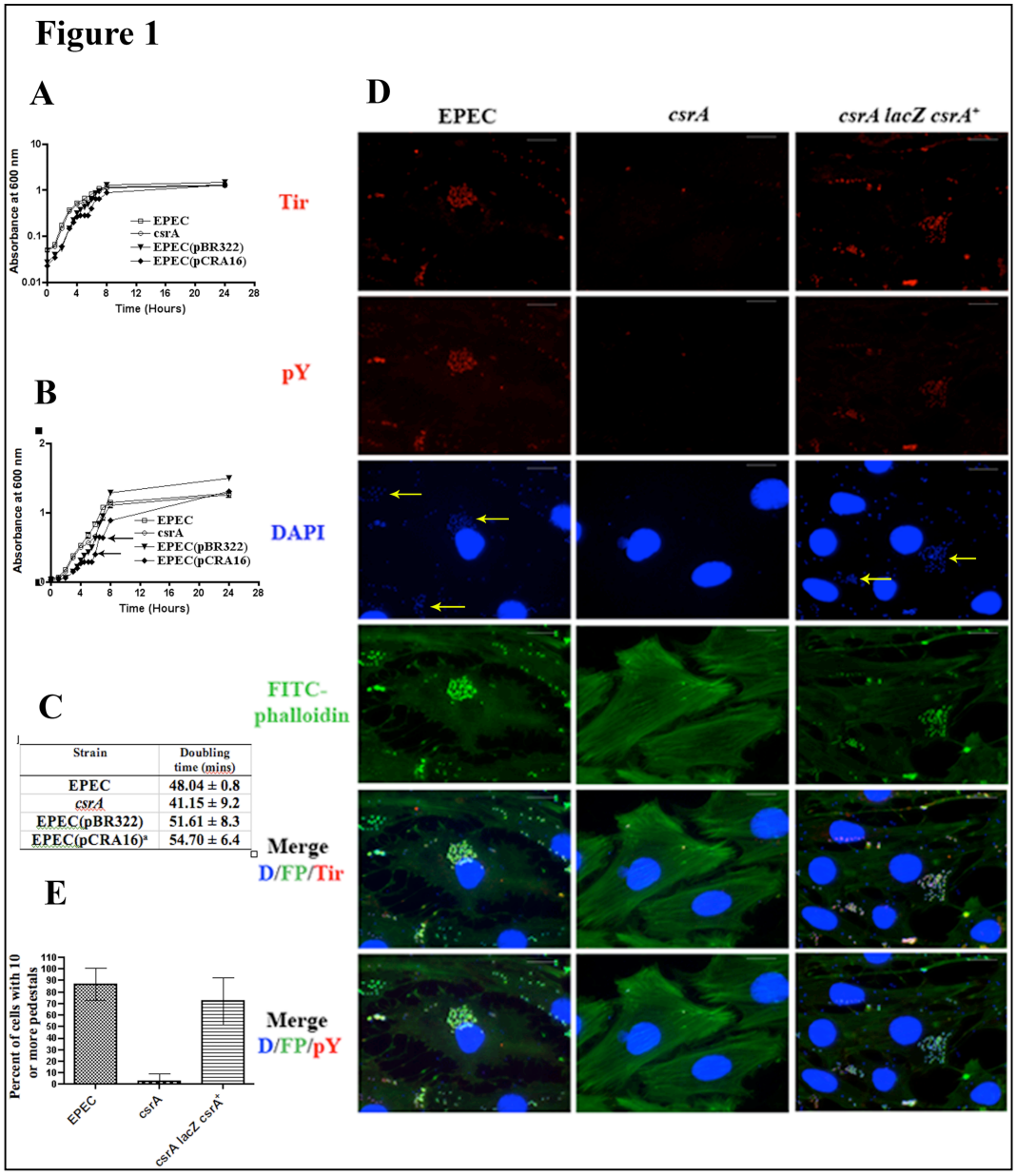

Figure 2.1 – Inactivation of *csrA* does not affect growth but profoundly diminishes adherence, pedestal formation and disruption of transepithelial resistance (TER) by EPEC

A & B – Overnight grown bacterial cultures were diluted in DMEM (lacking phenol red) to a starting OD_{600} of ~ 0.01 and the growth of the strains was measured hourly and

intermittently at half hours. Shown are the growth curves of the strains in the logarithmic (**A**) and linear scale (**B**). EPEC and its isogenic *csrA* mutant grow at similar rates. Furthermore, EPEC(pBR322) and EPEC(pCRA16) also grow at the same rate to an optical density of ~ 0.25-0.3 after which the multicopy *csrA* expressor grows at a slower rate.

C – The doubling time for EPEC, *csrA*-, EPEC(pBR322) and EPEC(pCRA16) were determined from at least two independent experiments with each strain assayed in duplicate per experiment. The values represent the mean ± standard deviation from all the experiments. **a** – The doubling time for EPEC(pCRA16) was determined in the early log phase (OD_{600} < 0.25) prior to the first biphasic lag (lower black arrow) displayed by the strain.

D - 3T3 cells were infected for 5 hours with equal number of bacteria. Cells were then fixed with formaldehyde and permeabilized with Triton-X. Pedestal formation was visualized by Immunofluorescence microscopy by using antibodies against Tir and phosphotyrosine. FITC-phalloidin was used to detect filamentous actin beneath the adherent bacteria. DAPI was used to detect adherent bacteria and the cellular nucleus. Adherence was diminished in the *csrA* mutant as evident from the relatively low number of DAPI stained mutant bacteria compared to the wild type or the complemented strain (Compare column 2 row 3 (*csrA*-) with column 1 row 3 (EPEC) or column 3 row 3 (*csrA-lacZ-csrA*+). Yellow arrows represent clustering of bacteria that was routinely observed in the wild type and complemented strain but not in the *csrA* mutant. The ability to form

pedestals was also reduced in the *csrA* mutant as manifested by diminished Tir, phosphotyrosine (pY), and polymerized actin staining. Pedestal assays were performed on 3 separate occasions from independent bacterial cultures with similar results obtained in each experiment. Images are representative of one such experiment. Scale bars represent 20 microns.

E - The extent of pedestal formation was quantified by counting a total of 100 cells from at least 10 different frames and the percent of cells with 10 or more pedestals per cell was determined. The values and error bars represent the standard deviation of the mean from one such experiment. ** denotes a p-value < 0.001.

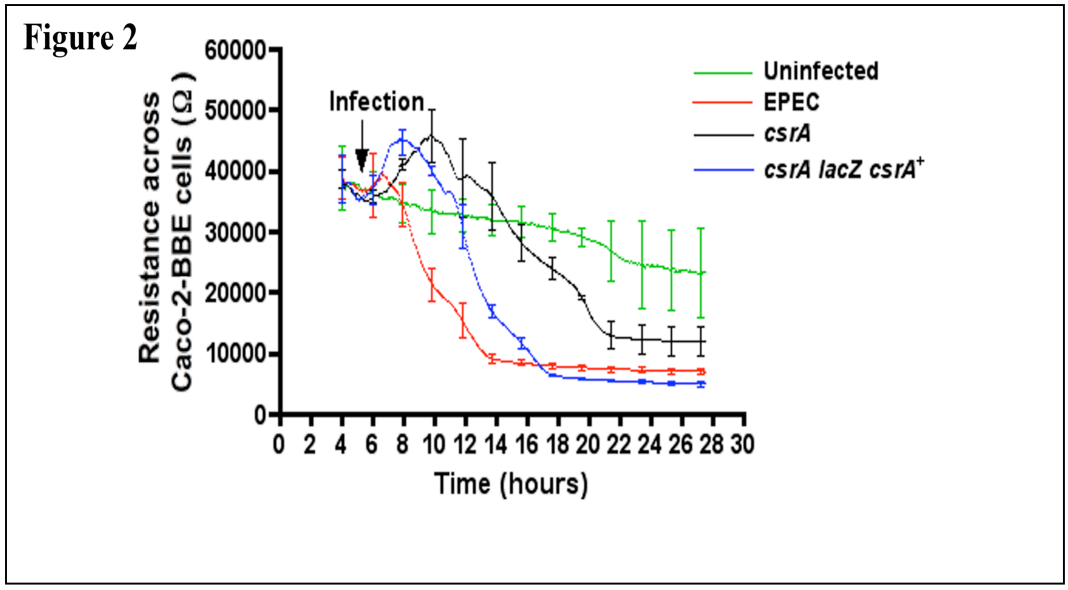

Figure 2.2 – *csrA* is necessary for the disruption of TER across Caco-2 BBE cells

Caco-2-BBE cells were seeded in ECIS 8W1E electrodes (5 x 10^5 cells/400 µl/electrode) and kept at 37˚C in a 5% CO_2 incubator with 90% humidity. Overnight grown bacterial cultures were used to infect the cells at an MOI of 10. Transepithelial resistance was measured continuously post-infection. Disruption of *csrA* delayed the onset and reduced the rate and extent of membrane depolarization. Each experiment was performed on three separate occasions with triplicate samples being assayed every experiment. Error bars represent standard deviation of the mean values obtained at the designated time points from one such experiment. The start of infection of the cells by the bacterial strains is indicated by the word “Infection” on the graph with a black arrow pointing downwards.

Figure 3

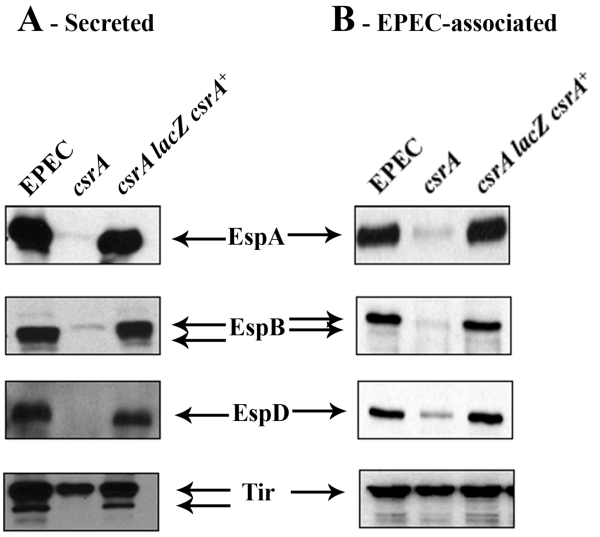

Figure 2.3– Secretion of EspA, EspB, EspD and Tir is diminished in the *csrA* mutant

A & B - EPEC, *csrA*- and the monocopy complemented strain *csrA-lacZ-csrA*+ were grown in DMEM lacking phenol red at 37˚C in a 5% CO_2 incubator to an optical density of ~ 1.0. Western blotting for the secreted (**A**) and EPEC-associated (**B**) EspA, EspB, EspD and Tir was performed using polyclonal antibodies as described in materials and methods. Whereas both the secreted and the bacterium-associated levels of EspA, EspB, and EspD were reduced only the secretion of Tir was reduced in the *csrA* mutant. Western blot experiments were conducted on at least three separate occasions with similar results. The image is a representation of one such experiment.

Figure 4

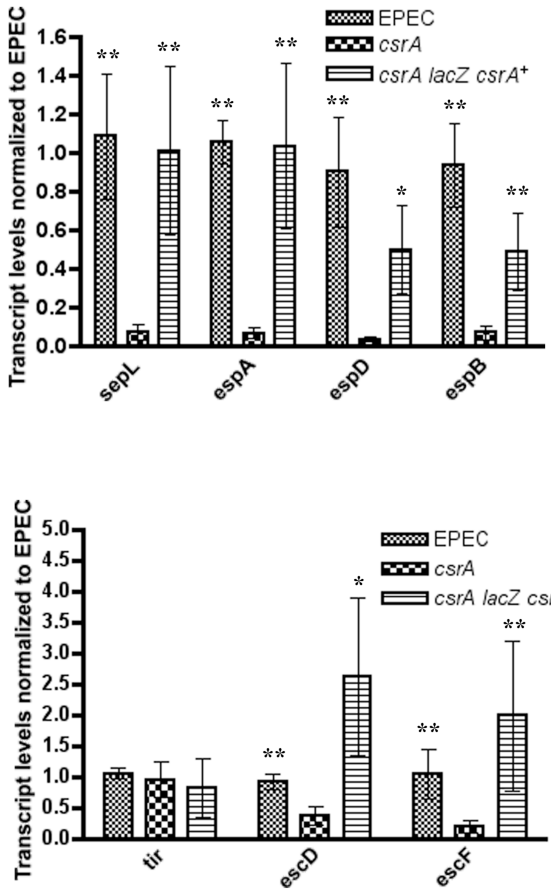

Figure 2.4 - *sepL*, *espA*, *espD, espB*, *escD* and *escF*, but not *tir*, transcript levels are reduced in the *csrA* mutant

A & **B** - RNA was isolated from bacterial cultures grown to an optical density of ~1.0 and RT-qRT-PCR was performed as described in materials and methods. Shown are the relative transcript levels of *sepL*, *espA*, *espD*, *espB* (**A**), *tir*, *escD*, and *escF* (**B**) in *csrA*- and *csrA-lacZ-csrA+* strains normalized to wild type EPEC. Results are means ± standard deviation from triplicate experiments with each sample being assayed in duplicate per

experiment. The unpaired student's *t*-test was used to assay for statistical significance between the *csrA*- mutant and EPEC or *csrA-lacZ-csrA*+ strain. A *p*-value <0.01 was considered statistically significant. ** denotes a *p*-value < 0.001 whereas * denotes a *p*-value < 0.005

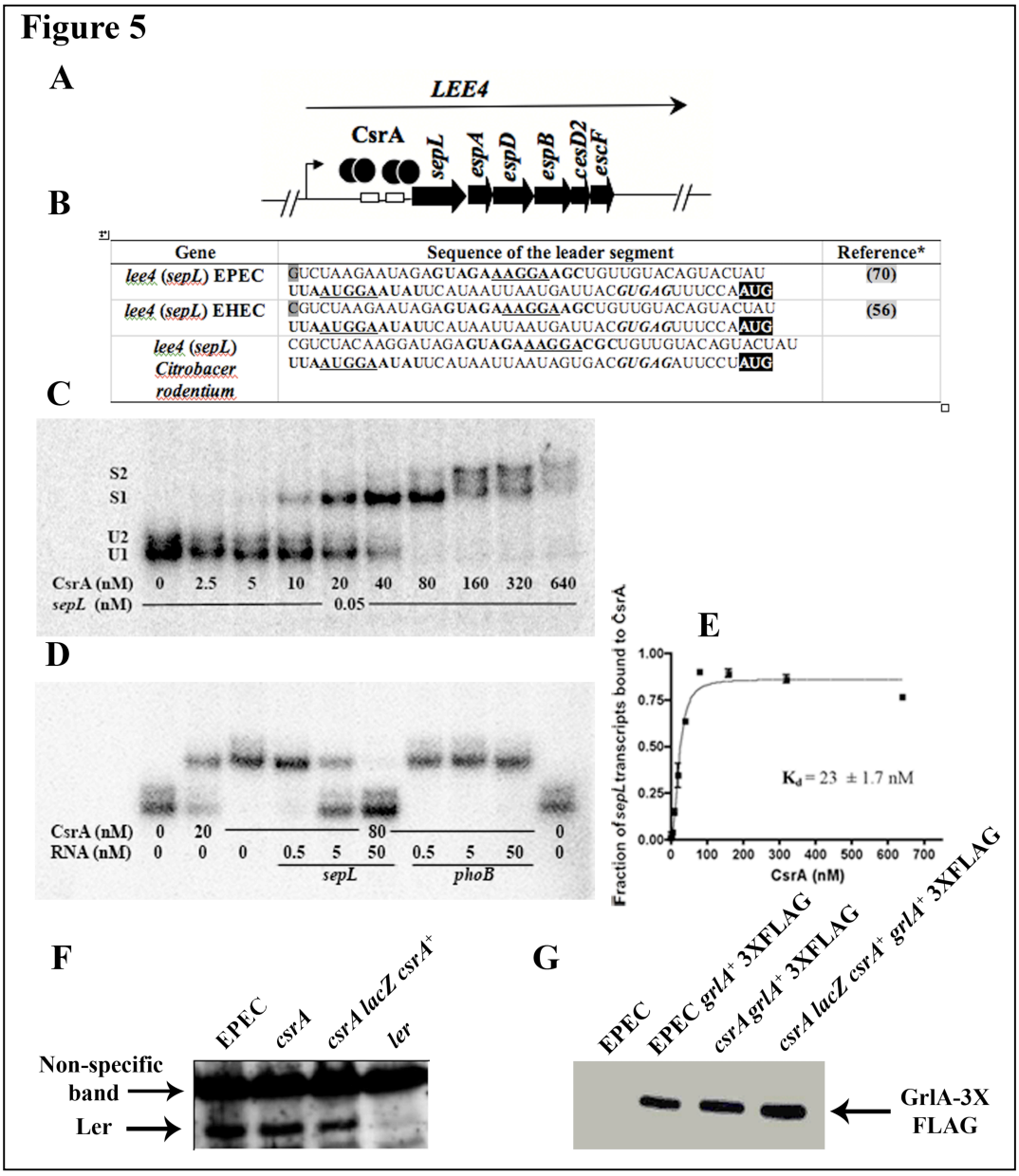

Figure 2.5 – CsrA binds to the leader segment of the *LEE4* operon

A - A cartoon depicting the location of putative CsrA binding sites in the leader segment of the *LEE4* transcript. The thick black arrows depict the genetic organization of *sepL*, *espA*, *espD*, *espB*, *cesD2* and *escF*. Two putative CsrA binding sites identified

upstream of *sepL* are depicted by white rectangles. The vertical line with an arrow at the apex indicates the transcription start site for the *LEE4* operon in EPEC.

B - The primary sequence in the leader segment of the *sepL* transcript of EPEC, EHEC and *Citrobacter rodentium* was scanned to determine the presence of putative CsrA binding sites by comparing the sequence to the SELEX [Selected evolution of ligands by exponential enrichment] determined CsrA consensus sequence [A/GUAC**AA/GGGA**UGU] (Dubey et al., 2005). The σ^{70} transcriptional start site for *sepL* is highlighted in grey. The predicted Shine-Dalgarno (S-D) sequence for the transcript is in bold and italicized. The predicted CsrA-binding sites are in boldface. Note that each of the predicted CsrA binding site contains the highly conserved ANGGA motif (boldface and underlined) present in known CsrA-regulated transcripts (Babitzke & Romeo, 2007, Lucchetti-Miganeh et al., 2008). The highlighted AUG represents the translation initiation codon. Reference* - Reference for the transcriptional start site and/or the predicted S-D sequence.

C - RNA-EMSA was performed using purified His-tagged CsrA and ^{32}P labeled *sepL* leader RNA as described in materials and methods. CsrA bound to the leader segment of *sepL* at a concentration of 10 nM (S1). Higher molecular weight ribonucleoprotein complexes were observed with increasing CsrA concentration ≥ 80 nM (S2). U1 and U2 represent the unbound transcript whereas S1 and S2 represent shifted ribonucleoprotein complexes.

D - Binding to the *sepL* transcript was specific because unlabeled *sepL* but not the non-competitor RNA, *phoB*, successfully competed with the labeled transcript. CsrA, *sepL* and *phoB* concentrations are indicated at the bottom of each lane.

E - The calculated apparent equilibrium binding constant of CsrA for *sepL* was 23 ± 1.7 nM. Error bars represent standard error of the means determined from two independent experiments.

F & **G** - The steady-state protein levels of Ler in EPEC, *csrA*- and *csrA-lacZ-csrA*+ (**F**) or chromosomally 3X-FLAG tagged GrlA in EPEC *grlA*3XFLAG, *csrA-grlA*3XFLAG and *csrA-lacZ-csrA*+*grlA*3XFLAG (**G**) were determined by western blotting. Ler and 3X-FLAG tagged GrlA protein levels were unaffected in the *csrA* disruptant.

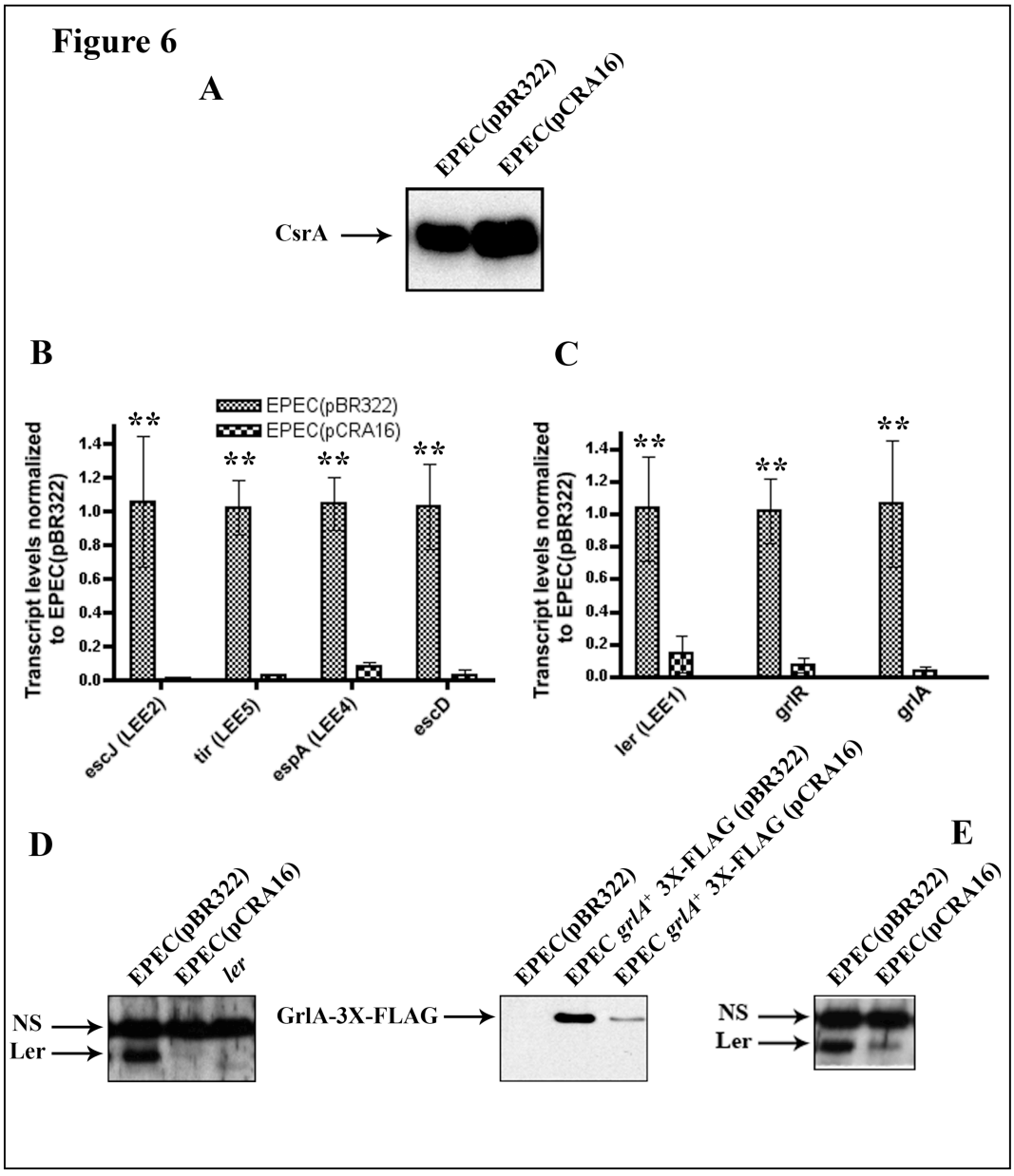

Figure 2.6 – Expression of *csrA* from a multicopy plasmid globally represses the expression from the LEE via GrlA

A - Overnight grown cultures of EPEC(pBR322) and EPEC(pCRA16) were diluted in DMEM and allowed to grow to an optical density of ~ 1.0. CsrA protein levels were

elevated by ~ 1.5 ± 0.1 in the overexpressor [EPEC(pCRA16)] compared to the empty vector containing strain [EPEC(pBR322)].

B & **C**- RNA was isolated from EPEC(pBR322) and EPEC(pCRA16) grown to an optical density of ~1.0 and RT-qRT-PCR for *escJ* (*LEE2*), *tir* (*LEE5*), *espA* (*LEE4*), *escD* (**B**), and *ler* (*LEE1*), *grlR* and *grlA* (**C**) transcripts were performed as described above. Multicopy expression of *csrA* repressed the transcript levels from all the tested *LEE* operons. Error bars represent standard deviation of the mean from two separate experiments with triplicate samples assayed every experiment. The unpaired student's *t*-test was used to assay for statistical significance between EPEC(pBR322) and EPEC(pCRA16). A p-value < 0.01 was considered statistically significant. ** denotes a p-value < 0.001.

D - Western blotting for the steady-state protein levels of Ler and 3X-FLAG tagged GrlA were performed on cell lysates of EPEC(pBR322) and EPEC(pCRA16) grown to an optical density of ~ 0.5. Multicopy expression of *csrA* repressed the steady-state protein levels of Ler and GrlA-3X-FLAG. NS refers to a cross-reacting non-specific band.

E – The steady-state Ler levels were reduced in the overexpressor even when both the strains were growing at the same rate (OD_{600} of ~ 0.2) suggesting that reduced expression is not the result of altered growth kinetics of the overexpressor. NS refers to a cross-reacting non-specific band. EPEC(pBR322) and EPEC(pCRA16) grow at comparable

rates to an $OD_{600} \sim 0.25$ after which the overexpressor grows at a slightly slower rate (**Figure 1A**, **B**, and **C**).

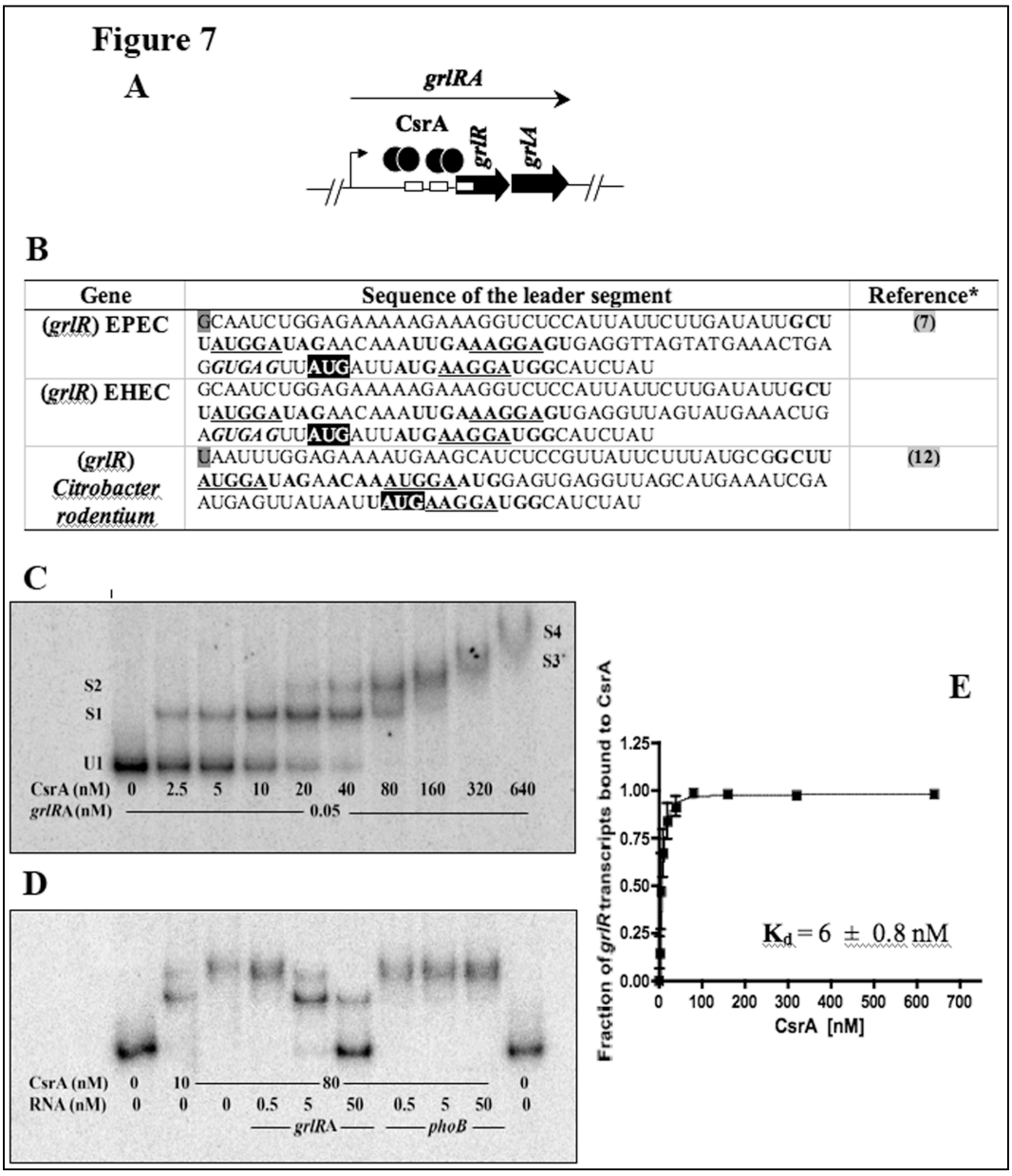

Figure 2.7– Purified CsrA binds to the leader segment of the *grlRA* transcript

A - A cartoon depicting the location of putative CsrA binding sites in the leader segment of the *grlRA* transcript. The thick black arrows depict the genetic organization of *grlR* and *grlA* respectively in the *grlRA* operon. The three white rectangles in the leader segment and the 5' region of the *grlR* ORF represent the predicted sites to which CsrA

dimers (bound black balls) might bind. The vertical line with an arrow at the apex indicates the transcription start site.

B - The primary sequence in the leader segment of the *grlRA* transcript of EPEC, EHEC and *Citrobacter rodentium* was scanned to determine the presence of putative CsrA binding sites. The σ^{70} transcriptional start site for the *grlRA* operon is highlighted in grey. The predicted Shine-Dalgarno sequence for the transcript is highlighted in bold and italicized. The predicted CsrA-binding sites are in boldface. Note that each of the predicted CsrA binding site contains the highly conserved ANGGA motif (boldface and underlined) (Lucchetti-Miganeh et al., 2008, Babitzke & Romeo, 2007). The highlighted trinucleotide AUG represents the translation initiation codon. Reference * - reference for the transcriptional start site.

C - RNA-EMSA using purified His-tagged CsrA and ^{32}P labeled *grlRA* leader RNA was performed as described above. The binding of CsrA to the leader segment of the *grlRA* transcript began to occur at a concentration of 2.5 nM. Higher molecular weight ribonucleoprotein complexes were observed beginning at concentrations of 20 nM (S2, S3 and S4). CsrA, and *grlRA* concentrations are indicated at the bottom of each lane. U1 represents the unbound transcript whereas S1, S2, S3 and S4 represent shifted ribonucleoprotein complexes.

D - Binding to *grlRA* was specific because high concentrations of unlabeled *grlRA*, but not the non-competitor RNA, *phoB*, successfully competed with the labeled transcripts.

E - The apparent equilibrium-binding constant of CsrA for the *grlRA* transcript was 6 nM. The error bars represent standard error of the means determined from two independent experiments.

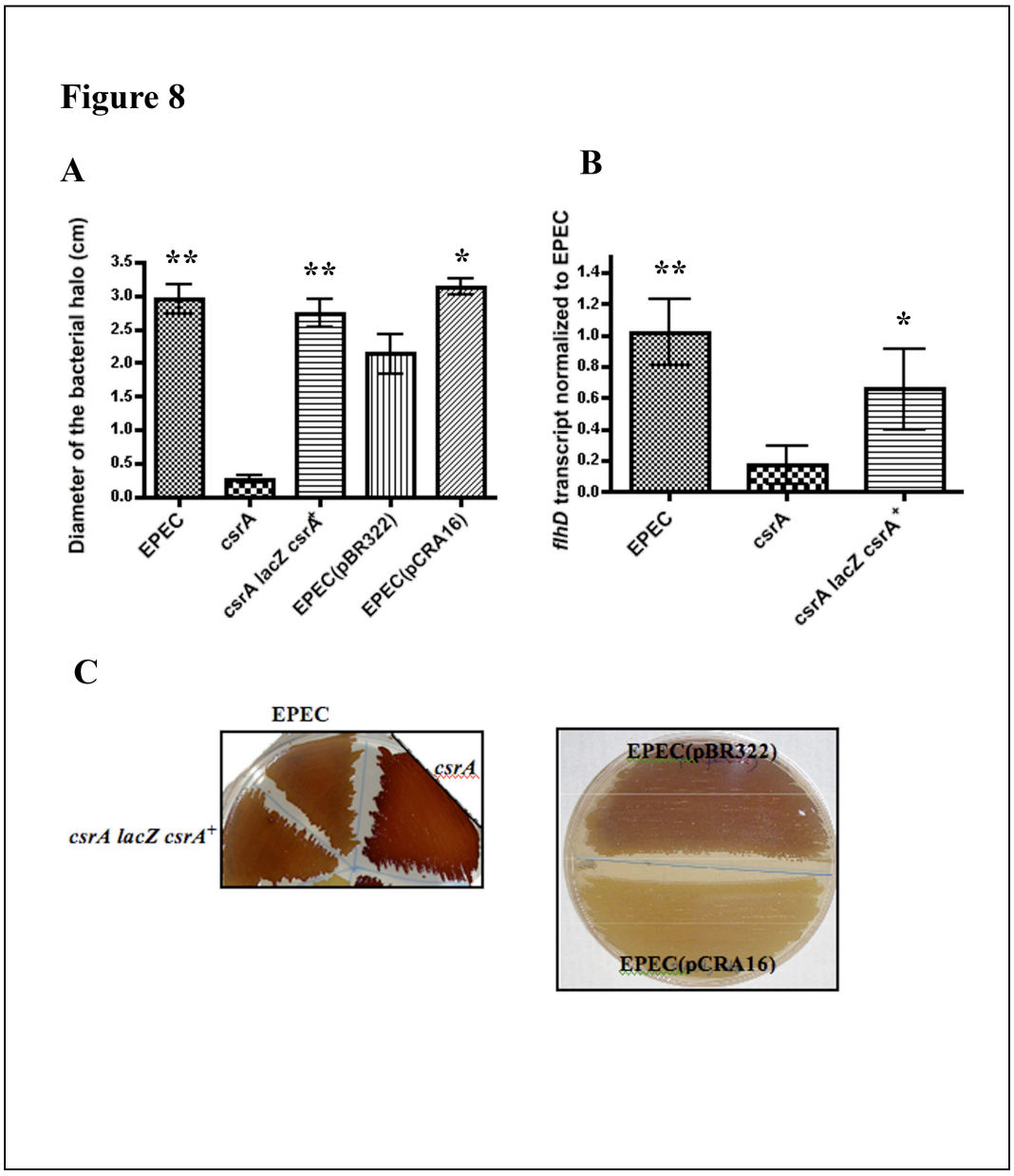

Figure 2.8 – *csrA* activates motility and represses glycogen biosynthesis in EPEC

A - For motility assays overnight grown cultures were diluted in LB or LB supplemented with the appropriate antibiotic and allowed to grow to an optical density of 1.0. 1 µl of the culture was stabbed onto 0.3% 0.5 X DMEM agar plates and incubated for 30 hours at 37˚C. Motility assays were performed in triplicate and the diameter of the bacterial halo was measured as an indicator of bacterial motility. The unpaired student's

t-test was used to assay for statistical significance between the *csrA* mutant and EPEC or *csrA-lacZ-csrA+* or between EPEC(pBR322) and EPEC(pCRA16). A p-value < 0.01 was considered statistically significant. ** denotes a p-value < 0.001 and * denotes a p-values < 0.01.

B - RT-qRT-PCR for *flhD* [Master regulator of the flagella] was performed as described above. *flhD* transcript levels were substantially reduced in the *csrA-* mutant and restored when the mutant was complemented with *csrA* in monocopy (B). ** denotes a p-value < 0.001 whereas * denotes a p-value < 0.005.

C – For measuring glycogen biosynthesis bacterial cultures were grown to an OD_{600} of ~1.0 and streaked onto Kornberg plates and incubated at 37˚C overnight after which the plates were exposed to iodine vapors. Glycogen levels were elevated in the *csrA* mutant compared to the wild type (EPEC) or the complemented strain (*csrA-lacZ-csrA+*) as evident from increased black-brown color formation in the mutant. *csrA* repressed glycogen biosynthesis in a dose-dependent manner because multicopy expression of *csrA* in the wild type background (EPEC(pCRA16)) did not lead to a color change in contrast to the brown color observed for the empty vector containing strain (EPEC(pBR322)) which contains a single copy of *csrA*.

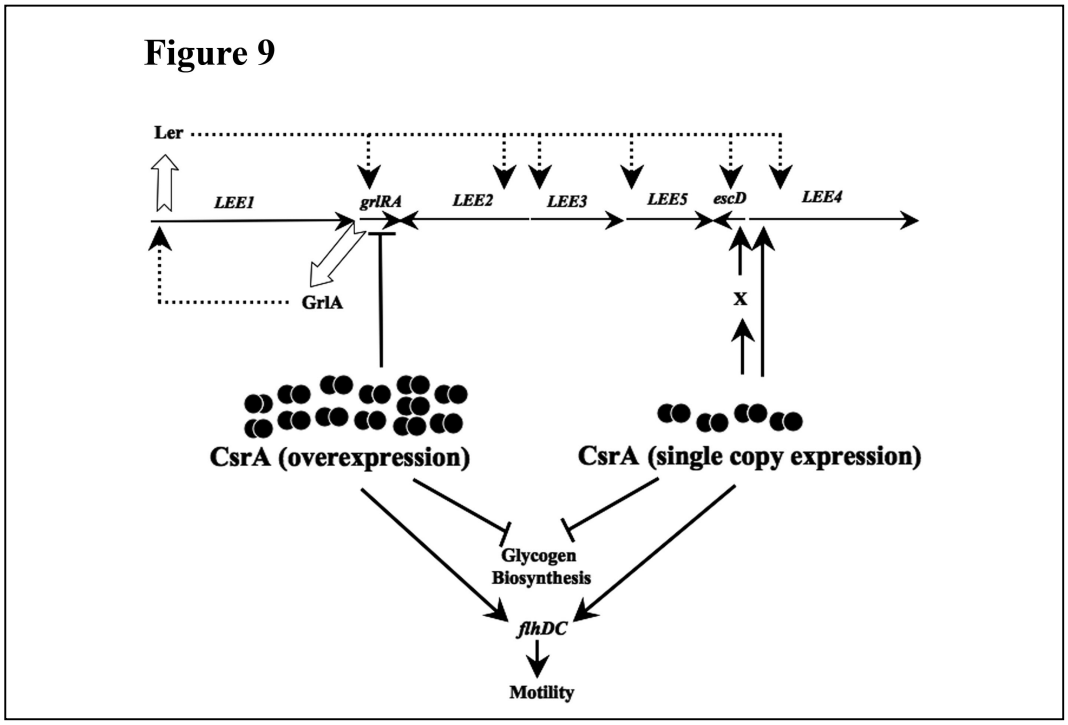

Figure 2.9 – Model for the CsrA mediated regulation of the LEE

When expressed in monocopy binding of CsrA to the *LEE4* operon encoding *sepLespADB* and *escF* transcript results in their increased steady-state transcript levels. However, activation of *escD* occurs indirectly via an intermediate regulator(s). For the purpose of simplicity the effect is shown to occur via an activator [X]. In contrast to the *csrA* mutant, overexpression of *csrA* globally represses the transcription from the LEE. This is achieved in part by CsrA binding to the leader segment and resulting in reduced *grlRA* transcript and consequent GrlA protein levels. Reduced GrlA protein levels lead to reduced Ler protein levels which in turn results in reduced transcription from the other LEE-encoded operons. *csrA* also promotes motility by upregulating the *flhDC* transcript levels in EPEC and represses glycogen biosynthesis. Dotted arrows represent positive genetic circuits that have been demonstrated previously, thick transparent arrows

represent the transcription activating gene product encoded in the *LEE1* and the *grlRA* operons, whereas thick filled arrows with shard ends (activated circuits) and thick filled arrows with blunted ends (repressed circuits) represent novel genetic circuits and/or phenotypes of EPEC identified in this paper.

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CHAPTER 3

CsrA and TnaB coregulate tryptophanase activity to promote exotoxin-induced killing of *Caenorhabditis elegans* by enteropathogenic *Escherichia coli*

Shantanu Bhatt[1,2], Akwasi Anyanful[1], and Daniel Kalman[1,2,†]

[1] Department of Pathology and Laboratory Medicine, Emory University, Atlanta, Georgia, USA 30322

[2] Microbiology and Molecular Genetics Program, Emory University, Atlanta, Georgia, USA 30322

† - This chapter consists of a manuscript that was published as –

Bhatt, S., A. Anyanful, & D. Kalman, (2011) CsrA and TnaB coregulate tryptophanase activity to promote exotoxin-induced killing of *Caenorhabditis elegans* by enteropathogenic *Escherichia coli*. ***Journal of Bacteriology*** **193: 4516-4522.**

Akwasi Anyanful conducted the paralysis and killing assay in Figure 3.1C & 3.1D. Shantanu Bhatt conducted the remainder of the experiments. Shantanu Bhatt and Daniel Kalman wrote the manuscript.

Abstract

Enteropathogenic *Escherichia coli* (EPEC) requires the *tnaA*-encoded enzyme, tryptophanase, and its substrate tryptophan to synthesize diffusible exotoxins that kill the nematode *Caenorhabditis elegans*. Here we demonstrate that the RNA-binding protein CsrA and the tryptophan permease TnaB coregulate tryptophanase activity, through mutually exclusive pathways, to stimulate toxin-mediated paralysis and killing of worms.

Introduction

Enteropathogenic *Escherichia coli* (EPEC) belongs to the attaching and effacing (A/E) family of pathogens, the other members of which include enterohemorrhagic *Escherichia coli* (EHEC) and *Citrobacter rodentium* (Luperchio *et al.*, 2000, Schauer & Falkow, 1993a, Schauer & Falkow, 1993b, Kaper *et al.*, 2004, Nataro & Kaper, 1998, Chen & Frankel, 2005, Mellies *et al.*, 2007, Bhatt *et al.*, 2011). Upon infection A/E pathogens bind to intestinal epithelia and destroy the cellular microvilli in their vicinity (Chen & Frankel, 2005, Mellies et al., 2007, Bhatt et al., 2011). Subsequently, the bacteria recruit several host factors that cooperate to promote the biogenesis of actin-filled membranous protrusions, termed "pedestals," beneath adherent bacteria (Chen & Frankel, 2005, Mellies et al., 2007, Kaper et al., 2004, Nataro & Kaper, 1998, Knutton *et al.*, 1989, Deng *et al.*, 2004). Pedestal formation is accompanied by severe diarrhea, which results in significant morbidity and mortality worldwide (Chen & Frankel, 2005, Senerwa *et al.*, 1989, Gomes *et al.*, 1991).

Penetrance of the A/E pathomorphology requires the pathogenicity island (PAI), locus of enterocyte effacement (LEE) that encodes for the regulators, structural components of a type III secretion system (T3SS), and several of its secreted effector molecules (Mellies et al., 2007, Elliott *et al.*, 2000, Deng et al., 2004, Mellies *et al.*, 1999, Bhatt et al., 2011, Sekiya *et al.*, 2001, Daniell *et al.*, 2001, Wilson *et al.*, 2001, Kenny *et al.*, 1997, Croxen & Finlay, 2010, Kaper et al., 2004). The *LEE1*-encoded master regulator, Ler, orchestrates the coordinated transcription from the other LEE operons to promote morphogenesis of the T3SS that forms a continuous conduit between the bacterial and the host cytoplasm (Barba *et al.*, 2005, Bustamante *et al.*, 2001, Mellies et al., 2007, Elliott et

al., 2000, Deng et al., 2004, Mellies et al., 1999, Friedberg *et al.*, 1999, Sperandio *et al.*, 2000, Kenny et al., 1997). Subsequently, effectors including the translocated intimin receptor (Tir) are trafficked into the host (Kenny et al., 1997, Croxen & Finlay, 2010, Kaper et al., 2004). Tir integrates into the host plasma membrane where it serves as a receptor for its ligand, the adhesin intimin, located on the outer bacterial membrane (Kenny et al., 1997). Tir-intimin interactions initiate a signal transduction cascade that leads to actin polymerization and pedestals (Gruenheid *et al.*, 2001, Kalman *et al.*, 1999, Swimm *et al.*, 2004, Bommarius *et al.*, 2007, Kenny et al., 1997).

A significant obstacle towards elucidating the pathobiology of EPEC infections is that this bacterium is a human pathogen that neither colonizes nor causes disease in mice (Mundy *et al.*, 2006). Over the past decade, the capacity of bacterial pathogens, including EPEC, to kill the nematode *Caenorhabditis elegans* has been utilized to determine virulence determinants in the bacteria or host that may be relevant to pathogenesis in mammalian systems (Sifri *et al.*, 2005, Mellies & Lawrence-Pine, 2010). Its small size, rapid generation time, large brood size, amenability to genetic manipulation, and high degree of homology to humans and other mammals make *C. elegans* a useful experimental system with which to study bacterial toxins or infection (Aballay & Ausubel, 2002, Mellies & Lawrence-Pine, 2010, Sifri *et al.*, 2003, Sifri et al., 2005).

The morbidity and mortality of *C. elegans* by noxious microbes can be classified into two broad categories on the basis of whether the pathogen makes contact with the worm (Aballay & Ausubel, 2002, Mellies & Lawrence-Pine, 2010, Sifri et al., 2003, Sifri et al., 2005). Contact-dependent killing usually involves the detrimental colonization of the worm in the form of a biofilm (e.g. - *Yersinia pestis*) (Darby *et al.*, 2002, Tan & Darby,

2004), an invasive infection (e.g. - *Streptomyces albireticuli*) (Park *et al.*, 2002), or accumulation within the intestine (e.g. – EPEC) (Mellies *et al.*, 2006). The death of the nematode, as a consequence of colonization, typically occurs over several days and is referred to as "slow-killing" (Sifri et al., 2005). By contrast, contact-independent killing is mediated through structurally and functionally unrelated exotoxins that are secreted by diverse pathogens including EPEC (Anyanful *et al.*, 2005, Anyanful *et al.*, 2009), *Pseudomonas aeruginosa* (Mahajan-Miklos, 1999, Gallagher L.A., 2001), and *Burkholderia cenocepacia* (Kothe *et al.*, 2003), amongst others, that lead to toxicity in the nematode (Sifri et al., 2005). Intoxication of the worms is a relatively rapid pathophysiological process occurring over a period of hours and is referred to as "fast-killing" (Sifri et al., 2005). The utility of *C. elegans* as a surrogate host for mimicking bacterial infections has been repetitively substantiated by numerous studies in which novel virulence factors that were identified employing worm-based screens were subsequently shown to modulate virulence in mammalian systems (Anyanful et al., 2005, Sifri et al., 2005, Garsin *et al.*, 2001, Mahajan-Miklos, 1999, Tan, 1999a). In reciprocal studies, virulence factors originally implicated in mammalian and plant pathogenesis were demonstrated to coregulate pathogenesis in worms (Sifri et al., 2003, Tan, 1999b, Sifri et al., 2005).

EPEC is capable of killing *C. elegans* by contact-dependent and -independent means (Anyanful et al., 2005, Mellies et al., 2006). On minimal nematode growth media (NGM) EPEC kills *C. elegans* over a period of several days by colonizing its intestinal tract (Mellies et al., 2006). However, no virulence factors that contribute to the contact-dependent killing of the worm have thus far been discovered (Mellies et al., 2006).

Moreover, none of the pathogenicity determinants previously implicated in mammalian pathogenesis were necessary for nematocidal activity (Mellies et al., 2006). By contrast, on nutritionally rich media (Luria-Bertani [LB] or *E. coli* Direct Agar [ECD]) supplemented with tryptophan, EPEC synthesizes diffusible exotoxins that lead to rapid paralysis and subsequent death of the nematode within a few hours (Anyanful et al., 2005, Anyanful et al., 2009). Exotoxin-induced lethality requires the bacterial enzyme tryptophanase. Subsequently, it was shown that tryptophanase regulates the LEE in both EPEC and EHEC and consequently influences pedestal formation and mammalian pathogenesis (Anyanful et al., 2005, Hirakawa *et al.*, 2009). However, other than tryptophanase, the EPEC-*C. elegans* pathosystem has not been utilized to identify additional virulence determinants that contribute to morbidity in mammals.

In a previous study we reported that the RNA-binding protein, CsrA, is necessary for EPEC to form pedestals on mammalian cells (Bhatt *et al.*, 2009, Bhatt et al., 2011). CsrA and its ortholog, RsmA, recognize AGGA/ANGGA tracts in the 5'-untranslated leader segments of transcripts and modulate mRNA stability and/or translation (Bhatt et al., 2009, Bhatt et al., 2011, Dubey *et al.*, 2005, Romeo, 1998). The relaxed sequence specificity of CsrA/RsmA enables this posttranscriptional regulator to modulate a panoply of physiological traits such as carbon homeostasis (Romeo *et al.*, 1993, Romeo, 1998, Sabnis *et al.*, 1995, Baker *et al.*, 2002, Dubey *et al.*, 2003), peptide uptake (Dubey et al., 2003), biofilm formation (Jackson *et al.*, 2002, Wang *et al.*, 2005), motility (Bhatt et al., 2009, Lawhon *et al.*, 2003, Wei *et al.*, 2001, Yakhnin *et al.*, 2007, Burrowes *et al.*, 2006), quorum sensing (Lenz *et al.*, 2005, Cui *et al.*, 1995), colicin biosynthesis (Yang *et*

al., 2010), and virulence (Cui et al., 1995, Brencic & Lory, 2009, Goodman *et al.*, 2004, Bhatt et al., 2009, Lenz et al., 2005, Forsbach-Birk *et al.*, 2004, Kerrinnes *et al.*, 2009).

Results and Discussion

Here we have evaluated the role of CsrA in the toxin-mediated killing of *C. elegans.* Bioassays employing worms were conducted on LB agar plates containing or lacking tryptophan essentially as described previously with the modification that tryptophan was added to a final concentration of 1 mg/ml and 200 µl of the overnight inoculum was seeded onto plates (Anyanful et al., 2005). Disruption of *csrA* abolished the ability of EPEC to paralyze (Figure 3.1A) and kill *C. elegans* (Figure 3.1B). The *csrA* mutant regained its pathogenicity when complemented in *trans* with the plasmid pCRA16 that expresses *csrA* under its native promoters (Figure 3.1A & B) (Figure 3.1) (Wang *et al.*, 2004).

The observation that disruption of *csrA* genocopies the effect of deleting *tnaA* (Figure 3.1) suggested that the two genes might constitute components of the same regulatory pathway. In *E. coli tnaA* is the central gene within a tricistronic operon that includes the upstream regulatory gene *tnaC* and the downstream structural gene *tnaB* (Gong & Yanofsky, 2002b, Gong & Yanofsky, 2002a, Deeley & Yanofsky, 1981, Deeley & Yanofsky, 1982). *tnaC* encodes a *cis*-acting regulatory peptide that governs the expression of *tnaA* and *tnaB* in response to tryptophan accumulation (Gong & Yanofsky, 2001, Gong *et al.*, 2001, Stewart & Yanofsky, 1985). *tnaA* encodes for the catabolic enzyme tryptophanase which catalyzes the hydrolysis of tryptophan into indole, pyruvate and ammonia whereas *tnaB* specifies a low-affinity tryptophan permease that facilitates

the import of tryptophan into the bacterium (Deeley & Yanofsky, 1981, Yanofsky *et al.*, 1991, Sarsero *et al.*, 1991, Snell, 1975, Lee & Phillips, 1995). To elucidate the regulatory hierarchy of *csrA* and *tnaA*, each gene was expressed from a multicopy plasmid in the mutant background of the other. Whereas multicopy expression of *csrA* failed to restore virulence to the *tnaA* mutant, overexpression of *tnaA*, from the medium copy number plasmid pMD6 (Figure 3.1), suppressed the attenuated phenotype of the *csrA* mutant and restored its ability to paralyze (Figure 3.1C) and kill *C. elegans* (Figure 3.1D). The observation that increased expression of *tnaA* circumvents the requirement for a functional *csrA* allele raised the possibility that *tnaA* might act downstream of CsrA in a putative regulatory pathway. To test this possibility, we assayed tryptophanase activity by measuring the hydrolysis of the chromogenic tryptophan analogue S-O-nitrophenyl-L-cysteine to O-nitrothiophenolate in bacterial lysates that had been precultivated on agar plates containing or lacking tryptophan, essentially as described previously (Anyanful et al., 2005). Accordingly, tryptophanase activity was dramatically reduced in the *csrA* mutant (Figure 3.1E). Moreover, this effect occurred independently of the addition of exogenous tryptophan (Figure 3.1E). Collectively, these results suggest that the inability of the *csrA* mutant to paralyze and kill the nematode results from reduced tryptophanase activity, and that *tnaA* acts distally to *csrA*.

In *E. coli* the *tnaCAB* operon is subject to transcriptional as well as posttranscriptional control (Blankenhorn *et al.*, 1999, Bordi *et al.*, 2003, Botsford & DeMoss, 1971, Chant & Summers, 2007, Gong & Yanofsky, 2002b, Gong & Yanofsky, 2002a, Stewart *et al.*, 1986). The nascent leader peptide, TnaC, while translocating through the exit tunnel of the ribosome transduces conformational alterations in the ribosome to generate an

stereospecific L-tryptophan-binding site near the peptidyltransferase center (Stewart, 2008). Bound tryptophan promotes ribosomal stalling, which in turn masks the *boxA-rut* riboelement of the transcriptional terminator Rho that overlaps the C-terminus as well as the segment immediately downstream of the *tnaC* ORF (Gong & Yanofsky, 2001, Gong et al., 2001, Stewart & Yanofsky, 1985). Consequently, Rho does not bind to the transcript and RNA polymerase is not offloaded and continues to transcribe the downstream genes, *tnaA* and *tnaB* (Gong & Yanofsky, 2001, Gong et al., 2001, Gong & Yanofsky, 2002b, Gong & Yanofsky, 2002a, Gong & Yanofsky, 2003a, Gong & Yanofsky, 2003b). Thus, tryptophan posttranscriptionally induces the expression from the *tnaCAB* operon in *E. coli* (Stewart, 2008). The primary structure of the TnaC leader peptide as well as the nucleotide sequence of the *boxA-rut* site within the *tnaCAB* operon of EPEC and EHEC are identical to that of *E. coli* K-12, suggesting that tryptophan-mediated stimulation of the *tna* operon is likely conserved (Figure 3.2A). Consistent with this bioinformatic observation, a modest but reproducible increase in tryptophanase activity was observed upon addition of tryptophan to LB media (Figure 3.1E & 2D). LB media is naturally replete with tryptophan in the form of tryptone and thus its presence likely masks the actual induction in tryptophanase activity by exogenously added tryptophan.

Because uptake of tryptophan is necessary for killing of *C. elegans* we reasoned that tryptophan importers might also be necessary for toxin production. In *E. coli* three permeases, *tnaB*, *aroP*, and *mtr*, are responsible for importing tryptophan into the bacterium (Yanofsky et al., 1991). Orthologs of all the three transporters are present in EPEC (data not shown). Using lambda Red mediated recombineering we substituted *mtr*

and *aroP* with a *cat* cassette as described previously (Bhatt et al., 2009, Datsenko & Wanner, 2000, Murphy & Campellone, 2003), and evaluated the roles of each of the permeases in toxin production and pathogenesis in *C. elegans*. Inactivation of *mtr* or *aroP* did not compromise the ability of EPEC to paralyze or kill *C. elegans* (Figure 3.2B & C). By contrast, inactivation of *tnaB* was sufficient to completely abolish EPEC-induced paralysis and killing of *C. elegans* (Figure 3.2B & C). The *tnaB* mutant regained its pathogenicity when complemented with a functional *tnaB* allele that was expressed under a heterologous promoter from the low copy plasmid ptnaB (Figure 3.2B & C) (Figure 3.1). The attenuated phenotype of the *tnaB* mutant correlated with reduced tryptophanase activity (Figure 3.2D). Moreover, the tryptophan-mediated induction of *tnaA* was no longer evident when *tnaB* was inactivated (Figure 3.2D). Taken together, these results suggest that on LB agar TnaB is the primary permease responsible for importing tryptophan into the bacterium, which subsequently induces *tnaA*. Besides inducing *tnaA*, tryptophan is also one of the natural substrates of tryptophanase (Newton *et al.*, 1965, Newton & Snell, 1964). Because overexpression of *tnaA* in LB media, without added tryptophan, is insufficient for worm killing, tryptophan must play an important role as a tryptophanase substrate and as a precursor for exotoxin synthesis. Interestingly, inactivation of *csrA* does not disrupt the tryptophan-mediated stimulation of tryptophanase (Figure 3.1E), suggesting that the import of the inducer remains unhindered in the *csrA* mutant. This corroborates the observation that overexpression of *tnaA*, without *tnaB*, is sufficient to restore virulence to the *csrA* mutant when cultivated on LBW (Figure 3.1C & 1D). Curiously, tryptophan repressed tryptophanase activity when *csrA* was overexpressed (Figure 3.1E). Biochemical studies with tryptophanase

from *E. coli* suggest that the degradative product of tryptophan, indole, exerts a dose-dependent, feedback inhibitory effect on the enzymatic activity by competing with its substrates for the catalytic site (Gooder & Happold, 1954). Moreover, derivatives of indole have also been demonstrated to silence the expression of *tnaA* (Lee *et al.*, 2007). Thus, the observed phenotype likely stems from the repressive effect of elevated indole levels on the expression and/or activity of tryptophanase. In summary, our results suggest that CsrA and TnaB exert their effects via parallel pathways that converge at the level of regulation of *tnaA* to synthesize exotoxins that enable EPEC to paralyze and kill *C. elegans* (Figure 3.3).

Herein we provide evidence that the dual metabolic and virulence regulator, CsrA, previously shown to regulate the virulence of EPEC in mammals (Bhatt et al., 2009), also contributes to pathogenicity in nematodes. Our results also suggest that toxin-based bioassays employing *C. elegans* can be effectively utilized to identify novel virulence factors of A/E pathogens with relevance to mammalian pathogenesis. Future experiments utilizing a saturated transposon mutagenized library will provide invaluable insight into evolutionarily conserved virulence determinants of EPEC. Moreover, using worm killing as readout we were able to determine the metabolic requirement of the different tryptophan importers in the nematocidal activity of EPEC. Thus it may be possible to adapt the toxin-based assay to study alternative metabolic pathways and design screens to identify virulence factors for other pathogens. For instance, the murine A/E pathogen *C. rodentium* lacks *tnaA*. However, the closely related enzyme, tyrosine phenol lyase (*tpl*) is present in the genus *Citrobacter* (Iwamori *et al.*, 1991, Faleev *et al.*, 1988b, Faleev *et al.*, 1988a). Both the enzymes utilize the same cofactors and display remarkable conservation

of key residues (Barbolina *et al.*, 2000). Tpl enzymatically cleaves tyrosine to yield phenol, pyruvate and ammonia. Because phenolic compounds are nematotoxic (Kohra *et al.*, 2002, Tominaga *et al.*, 2003), substitution of tryptophan with tyrosine in the media may facilitate in evaluating the toxicity of *C. rodentium* towards *C. elegans* and identifying virulence factors that may also induce pathology in mammals.

Acknowledgements

We would like to thank Dr. Charles Yanofsky and Dr. Tony Romeo for their generous gifts of the plasmids pMD6 and pCRA16 respectively. This work was supported by NIH grants R01DK074731-01 and R01-A1056067-01 to DK. SB is the receipient of the National Science Foundation award 0450303 subaward I-66-606-63 to Emory University.

Table 3.1 – Bacterial strains and plasmids used in this study

Strain	Relevant genotype / phenotype	Reference or source
EPEC	Prototypical EPEC 2348/69 serotype O127:H6	Jim Kaper
EPEC *csrA*	EPEC 2348/69 Ω*csrA::cat* / *cat*^{r}	(Bhatt et al., 2009)
EPEC *tnaA*	EPEC 2348/69 Δ*tnaA::cat* / *cat*^{r}	(Anyanful et al., 2005)
EPEC *csrA* (pCRA16)	EPEC 2348/69 Ω*csrA::cat* transformed with the plasmid pCRA16 / *cat*^{r} Tc^r	This study
EPEC *csrA* (pMD6)	EPEC 2348/69 Ω*csrA::cat* transformed with the plasmid pMD6 / *cat*^{r} Ap^r	This study
EPEC *tnaA* (pCRA16)	EPEC 2348/69 Δ*tnaA::cat* transformed with the plasmid pCRA16 / *cat*^{r} Tc^r	This study
EPEC *tnaB*	EPEC 2348/69 Ω*tnaB::*Tn*5-kan* / Km^r	This study
EPEC *tnaB* (ptnaB)	EPEC 2348/69 Ω*tnaB::*Tn*5-kan* transformed with the plasmid ptnaB / Km^r Ap^r	This study
EPEC *mtr*	EPEC 2348/69 Δ*mtr::cat* / *cat*^{r}	This study
EPEC *aroP*	EPEC 2348/69 Δ*aroP::cat* / *cat*^{r}	This study
Plasmids		
pKD3	pANTSγ - (FRT-*cat*-FRT) R6Kγ*oriV* / Ap^r *cat*^{r}	(Datsenko & Wanner, 2000)
pCRA16	pBR322 – Ω*bla::*(P_{csrA} – $csrA^+_{K\text{-}12}$) / Tc^r	(Suzuki *et al.*, 2002)
pMD6	pBR322 – (P_{tnaCAB} – $tnaCA_{K\text{-}12}$) / Ap^r	(Deeley & Yanofsky, 1981)
ptnaB	An *Eco*RI-*Pst*I restricted amplicon containing the *tnaB* ORF from EPEC 2348/69 cloned downstream of the P_{araBAD} promoter of the identically restricted plasmid, pBAD24 / Ap^r	This study

Table 3.2 – Oligonucleotides used in this study

Primer	Sequence
5'-aroP-P2-Wanner-EPEC	CCGCCACATACAGCTTATCGCGCTGGGAGGCGCGATAGGG ACAGGCATATGAATATCCTCCTTA
3'-aroP-P1-Wanner-EPEC	TACCTAACACGATCAGCCATACCGGGATCAGGTATACCGAA ATCGGTGTAGGCTGGAGCTGCTTC
5'-mtr-P2-Wanner-EPEC	TTATCGGCGGCACCATTATTGGCGCAGGGATGTTTTCTCTG CCAGCATATGAATATCCTCCTTA
3'-mtr-P1-Wanner-EPEC	CATTGTGTAGGCAGCAGAAATGTCGGATAAGGCACCGCTG ATTACGTGTAGGCTGGAGCTGCTTC
5'-tnaB-*Eco*RI-pBAD24	gcggcc<u>GAATTC</u>CCTCTAAAGGTGGCATCATGACTG
3'-tnaB-*Pst*I-pBAD24	gcggcc<u>CTGCAG</u>AAAGCGGGACATGGGCTAAAG
c1[a]	TTATACGCAAGGCGACAAGG
c2[a]	GATCTTCCGTCACAGGTAGG

a - Reference for c1 and c2 (Datsenko & Wanner, 2000). Underlined sequences indicate restriction sites

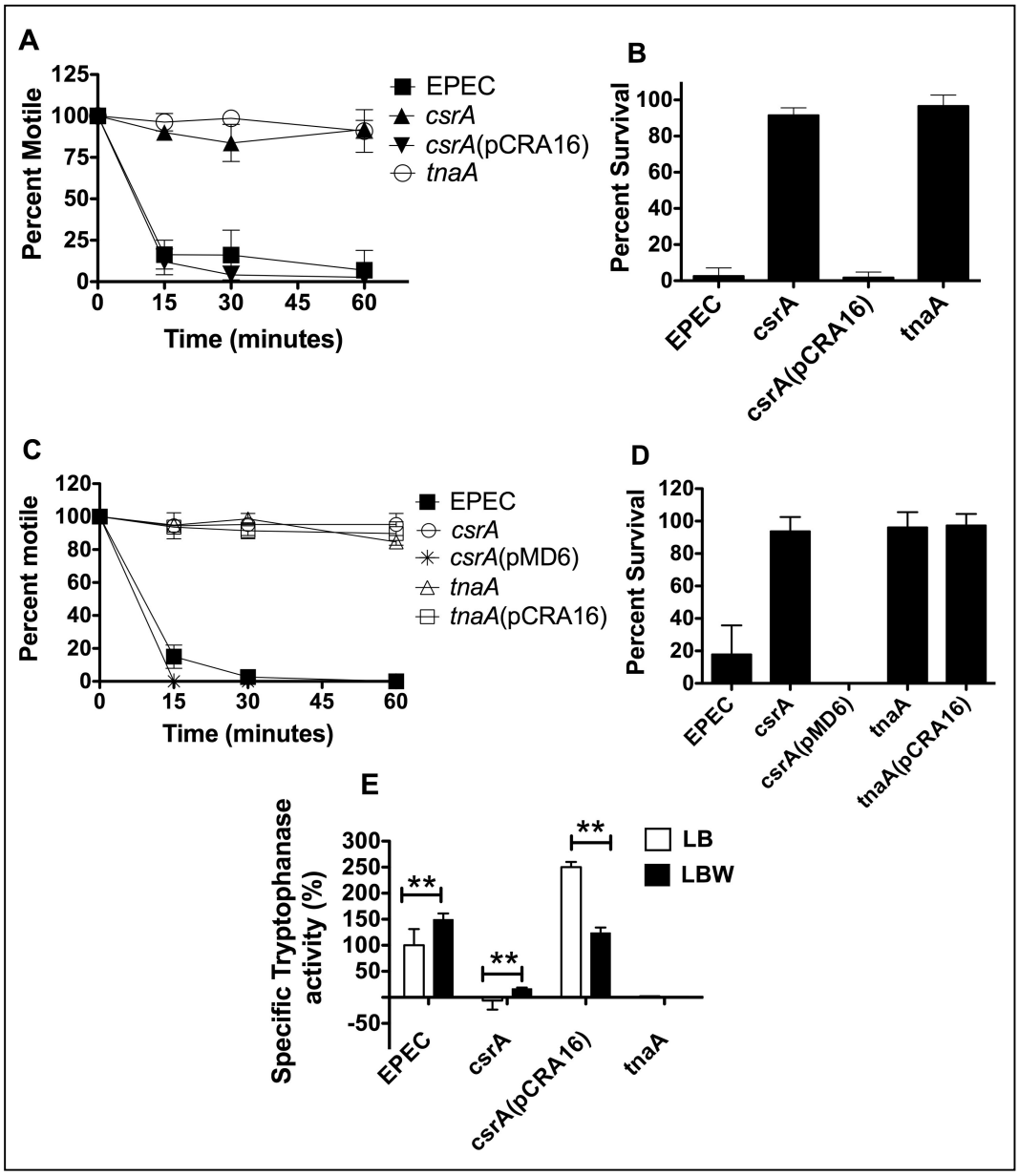

Figure 3.1 A-E – *csrA* regulates tryptophanase activity to promote the toxin-mediated killing of *C. elegans* by EPEC

A – B - Young adult worms were exposed to confluent lawns of EPEC, *csrA* mutant, *csrA* mutant complemented with a functional *csrA* allele expressed from a multicopy plasmid [*csrA*(pCRA16)]), and *tnaA* mutant and monitored for paralysis (**A**) and killing

(**B**) on LB agar supplemented with tryptophan (LBW). Worms were considered paralyzed if they failed to traverse an entire body length on prodding. Worm mortality was assayed by transferring the pathogen-exposed worms onto NGM plates containing nonpathogenic *E.coli* OP50 and assaying for motility 24 hours later. Error bars indicate standard deviation from at least three independent experiments with each employing at least two biological replicates. A one-way ANOVA was used to assess for statistical significance. A *p*-value cutoff < 0.05 was considered statistically significant. The calculated *p*-values for both the paralysis and killing assays were < 0.02.

C – D - Paralysis (**C**) and killing (**D**) of the nematodes was assayed in the presence of EPEC, *csrA* mutant, *csrA* mutant overexpressing *tnaA* [*csrA*(pMD6)], *tnaA* mutant, and *tnaA* mutant overexpressing *csrA* [*tnaA*(pCRA16)] essentially as described above. A one-way ANOVA was used to assess for statistical significance. The calculated *p*-values for both the paralysis and killing assays were < 0.02.

E - Tryptophanase activity was measured from lysates of bacteria cultivated on agar plates. The rate of hydrolysis of SOPC, a chromogenic tryptophan analogue, to ONTP was measured as described previously (Anyanful et al., 2005). Error bars indicate standard deviation from at least two independent experiments each with at least three replicates. The unpaired student's *t*-test was employed to assay for statistical significance between the indicated samples. A *p*-value cutoff < 0.01 was considered statistically significant. ** - denotes a *p*-value < 0.01.

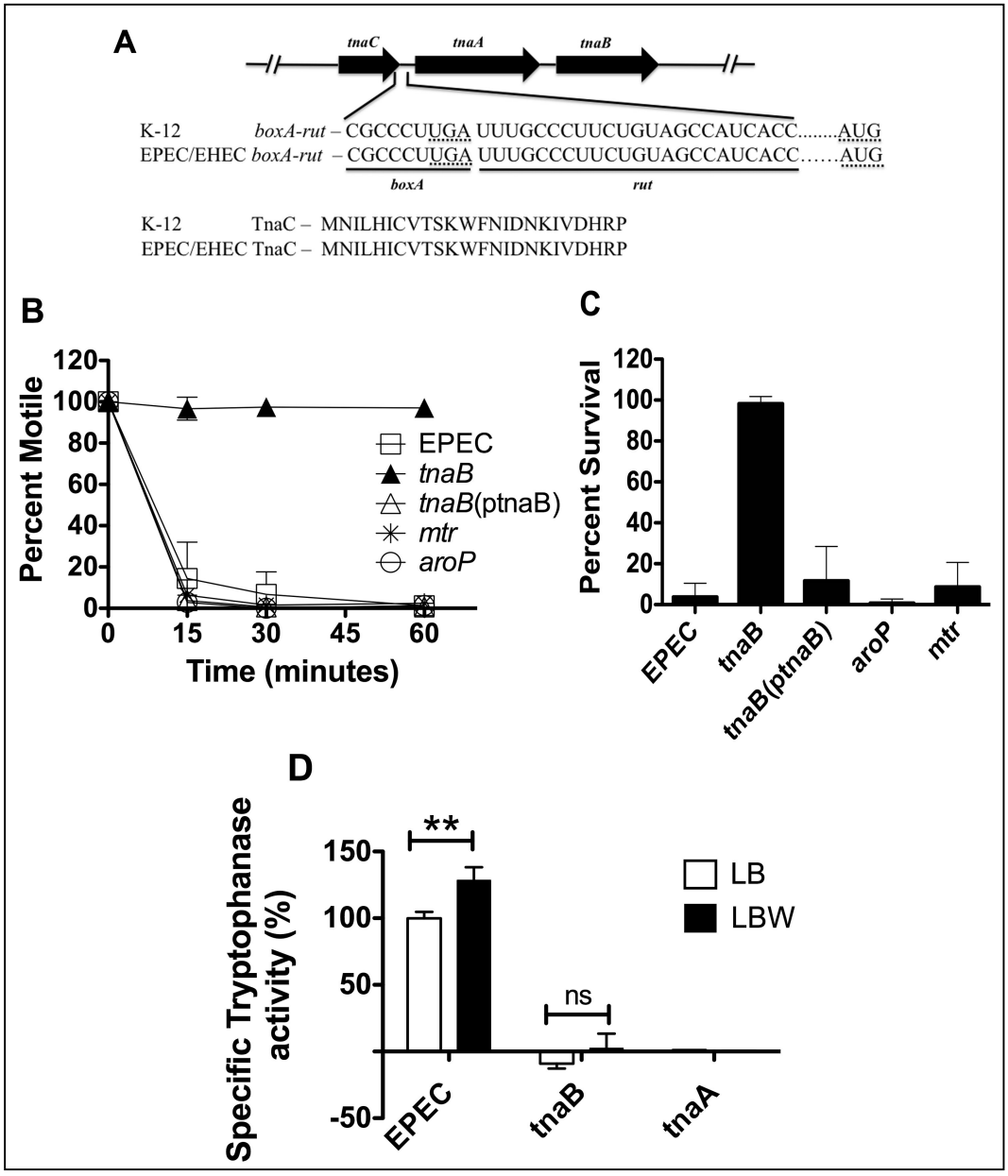

Figure 3.2 A-D – TnaB, but not AroP or Mtr, imports tryptophan into EPEC to stimulate toxin-dependent killing of *C. elegans*

A - Comparative analysis of the primary structure of the *boxA-rut* riboelement and the TnaC leader peptide of the *tnaCAB* operon in *E. coli* K-12 and EPEC/EHEC. The translation termination codon of *tnaC* and the translation initiation codon of *tnaA* are

indicated by dashed underlines. The intersite distance between the terminal nucleotide of *rut* and the translational initiation nucleotide of *tnaA* is 197 bases.

B – **C** – Young adult worms were exposed to EPEC and its congenic mutant derivatives, *tnaB*, *mtr*, *aroP*, and the *tnaB* complemented strain [*tnaB*(ptnaB)], and assayed for paralysis (**B**) and killing (**C**). Error bars indicate the standard deviation from at least three independent experiments with each using at least two replicates. A one-way ANOVA was used to assess for statistical significance. A *p*-value cutoff < 0.05 was considered statistically significant. The calculated *p*-values for both the paralysis and killing assays were < 0.02.

D - Specific tryptophanase activity was assayed in the *tnaB* mutants as described above. The unpaired student's *t*-test was employed to assay for statistical significance between the indicated samples. A *p*-value cutoff < 0.01 was considered statistically significant. ** - denotes a *p*-value < 0.01 whereas ns indicates no statistically significant difference.

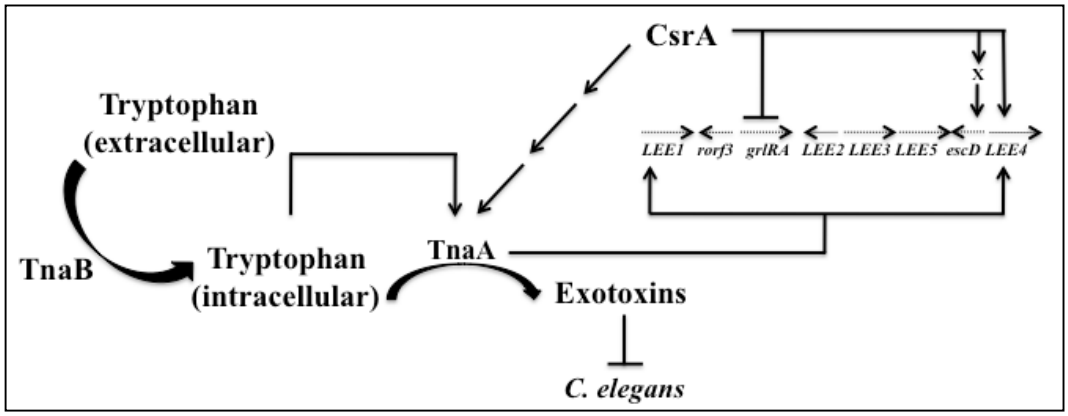

Figure 3.3 – Model for the role of CsrA and TnaB in the regulation of *tnaA* and toxin production

CsrA positively regulates tryptophanase activity independently of the tryptophan permease TnaB. TnaB, is the primary importer of tryptophan when EPEC is cultivated on LB media. Imported tryptophan stimulates the expression of *tnaA* as evident by elevated tryptophanase activity. In turn, tryptophanase catabolizes tryptophan to synthesize exotoxins, which paralyze and kill *C. elegans*. *csrA* and *tnaA* also regulate the *LEE* in A/E pathogens. Activating and repressive circuits are depicted as thin lines with arrowheads and blunt ends respectively. Dashed arrows represent the LEE. Thick curved arrows indicate catalytic reactions.

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CHAPTER 4

Honing the Message: Posttranscriptional and Posttranslational Control in Attaching and Effacing Pathogens

Shantanu Bhatt[1,2], Tony Romeo[1,3] and Daniel Kalman[1,2,†]

[1] Microbiology and Molecular Genetics Program, Emory University, Atlanta, Georgia, USA 30322

[2] Department of Pathology and Laboratory Medicine, Emory University, Atlanta, Georgia, USA 30322

[3] Department of Microbiology and Cell Science, P.O. Box 110700, University of Florida, Gainesville, Florida 32611-07004

† - This chapter consists of a manuscript that was published as –

Bhatt, S., T. Romeo & D. Kalman, (2011) Honing the message: post-transcriptional and post-translational control in attaching and effacing pathogens. ***Trends Micro.*** **19**: **217-224**.

Scanning electron micrograph – courtesy of Dr. Jorge Giron (jagiron@ufl.edu). Shantanu Bhatt created all other figures. Shantanu Bhatt, Tony Romeo, and Daniel Kalman wrote the manuscript.

Abstract

Bacteria evolve the capacity to cause disease by acquiring virulence genes that are usually clustered in discrete genetic modules termed pathogenicity islands (PAI). Stable integration of PAIs into preexisting transcriptional networks coordinates expression from PAIs with ancestral genes, in response to diverse environmental cues. Such transcriptional controls are evident in the regulation of the locus of enterocyte effacement (LEE), a PAI of enteropathogenic and enterohemorrhagic *Escherichia coli*. However, recent reports indicate that global posttranscriptional and posttranslational regulators, including CsrA, Hfq and ClpXP, fine-tune the transcriptional output from the LEE. Here, we highlight recent advances in understanding posttranscriptional and posttranslational regulation in A/E pathogens. Moreover, we consider how coordinate regulation of ancestral and newly acquired traits contributes to morbidity and mortality.

What are EPEC and EHEC?

Enteropathogenic *Escherichia coli* (EPEC) and enterohemorrhagic *Escherichia coli* (EHEC) cause significant morbidity and mortality worldwide (Mellies *et al.*, 2007, Karch *et al.*, 2005). EPEC is a waterborne pathogen that causes diarrhea, primarily amongst infants, in developing countries (Mellies et al., 2007). EHEC is spread via contaminated food and water and affects both infants and adults in developed countries (Mellies et al., 2007). Certain EHEC strains harbor Shiga toxins that cause bloody diarrhea and hemolytic uremic syndrome (HUS), a disease characterized by hemolytic anemia, thrombocytopenia, and acute renal failure (Karch et al., 2005).

Upon infection, EPEC and EHEC compete with the native microflora for limited nutrients, respond to stressors from the host immune system, and migrate to sites within the small or large intestine respectively, where they colonize and cause disease (Mellies et al., 2007). They belong to the attaching and effacing (A/E) family of pathogens, because they adhere intimately to intestinal cells (attachment), and promote the destruction of microvilli (effacement). Upon attachment, EPEC and EHEC recruit host cytoskeletal proteins to form characteristic actin-filled membraneous protrusions, termed "pedestals", beneath themselves (Figure 4.1A-B). Mutational analysis has revealed that the locus of enterocyte effacement (LEE), a 35-42 kb pathogenicity island that encodes transcriptional regulators, a functional type III secretion system (T3SS), and various exported translocators and effectors, is necessary for pedestal formation and disease (Mellies et al., 2007) (Figure 4.2).

Coordinated regulation of virulence

During infection, A/E pathogens encounter changes in pH, osmolarity, ferric nitrate [$Fe(NO_3)_3$], Ca^{2+}, temperature, quorum sensing, and bicarbonate (HCO_3^-), among others, and respond by coordinately regulating virulence gene expression in conjunction with other physiological processes (Mellies et al., 2007, Kenny *et al.*, 1997). Whereas transcriptional control of the LEE has been rigorously characterized, recent evidence indicates that posttranscriptional and posttranslational factors play key roles in fine-tuning the transcriptional output from the LEE, and in addition may integrate virulence with other physiological processes. Here we discuss such regulatory mechanisms, and highlight the physiological and evolutionary benefits of posttranscriptional and posttranslational regulation as a complement to transcriptional control. Moreover, we suggest that coordinate regulation of ancestral traits such as motility and metabolism together with virulence traits is a key determinant of morbidity in A/E pathogens.

Intrinsic transcriptional control of the LEE

The LEE is organized into five major polycistronic operons (*LEE1*-*LEE5*), the bicistronic operon (*grlR*-*grlA*) and numerous monocistronic genes (*grlA*, *escD*, *map*, *espG*, *cesF*, *rorf3*) (Mellies *et al.*, 1999, Mellies et al., 2007, Deng *et al.*, 2004, Sanchez-SanMartin *et al.*, 2001, Tauschek *et al.*, 2010) (Figure 4.2). Expression from the *LEE1*, *LEE2*, and *LEE3* operons and *escD* results in synthesis and assembly of a T3SS, which traverses the inner and outer membranes, and the peptidoglycan layer (Mellies et al., 2007, Deng et al., 2004). The *LEE4* operon primarily encodes the extracellular components of the T3SS including EscF, EspA, EspB and EspD. EscF forms the needle tip of the T3SS, and EspA monomers polymerize to form a hollow filament that connects

EscF to the host cell membrane (Sekiya *et al.*, 2001, Wilson *et al.*, 2001, Daniell *et al.*, 2001). Subsequently, EspB and EspD translocate through the EspA filament and integrate into the host plasma membrane thereby forming a contiguous pore between the bacterium and the host. Effectors, including Tir, enter the host cell through the portal and mediate the formation of pedestals (Shaw *et al.*, 2001, Wachter *et al.*, 1999, Wolff *et al.*, 1998, Ide *et al.*, 2001, Kenny, 1999, Mellies et al., 2007) (Figure 4.1A).

Under refractory conditions, the global regulator H-NS binds to *cis*-regulatory sequences located upstream of *LEE1-3*, *rorf3*, *grlR, grlA* and *LEE5* and inhibits transcription initiation (Mellies et al., 2007, Barba *et al.*, 2005, Jimenez *et al.*, 2010, Haack *et al.*, 2003) (Figure 4.2). H-NS-dependent transcriptional regulation is governed by diverse environmental cues including temperature and HCO_3^- ions, and it appears to be the most prominent repressor of the LEE (Umanski *et al.*, 2002, Mellies et al., 2007) (Figure 4.2). Under conditions conducive for pedestal formation H-NS-mediated repression of *LEE1* is relieved and transcription of *ler* is induced (Umanski et al., 2002, Mellies et al., 2007). Consequently, Ler activates transcription from the other LEE-encoded transcriptional units, including *grlRA*, *LEE2*, *LEE3*, *LEE4*, *LEE5*, *map* and *espG*, primarily by competing with H-NS for overlapping binding sites (Mellies et al., 2007, Sperandio *et al.*, 2000, Haack et al., 2003, Bustamante *et al.*, 2001, Barba et al., 2005, Sanchez-SanMartin et al., 2001) (Figure 4.2). The *grlRA* operon encodes a global transcriptional repressor of the LEE, GrlR, and an associated activator, GrlA (Deng et al., 2004). Ler, GrlR, and GrlA are components of an autoregulatory loop in which GrlA further enhances transcription of *ler* (Huang & Syu, 2008, Barba et al., 2005, Jimenez et

al., 2010), whereas GrlR binds to and inactivates GrlA, thereby limiting transcription of *ler* (Jobichen *et al.*, 2007, Jimenez et al., 2010).

Besides transcriptional control, several recent studies have highlighted the importance of posttranscriptional and posttranslational mechanisms in refining gene expression from the LEE (Bhatt *et al.*, 2009, Hansen & Kaper, 2009, Shakhnovich *et al.*, 2009, Laaberki *et al.*, 2006, Iyoda & Watanabe, 2005, Lodato & Kaper, 2009, Tomoyasu *et al.*, 2005, Roe *et al.*, 2003, Grant *et al.*, 2003, Campellone *et al.*, 2007). Importantly, many of these mechanisms were originally identified in *E. coli* (Romeo, 1998, Timmermans & Van Melderen, 2010, Jousselin *et al.*, 2009, Carpousis, 2007, Gottesman *et al.*, 1993), but are ubiquitously used by other bacterial pathogens including EPEC and EHEC (Timmermans & Van Melderen, 2010, Jousselin et al., 2009, Ingmer & Brondsted, 2009).

Why control the LEE at the posttranscriptional and posttranslational levels?

There are several reasons why posttranscriptional and posttranslational controls may have evolved to complement transcriptional control. First, the operon organization of bacterial genes limits the capacity of transcription factors to differentially modulate genes within the same transcription unit. For instance, Ler activates the transcription of all the genes encoded within *LEE2, LEE3*, *LEE5* and *LEE4* operons without selectively affecting the expression of genes within individual transcription units (Elliott *et al.*, 2000, Mellies et al., 2007). Moreover, such a genetic organization is particularly constraining when gene products required in different stoichiometric ratios are encoded on the same transcript. For instance, the *LEE4*-encoded polycistronic transcript encodes a regulator (SepL), the structural components of a mature T3SS (EspA, EspD, EspB, and EscF),

chaperones (CesD2 and L0017), and an effector (EspF), (Lodato & Kaper, 2009). While most of the genes in the transcript contribute to T3SS in general, they are required in different concentrations; the translocators EspA, EspB and EspD are made in excess relative to the regulator SepL (Lodato & Kaper, 2009). In EHEC, this is accomplished by posttranscriptional processing within the intracistronic segment of *sepL*, followed by the selective degradation of *sepL* and the concomitant stabilization of *espA*, *espD*, *espB* (Lodato & Kaper, 2009, Roe et al., 2003).

Second, posttranscriptional and posttranslational mechanisms allow bacteria to conrol gene expression over a wide dynamic range, degrading some transcripts or proteins when they are no longer needed, or retaining others in abeyance for rapid mobilization at a later time (Waters & Storz, 2009). For instance, the RNA-binding protein CsrA represses translation from some transcripts by promoting their degradation (e.g. *pgaABCD*) (Wang *et al.*, 2005) or without affecting transcript stability (e.g. *hfq*) (Baker *et al.*, 2007). Likewise, the adaptor protein RssB can target the alternative stationary phase sigma factor, RpoS, for degradation by the ClpXP protease or simply bind to and sequester it, consequently repressing the expression of RpoS-activated genes (Hengge, 2009). Thus, by affecting substrate activity, stability and/or abundance (Waters & Storz, 2009, Nogueira & Springer, 2000, Hengge, 2009, Hengge & Turgay, 2009, Deng *et al.*, 2005, Wang *et al.*, 2008), posttranscriptional and posttranslational mechanisms fine-tune gene expression in a way not easily accomplished by transcriptional controls alone.

Third, posttranscriptional and posttranslational controls of gene regulation provide a means to rapidly and globally adapt to diverse environmental stimuli. The use of global regulators of ancestral processes for this purpose allows bacterial pathogens to coordinate

virulence with other physiological processes. Examples of such global regulatory factors include CsrA, Hfq, DsrA, and ClpXP, whose activities are described in detail below.

Lastly, posttranscriptional regulation is energetically efficient because sRNAs governing this process, such as DsrA and CsrB, or small proteins such as CsrA and Hfq, which exert much of the posttranscriptional control, can be synthesized quickly and with less energy compared to the relatively larger transcription factors (Waters & Storz, 2009). In summary, posttranscriptional and posttranslational mechanisms provide a complement to transcriptional control for highly plastic regulatory responses to diverse environmental stimuli (Waters & Storz, 2009).

RNA-binding proteins regulate the LEE

Carbon storage regulator A (CsrA), also called RsmA, was originally identified as a repressor of glycogen biosynthesis and biofilm formation in nonpathogenic *E. coli* (Romeo *et al.*, 1993). Since then, CsrA has been found to regulate diverse physiological processes including carbon homeostasis, quorum sensing, peptide uptake, morphogenesis of flagella, and T3SS (Timmermans & Van Melderen, 2010, Romeo, 1998).

CsrA is highly conserved across diverse bacteria but has been most extensively characterized in *E. coli* (Timmermans & Van Melderen, 2010, Romeo, 1998). CsrA exists as a homodimer and binds to single-stranded tracts in the leader segment of transcripts containing a core motif, AGGA or ANGGA and influences messenger RNA (mRNA) stability and/or translation (Schubert *et al.*, 2007, Dubey *et al.*, 2005, Timmermans & Van Melderen, 2010, Romeo, 1998). The regulatory small RNAs (sRNAs), CsrB and CsrC contain repetitive AGGA/ANGGA tracts that sequester

multiple CsrA molecules, and reduce the effective concentration and activity of CsrA (Dubey et al., 2005, Liu *et al.*, 1997, Weilbacher *et al.*, 2003). Orthologs of the Csr genes are highly conserved in A/E pathogens (Bhatt et al., 2009).

In EPEC, CsrA acts as an activator or repressor of the LEE in a manner that depends upon its concentration (Bhatt et al., 2009) (Figure 4.2). CsrA binds to two ANGGA motifs in the untranslated leader segment of the *LEE4* transcript, and increases the steady-state levels, likely by stabilizing the message. Additionally, CsrA also activates the expression of the inner membrane protein of the T3SS, EscD, through an intermediate regulator. As a consequence, CsrA facilitates pedestal formation (Bhatt et al., 2009). By contrast, high concentrations of CsrA globally inhibit gene expression from the LEE by reducing *grlRA* transcript levels (Bhatt et al., 2009). Moreover, CsrA binds to as many as three sites within the *grlRA* transcript, one of which is in close vicinity of the Shine-Dalgarno sequence, a topological feature generally conserved in repressed transcripts (Timmermans & Van Melderen, 2010, Romeo, 1998). Taken together, these observations suggest that the CsrA-mediated repression of *grlRA* might result from reduced transcript stability (Bhatt et al., 2009).

Besides CsrA, the highly conserved RNA-chaperone Hfq has recently been identified as an important posttranscriptional regulator of the LEE in EPEC and EHEC (Shakhnovich et al., 2009, Hansen & Kaper, 2009). Inactivation of *hfq* renders *E. coli* hypersensitive to a plethora of environmental stressors (Tsui *et al.*, 1994). Hfq has since been recognized to play a key role in stress responses and virulence of diverse bacteria (Chao & Vogel, 2010). Hfq exists as a homohexamer and assumes a toroidal conformation that contains distinct proximal and distal RNA-binding surfaces (Link *et*

al., 2009, Valentin-Hansen *et al.*, 2004). Structural studies with Hfq from *E. coli* reveal that the proximal face binds A/U rich sequences, whereas the distal face simultaneously recognizes tandem poly-(A-R-E) tracts (Link et al., 2009, Soper *et al.*, 2010, Valentin-Hansen et al., 2004). This enables Hfq to stabilize base-pairing between sRNAs and target mRNAs, which have limited complementarity, and affect transcript stability and/or translation (Valentin-Hansen et al., 2004).

In EHEC, Hfq represses the LEE via two pathways and consequently affects pedestal formation (Hansen & Kaper, 2009, Shakhnovich et al., 2009) (Figure 4.2). In the exponential phase, Hfq destabilizes the *grlRA* transcript resulting in reduced expression of GrlA. This, in turn, causes a reduction in levels of Ler, and global silencing of the LEE (Hansen & Kaper, 2009). By contrast, in the stationary phase, Hfq-mediated repression of the LEE remains largely independent of *grlRA* and instead occurs by translational repression of Ler (Shakhnovich et al., 2009, Hansen & Kaper, 2009) (Figure 4.2). In both EPEC and EHEC the leader segment of the *grlRA* transcript possesses a canonical poly-(A-R-E) motif (5'-AGA AAA AGA AAG-3'), raising the possibility that the transcript binds to the distal face of Hfq. By contrast, the 5' untranslated leader segment of *ler* is A/U rich (~72%), and may interact with the proximal face of Hfq (S.B. and D.K., unpublished observations).

Besides non-catalytic RNA-binding proteins, the single-strand specific endoribonuclease RNase E also controls gene expression from the LEE of EHEC (Lodato & Kaper, 2009). RNase E is a component of the bacterial degradosome, a multiprotein complex involved in the maturation or degradation of heterogeneous RNA species (Carpousis, 2007). In *E. coli* RNase E displays relaxed sequence specificity with a

preference for A/U-rich transcripts (Jousselin et al., 2009). Consequently, RNase E and Hfq co-regulate several transcripts antagonistically (Jousselin et al., 2009). RNase E is responsible for the posttranscriptional processing of the *LEE4*-encoded *sepLespADB* transcript in EHEC (Lodato & Kaper, 2009) (Figure 4.2). The 5' fragment containing *sepL* undergoes rapid degradation rendering SepL undetectable. By contrast, the 3' segment spanning *espA* and the downstream genes remains stable (Lodato & Kaper, 2009). Additionally, the leader segment of *sepL* possesses a noncanonical ribosome-binding site (RBS) that is highly divergent from the near-consensus RBS observed in the leader segment of *espA*. Lodato and Kaper propose that inefficient recruitment of the ribosome to the RBS of *sepL* renders the *sepL* segment of the transcript sensitive to RNase E; by contrast, proficient binding of the ribosome to the *espA* segment and ensuing translation might sterically hinder binding by ribonucleases (Lodato & Kaper, 2009). Such a mechanism not only accounts for the stoichiometric difference between the abundance of the regulator SepL and the translocators (EspA, EspB and EspD), but also might promote the transition from the export of translocators, which connect the T3SS to the host cell, to that of the effectors (e.g. Tir) (Wang et al., 2008). Thus, upon appropriate environmental cues, degradation of the *sepL* transcript and consequent reduction in SepL levels may permit the secretion of effectors, which are otherwise sequestered by SepL (Wang et al., 2008, Lodato & Kaper, 2009). However, it remains to be determined whether posttranscriptional processing of *sepLespADB* occurs in other A/E pathogens. Furthermore, the physiological relevance of maturation of the *LEE4* transcript during synthesis of the T3SS needs to be evaluated by selective mutation of the RNase E cleavage sites in *sepL*.

sRNA-mediated regulation of the LEE

Regulatory sRNAs are ubiquitously conserved in the three taxonomic domains of life where they affect all steps of gene regulation (Toledo-Arana *et al.*, 2007). In *E. coli*, DsrA exists as an 87-nucleotide, untranslated transcript that modulates gene expression by antisense base-pairing with its target mRNAs in the presence of the RNA-chaperone Hfq (Sledjeski *et al.*, 2001, Lease & Belfort, 2000). DsrA binds to *hns* and *rpoS* mRNAs, destabilizing the former but promoting translation of the latter (Majdalani *et al.*, 1998, Lease *et al.*, 1998). Riboregulation of these global regulators enables DsrA to regulate responses to an array of environmental stressors (Lease & Belfort, 2000). In EHEC, overexpression of *dsrA* activates *ler* in an *hns*- and *rpoS*-dependent manner (Laaberki et al., 2006) (Figure 4.2). While details of this regulation have not been elucidated, it is likely that the observed phenotype results from a mechanism similar to that observed in *E. coli*. Thus, high levels of DsrA may result in loss of H-NS-mediated repression of the LEE and, consequently, activation of Ler. DsrA-mediated activation of Ler also requires a functional *rpoS* allele. Paradoxically, RpoS has also been recognized as a repressor of Ler, when DsrA is expressed in single copy, and RpoS levels are low (Laaberki et al., 2006, Iyoda & Watanabe, 2005). However, increasing DsrA concentrations may promote translation of RpoS, and lead to activation of Ler (Laaberki et al., 2006). Thus, RpoS may act as a dose-dependent repressor or activator of the LEE, in a manner that depends on DsrA levels (Figure 4.2).

Intriguingly, the effects of DsrA are pathovar-specific, as overexpression has negligible effects on LEE gene expression in EPEC (Laaberki et al., 2006). One possible

explanation is that the basal levels of the DsrA transcript might be inherently higher in EPEC compared to EHEC (Laaberki et al., 2006). Thus, DsrA may contribute to the higher overall level of expression from the LEE of EPEC compared to EHEC. A confounding factor in studies on EPEC and EHEC is that inactivation of *dsrA* was without effect. In this regard, riboregulators, including DsrA, often exhibit functional redundancy (Waters & Storz, 2009, Mandin & Gottesman, 2010). In *E. coli,* inactivation of *dsrA, rprA,* and *arcZ* is required to substantially reduce expression of *rpoS* under nutrient deprivation conditions (Mandin & Gottesman, 2010), and the same may be necessary in EPEC and EHEC to observe physiologically relevant changes in gene expression from the LEE.

Posttranslational control of the LEE

Information on how posttranslational factors affect virulence of A/E pathogens is more limited than for posttranscriptional regulators. The ATP-dependent protease ClpXP is one of the few posttranslational regulators whose role in the virulence of EHEC is well established (Iyoda & Watanabe, 2005, Tomoyasu et al., 2005). Substrates bind to ClpX in an ATP-dependent manner and are subsequently hydrolyzed in the inner cavity of the catalytic component ClpP (Grimaud *et al.*, 1998). In EHEC, ClpXP activates the transcription of *ler* in an *rpoS*- and *grlR*-dependent manner (Iyoda & Watanabe, 2005, Tomoyasu et al., 2005) (Figure 4.2). In *E. coli* K-12*,* the adaptor protein RssB is sufficient to target RpoS for degradation by ClpXP (Hengge, 2009). RssB is highly conserved in A/E pathogens suggesting that *clpXP*-mediated activation of the LEE might result in part from the direct degradation of RpoS by ClpXP. ClpXP effects also appear to

be mediated via GrlR because inactivation of *grlR* completely bypasses the requirement for functional *clpXP* (Iyoda & Watanabe, 2005). The observation that the stability and abundance of GrlR is elevated in a *clpXP* mutant of EHEC raises the possibility that GrlR may be a direct substrate of ClpXP (Iyoda & Watanabe, 2005). This is perhaps not surprising as intrinsic regulators of horizontally acquired genes frequently integrate into ancestral regulatory circuits, including those acting at the posttranscriptional and posttranslational level (Perez & Groisman, 2009, Chao & Vogel, 2010, Timmermans & Van Melderen, 2010).

Other extra-transcriptional mechanisms affecting the LEE

Posttranscriptional regulation has also been implicated in the phenotypic plasticity of EspADB translocons evident in different EHEC strains (Roe et al., 2003). High secretors exhibit more T3SSs on their surface than low secretors. Paradoxically, high-secretors export more translocator proteins but contain less mRNA in comparison to low-secretor strains (Roe et al., 2003). The regulatory factors and the corresponding networks mediating this response have yet to be elucidated, except in so far as the effect appears to be at the posttranscriptional level. In EHEC, the EspA translocon also functions as an adhesin (Mellies et al., 2007), and evidence in other systems suggests that adhesin levels can contribute to tissue tropism amongst related strains (Perez & Groisman, 2009). Such strain-specific effects raise the possibility that rewiring of posttranscriptional regulatory networks amongst EHEC strains might confer alternate sites of attachment within the intestinal tract and/or facilitate colonization of different tissues within the host.

A variety of other extra-transcriptional mechanisms have been described that regulate LEE expression, though little detailed mechanistic information is available. For example, the ribosome binding GTPase BipA induces gene expression from the LEE of EPEC and EHEC by promoting the steady-state transcript levels of *ler* (Grant et al., 2003). Moreover, BipA is also required for the proteolysis of intimin in EPEC (Grant et al., 2003) (Figure 4.2). In addition, epigenetic control via the DNA-modifying enzyme DNA adenine methyltransferase (Dam) has also been implicated in regulation of the LEE in EHEC (Heusipp *et al.*, 2007). The physiological role of Dam is to methylate the adenine residue located within the tetranucleotide tract, GATC (Geier & Modrich, 1979). The resulting methylation pattern globally affects numerous cellular processes including general gene expression, chromosomal replication, and methyl-directed mismatch repair (Heusipp et al., 2007). In EHEC, inactivation of *dam* induces gene expression from the LEE and promotes bacterial adherence and pedestal formation (Campellone et al., 2007) (Figure 4.2). This effect appears to be mediated at the translational or posttranslational level as the abundance of the LEE-encoded proteins, but not transcripts, is dramatically elevated in the mutant (Campellone et al., 2007). It is likely that this effect on the LEE is regulated through an intermediary because Dam specifically modifies DNA (Heusipp et al., 2007, Geier & Modrich, 1979).

Elucidating the posttranscriptional and posttranslational "virulence regulome" of A/E pathogens

The majority of posttranscriptional and posttranslational regulators that govern the LEE are ancestral factors shared between nonpathogenic *E. coli* and A/E pathogens. Such

factors affect a plethora of physiological processes. For instance, CsrA, Hfq and ClpXP control motility, metabolism and adaptation to stress in nonpathogenic and pathogenic *E. coli* (Hansen & Kaper, 2009, Bhatt et al., 2009, Iyoda *et al.*, 2006, Timmermans & Van Melderen, 2010, Chao & Vogel, 2010). The flagellum of EPEC and EHEC possesses adhesive properties and has been shown to facilitate bacterial colonization (Mellies et al., 2007, Erdem *et al.*, 2007). Moreover, the ability of EHEC to switch between glycolytic and gluconeogenic substrates is critical to its pathogenicity *in vivo* (Miranda *et al.*, 2004). Thus, the ability of A/E pathogens to cause disease is not exclusively the result of traits conferred by horizontally acquired PAIs. Rather, a successful infection requires the contribution of both ancestral and newly acquired traits, acting in a coordinated spatiotemporal manner. Techniques that permit the genome-wide identification of regulons and their corresponding traits are therefore important for elucidating the "virulence regulome" of A/E pathogens. In this regard, RNA immunoprecipitation (RIP) of RNA-binding proteins, such as CsrA and Hfq, coupled to the sequencing of the bound transcripts will be instrumental in identifying the direct targets of posttranscriptional factors. Moreover, the investigation of the transcriptome and proteome profiles of these regulators would facilitate discrimination between direct and indirect regulatory targets. At the posttranslational level, catalytically inactive variants may prove useful. For example, ClpXP variants that trap but do not degrade substrates would permit identification of direct targets. Likewise, indirect targets of ClpXP could be inferred by pairing substrate trapping with proteomic analysis. Such approaches have been successfully employed to identify regulons of posttranscriptional and posttranslational factors in nonpathogenic *E. coli* and other bacteria (Chao & Vogel, 2010, Flynn *et al.*,

2003). Furthermore, comparative metatranscriptomic and metaproteomic analyses may also shed light on the evolution of posttranscriptional and posttranslational regulatory networks in different A/E pathogens, as has been done for orthologous transcriptional networks in *Yersinia pestis* and *Salmonella*Typhimurium (Perez *et al.*, 2009, Perez & Groisman, 2009).

Lastly, even though *trans*-acting mutations are useful in identifying regulators of pathogenesis, such mutations affect several biological pathways, of which only some contribute to virulence. Therefore, construction of *cis*-regulatory mutations, for example in the binding sites of posttranscriptional and posttranslational regulators, to selectively affect particular pathways, will provide a better understanding of the relative contributions of these pathways to bacterial colonization and disease. Moreover, such analysis of *trans*- and *cis*-acting mutations should not be limited to *in vitro* studies, because predictions based on *in vitro* assays may not translate *in vivo*. This is particularly true for posttranscriptional regulators. For example, inactivation of *hfq* induces the expression of the T3SS and virulence-associated effectors in *Shigella sonnei*, *Vibrio cholerae*, and *Pseudomonas aeruginosa* (Shakhnovich et al., 2009, Sonnleitner *et al.*, 2003, Mitobe *et al.*, 2009, Mitobe *et al.*, 2008). However, the *hfq* mutant of each of these pathogens is dramatically attenuated *in vivo* (Chao & Vogel, 2010, Sonnleitner et al., 2003, Ding *et al.*, 2004, Mitobe et al., 2009). The *in vivo* pathogenicity profile of the *hfq* mutants is not unexpected because *hfq* mediates adaptation to a number of different stressors (Chao & Vogel, 2010). Thus, despite enhanced virulence *in vitro*, *hfq* mutants of EPEC and EHEC will likely be attenuated *in vivo* due to enhanced sensitivity to host stressors.

Concluding remarks

Posttranscriptional and posttranslational regulation significantly expand the regulatory flexibility of A/E pathogens and provide additional checkpoints to refine transcriptional output. However, our current knowledge of such regulatory mechanisms, which may be both specific to A/E pathogens and more generally applicable, is still in its infancy. Nonetheless, it is clear that such modes of regulation may be key factors coordinating the expression of newly acquired virulence genes with ancient metabolic processes. Finally, a comprehensive understanding of the extra-transcriptional honing of the transcriptional output is essential for the development of novel therapeutic measures to effectively combat A/E pathogens.

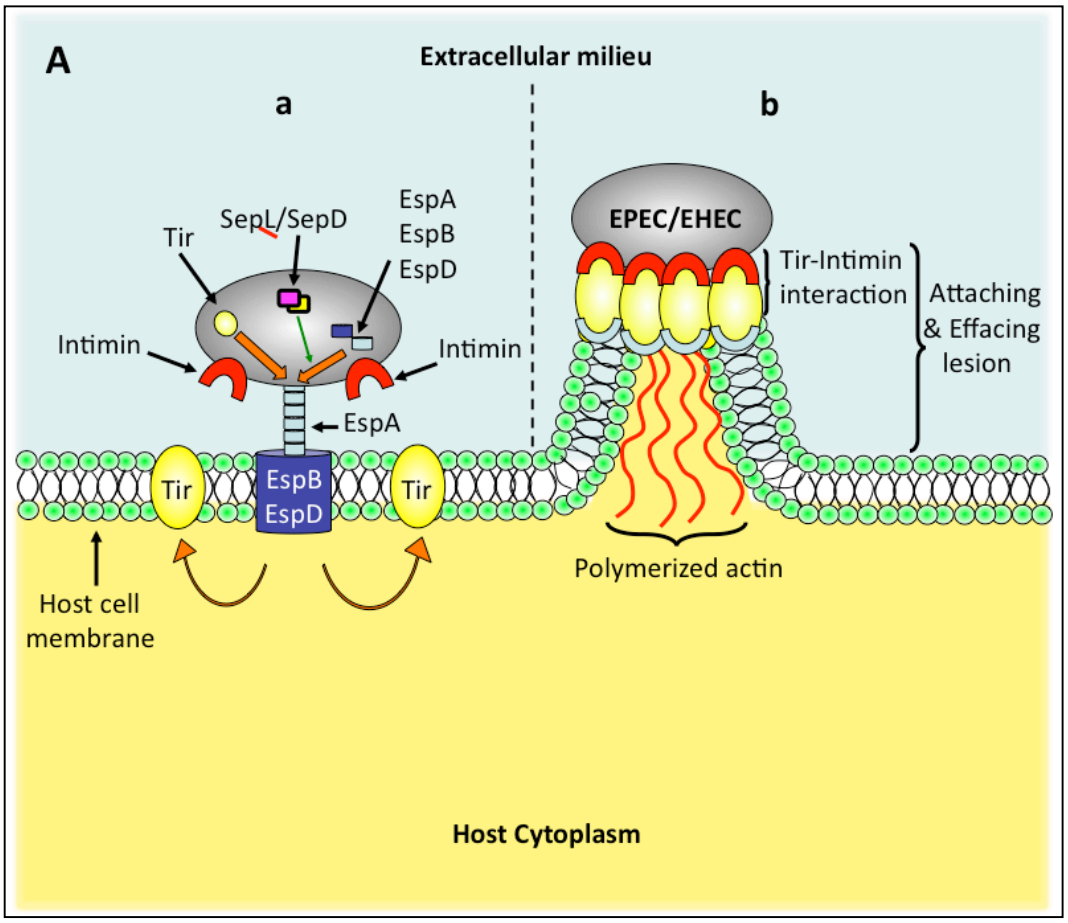
Outstanding Questions

1. How do environmental cues affect extra-transcriptional regulators of the LEE?
2. What genes comprise the virulence regulon of extra-transcriptional factors and what is the contribution of such regulation to the morbidity associated with A/E pathogens *in vivo*?
3. What is the hierarchical organization of the different posttranscriptional and posttranslational regulators that govern the LEE?
4. Can highly conserved extra-transcriptional factors serve as targets for broad-spectrum antibiotics?
5. How have ancestral extra-transcriptional networks evolved to coordinate expression of ancient processes with recently acquired pathogenic traits during development of virulence in A/E pathogens?

6. Are there posttranscriptional and posttranslational virulence factors that are specific to A/E pathogens, and do such regulators affect ancestral genes just as ancestral regulators modulate virulence genes?
7. Do orthologs of extra-transcriptional regulators with established roles in the virulence of other pathogens function similarly in A/E pathogens?

Acknowledgements

We regret that space limitations precluded citation of all the work in this area. We are grateful to the members of the Kalman and Romeo labs for helpful discussions. Work in our lab is supported by grants from the National Institute of Health - R01DK074731-01 and R01A1056067-01 (to D.K.) and R01-GM059969 (to T.R.). S.B is a recipient of the National Science Foundation award 0450303 subaward I-66-606-63 to Emory University.


A
Extracellular milieu
a
b
Tir
SepL/SepD
EspA
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Intimin
Intimin
EspA
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Host cell membrane
EPEC/EHEC
Tir-Intimin interaction
Attaching & Effacing lesion
Polymerized actin
Host Cytoplasm

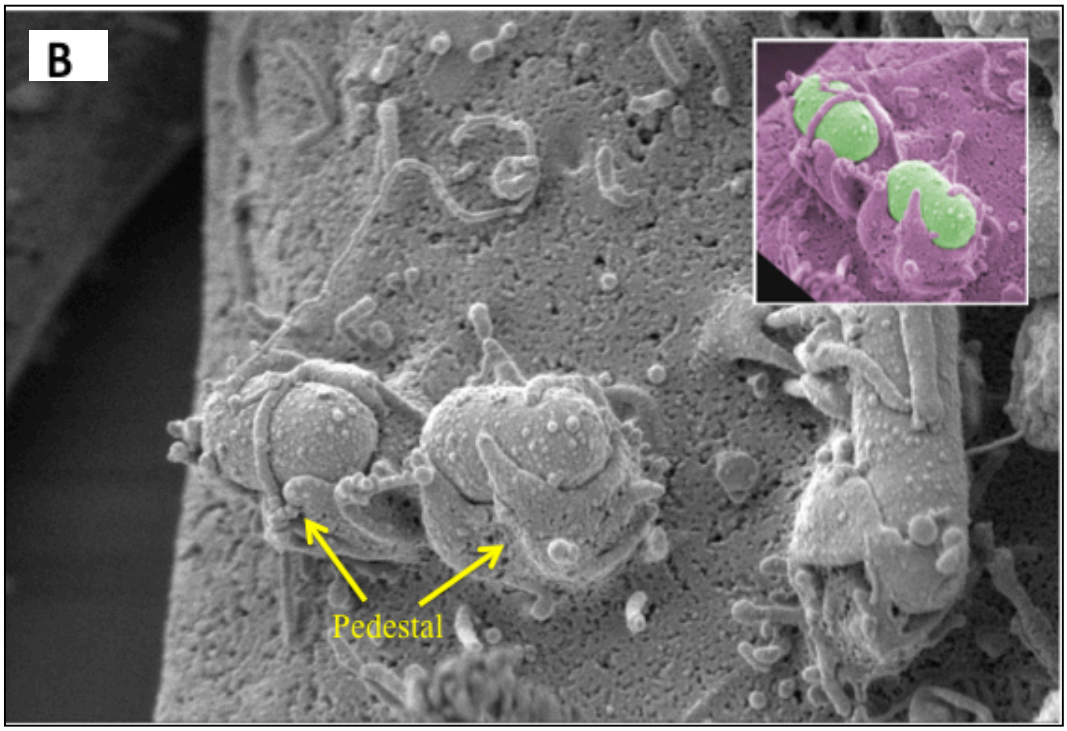
B
Pedestal

Figure 4.1 (A-B) Stages of pedestal formation by A/E pathogens –

A – Under inducible conditions the gatekeeper switch, composed of SepL and SepD, orchestrates the hierarchical secretion of translocators (EspA, EspB and EspD) over effectors (Tir and other effectors such as NleA, EspF and Map). While the EspADB translocon is being assembled, SepL directly binds to Tir and possibly other effectors and suppresses their export (**a**). After the maturation of the translocon, that connects the bacterium to the host, effectors are trafficked into the host's cytoplasm via the T3SS (**a**). Subsequently, Tir integrates into the host plasma membrane where it interacts with its ligand, intimin, located on the outer bacterial membrane. Tir-intimin interactions promote the clustering of Tir and initiate a signal transduction cascade that leads to the conscription and subsequent polymerization of actin to form membraneous protrusions, termed "attaching and effacing (A/E) pedestals", underneath adherent bacteria (**b**).

B – Scanning electron micrographic (SEM) image of EPEC forming pedestals on infected mammalian cells. Inset – Pseudocolored image of two EPEC bacteria (green) infecting HeLa cells (purple). EM Image courtesy of Jorge Giron.

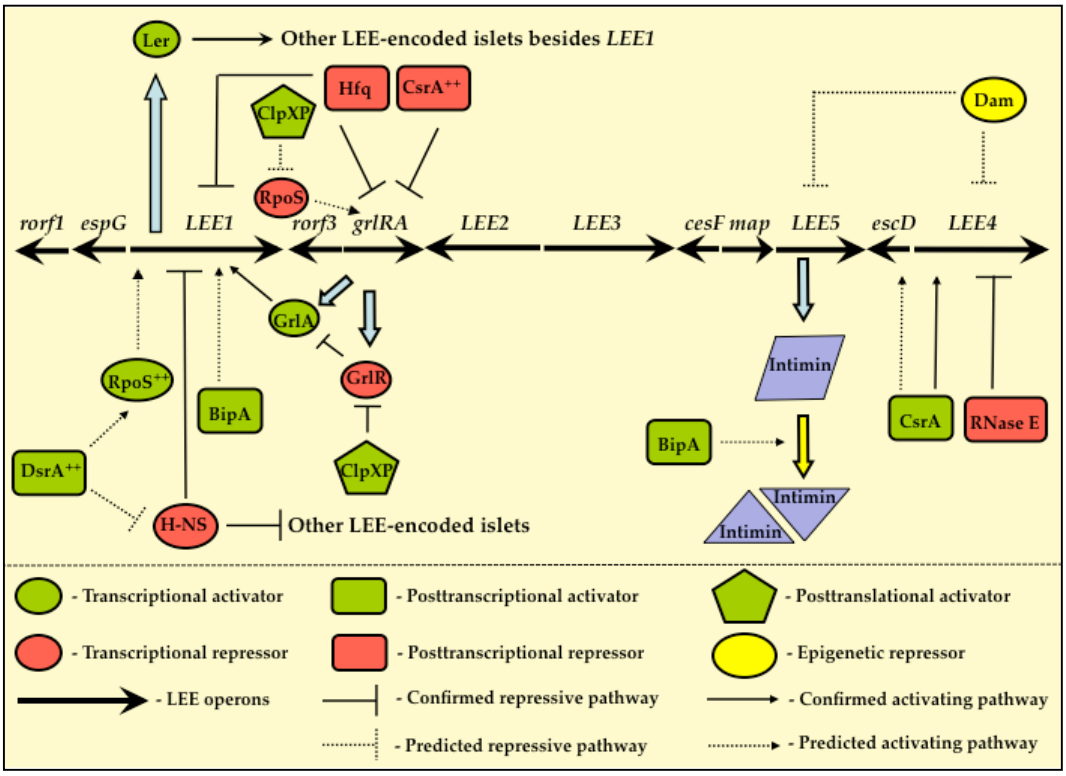

Figure 4.2 – Transcriptional and extratranscriptional control of the locus of enterocyte effacement (LEE)

The LEE is organized into five principal polycistronic operons, *LEE1*-5, the bicistronic operon, *grlRA*, and numerous monocistronic genes. Under inducible conditions, the H-NS-mediated repression of *LEE1* is relieved and transcription of *ler* is induced. Ler, an H-NS paralog, activates transcription from the other LEE operons primarily by counteracting the H-NS-mediated repression. Expression from the LEE leads to the synthesis and consequent assembly of a T3SS that connects the bacterial cytosol to the host cytoplasm and leads to pedestal formation and ensuing disease (refer to the text for details). The posttranscriptional factors DsrA, BipA, Hfq, and CsrA, as

well as the posttranslational factor ClpXP govern gene expression from the LEE by directly or indirectly influencing the abundance of the LEE-encoded regulator, Ler. Moreover, the regulators CsrA, BipA, Dam, and RNase E exert extra-transcriptional controls on the LEE-encoded lower tier genes within the Ler regulon (refer to the text for details).

Activators and repressors have been defined based on their capacity to globally induce or silence gene expression from the LEE respectively, and consequently affect pedestal formation. Thin filled lines with blunted ends or arrowheads depict regulatory pathways where the effect is direct and/or the mechanistic basis of gene expression has been defined, whereas thin dashed lines with blunted ends or arrowheads reflect presumptive circuits for which a detailed mechanism has not been established. ++ denotes overexpression of the gene product.

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CHAPTER 5

Overall Conclusions and Future Directions

In summary, our results suggest that *csrA* is required for the virulence of EPEC in multiple model systems. Inactivation and overexpression of *csrA* modulate gene expression from the LEE through distinct regulatory pathways. Whereas the *csrA* mutant exhibits reduced synthesis of the structural components of the T3SS, modest overexpression of *csrA* globally shuts of the LEE – the latter effect dependent on the LEE-encoded transcriptional activator, GrlA. Furthermore, CsrA also regulates other cellular processes such as motility and glycogen biosynthesis in EPEC. Besides mammalian pathogenesis, the *csrA* mutant was also attenuated in the exotoxin-dependent killing of the nonmammalian host *C. elegans*. This effect of *csrA* requires the *tnaA*-encoded catabolic enzyme tryptophanase as overexpression of *csrA* fails to paralyze and kill worms in the *tnaA* mutant. By contrast, tryptophanase activity is abolished in the *csrA* mutant and overexpression of *tnaA* suppresses the avirulence of the *csrA* mutant.

According to the prevailing model, we postulate that the binding of CsrA to the *sepLespADB* leader segment presumably stabilizes the transcript, whereas binding to the *grlRA* leader segment potentially destabilizes the transcript. However, this hypothesis must be substantiated by mRNA stability assays for CsrA often regulates its targets through multiple pathways that involve direct and/or indirect modes of regulation, as in the case of *hfq* (Baker *et al.*, 2007). Indole, the degradative product of tryptophan that is produced by tryptophanase, activates the *LEE4'-lacZ*$^{+}$ transcriptional fusion in EHEC (Hirakawa *et al.*, 2009). Because CsrA promotes tryptophanase activity, it is likely that CsrA affects the transcription from the *LEE4* operon in a *tnaA*-dependent manner. The LuxR-type transcriptional factor, SdiA, also represses transcription from the *LEE4*

operon in EHEC (Kanamaru *et al.*, 2000). Recently, *sdiA* was shown to be negatively regulated by CsrA in *E. coli* (Yakhnin *et al.*, 2011). If the observed regulatory architecture in *E. coli* is conserved in A/E pathogens, one would expect that CsrA would derepress the transcription from the *LEE4* operon by repressing translation from the *sdiA* encoding transcript. Additionally, the presumptive CsrA binding sites must be validated by site-directed mutagenesis to determine whether each or just a subset of the predicted sites participate in the observed CsrA-dependent regulation of *grlRA* and the *LEE4* operon. This is particularly important as experimental evidence suggests that the number of binding sites of regulatory factors are far more than the ones that are physiologically relevant(Perez & Groisman, 2009).

The inability of EPEC and EHEC to proliferate in mice limits the direct investigation of virulence factors in a surrogate mammalian host (Mundy *et al.*, 2006). Often, the natural murine pathogen, *Citrobacter rodentium*, which possesses the LEE, is recruited to evaluate the roles of orthologous virulence factors in mice. *C. rodentium* possesses a CsrA orthologue that is identical to that of EPEC and EHEC (unpublished observations). Thus, future studies with the *csrA* mutant of *C. rodentium* will be invaluable in exploring the role of CsrA in a mammalian host.

Accumulating evidence in EPEC and EHEC suggest that global posttranscriptional factors exhibit a greater degree of regulatory restructuring than transcriptional factors. For example, transcription factors that are conserved in EPEC and EHEC often perform similar functions in the two bacteria. The intrinsic LEE-encoded regulators, Ler and GrlA, activate the transcription from the LEE in both EPEC and EHEC (Mellies *et al.*, 2007, Mellies *et al.*, 1999, Elliott *et al.*, 2000, Islam *et al.*, 2011). Similarly, the non-LEE

encoded factor, QseA, promotes transcription from the *LEE1* operon in both EPEC and EHEC(Sperandio, 2002). By contrast, the regulatory sRNA, DsrA, activates the LEE of EHEC but not EPEC grown under similar conditions (Laaberki *et al.*, 2006). Similarly, the RNA chaperone Hfq represses the LEE in the EHEC strain EDL933 (Hansen & Kaper, 2009, Shakhnovich *et al.*, 2009), but activates it in the EHEC strain 86-24(Kendall *et al.*, 2011). Thus, a metacomparison of the regulons of CsrA will be informative on the extent of its regulatory remodeling in the different A/E pathogens.

Orthologs of CsrA are highly conserved and ubiquitously distributed amongst both the Gram-negative and Gram-positive phyla(Babitzke & Romeo, 2007). Thus, CsrA represents a potential target for designing broad-range pharmacological agents. This is particularly relevant for A/E pathogens, as recent reports have documented the emergence of muti-drug resistant strains of EPEC (Mitra *et al.*, 2011) and EHEC (de Castro *et al.*, 2003). Moreover, several DNA-damaging antibiotics, which are frequently prescribed against Gram-negative bacteria, actually potentiate the virulence of A/E pathogens (Mellies et al., 2007, Teel *et al.*, 2002, Zhang *et al.*, 2000).

Finally, our results demonstrate that EPEC factors that modulate nematotoxicity are also involved in mammalian pathogenesis (Anyanful *et al.*, 2005, Anyanful *et al.*, 2009, Bhatt *et al.*, 2011). Thus, the *C. elegans*-EPEC pathosystem provides a reliable and high throughput bioassay for the identification of virulence factors that may be potentially involved in the natural epidemiology of EPEC and EHEC infections.

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Appendix

Additional Publication (#1)

The rhomboid protease AarA cleaves TatA and is required for function of the twin-arginine translocase in *Providencia stuartii* ∇

Lindsay G. Stevenson[1], Kvido Strisovsky[2], Katy M. Clemmer[3], Shantanu Bhatt[1], Matthew Freeman[2] and Philip N. Rather* [1, 3]

[1] Department of Microbiology and Immunology, Emory University School of Medicine, [2]MRC Laboratory of Molecular Biology, Cambridge CB2 2QH, United Kingdom and [3] Laboratories of Bacterial Pathogenesis, Atlanta VA Medical Center, Atlanta, GA

* Corresponding author

Tel: (404) 728-5079

Fax: (404) 728-7780

e-mail prather@emory.edu

∇ - This chapter is part of the manuscript originally published as -

Stevenson, L.G., K. Strisovsky, K.M. Clemmer, S. Bhatt, M. Freeman & P.N. Rather, (2007) Rhomboid protease AarA mediates quorum-sensing in *Providencia stuartii* by activating TatA of the twin-arginine translocase. ***Proceedings of the National Academy of Sciences*** **104**: 1003-1008.

Shantanu Bhatt performed the western blots in Figure A1.2B.

Abstract

The *Providencia stuartii* AarA protein is a member of the rhomboid family of intramembrane serine proteases and required for the production of an unknown cell-cell signaling molecule. In a screen to identify rhomboid-encoding genes from *Proteus mirabilis*, *tatA* was identified as a multicopy suppressor and restored extracellular signal production to an *aarA* mutant. TatA is a component of the twin-arginine-translocase (Tat) protein secretion pathway and likely forms a secretion pore. The *tatA* gene from *P. mirabilis* restored additional *aarA*-dependent phenotypes in *P. stuartii,* such as growth on MacConkey plates, anaerobic growth with trimethylamine N-oxide (TMAO), normal cell division and pigment production. Unexpectedly, the native *tatA* gene of *P. stuartii* in multicopy did not suppress an *aarA* mutation and encoded a protein with a 7 amino acid N-terminal extension that was atypical of TatA proteins from other bacteria. In *P. stuartii,* TatA was cleaved both in-*vivo* and in-*vitro* in an AarA-dependent manner. Both cleavage sites occurred between amino acids 8 and 9 at the N-terminus. A *P. stuartii* TatA protein missing the first 7 amino acids restored the ability to rescue the *aarA*-dependent phenotypes. To verify that loss of the Tat system was responsible for the various phenotypes exhibited by an *aarA* mutant, a *tatC* null allele was constructed. The *tatC* mutant exhibited the same phenotypes as an *aarA* mutant and was epistatic to *aarA,* suggesting that AarA was required for Tat function. TatA represents the first authentic prokaryotic substrate for a rhomboid protease.

Introduction

Bacterial cells are able to communicate information to each other by using diffusible chemical signals called autoinducers or pheromones (Fuqua *et al.*, 1996, Dunny & Leonard, 1997, Miller & Bassler, 2001). Previous studies in *Providencia stuartii* identified genes that were regulated by a protease-sensitive, extracellular signaling molecule present in culture supernatants (Rather *et al.*, 1999, Rather *et al.*, 1997). Production of this extracellular signal required AarA, a member of the rhomboid family of intramembrane serine proteases (Gallio *et al.*, 2002, Rather et al., 1999, Urban *et al.*, 2001, Urban *et al.*, 2002). In *Drosophila*, Rhomboid proteins cleave additional membrane-bound proteins to release fragments that act as ligands for the epidermal growth factor (EGF) receptor (Urban et al., 2002). Both Rhomboid-1 and AarA are able to functionally substitute for each other, indicating that the specificity of at least some rhomboids has been conserved throughout evolution (Gallio et al., 2002).

In bacteria, the Tat (twin-arginine translocase) system for protein export transports pre-folded, cofactor-containing proteins with a twin-arginine motif within the signal sequence (Santini *et al.*, 1998, Sargent *et al.*, 1998, Weiner *et al.*, 1998). In *E. coli,* five proteins, TatA-E, comprise the Tat system, although only three subunits, TatA-C, are required for protein export (Sargent et al., 1998, Wexler *et al.*, 2000). The Tat system also appears to be required for the insertion of certain proteins into the cytoplasmic membrane (Hatzixanthis *et al.*, 2003). Comprehensive reviews on the Tat export pathway have been published (Wu *et al.*, 2000, Berks *et al.*, 2000, Lee *et al.*, 2006, Rather & Orosz, 1994, Clemmer *et al.*, 2006, Bogsch *et al.*, 1998, Stanley *et al.*, 2001,

Lemberg *et al.*, 2005, Ize *et al.*, 2004, Bolhuis *et al.*, 2001, Sargent *et al.*, 2001, de Leeuw *et al.*, 2002, Porcelli *et al.*, 2002, Bolhuis *et al.*, 2000).

In a search for *P. mirabilis* genes that can complement the loss of *aarA* in *P. stuartii,* the *tatA* gene in multicopy was found to rescue *aarA*-dependent phenotypes. Unexpectedly, the native *tatA* gene from *P. stuartii* did not rescue the phenotypes of an *aarA* mutant. In this study, we demonstrate that AarA is required for cleaving the amino terminus of TatA, a modification that is essential for function. The TatA protein represents the first authentic prokaryotic substrate for a rhomboid protease.

Results

Identification of a high copy suppressor that restores extracellular signal production to an *aarA* mutant of *P. stuartii*.

P. stuartii aarA mutants are pleiotropic and the resulting phenotypes include: loss of an extracellular signaling molecule, abnormal cell division (cell chaining), inability to grow on MacConkey agar and loss of a diffusible yellow pigment (Rather et al., 1999, Rather & Orosz, 1994, Clemmer et al., 2006). Wild-type *P. stuartii* cells expressing ß-galactosidase form green colonies on X-gal plates due to the combined reaction of the diffusible yellow pigment and the blue indigo product of X-gal cleavage. However, *aarA* mutants form a distinct blue colony due to the lack of yellow pigment. In a search for rhomboid-like proteins in the related organism *P. mirabilis,* a pACYC184-based genomic library from *P. mirabilis* was electroporated into *P. stuartii* XD37.A Δ*aarA cma37-lacZ* and colonies with restored production of yellow pigment were identified based on their green color. Plasmids conferring this phenotype contained related inserts and sequence analysis indicated the only complete genes present were *tatA* alone or *tatA* and *tatB,* which both encode products involved in the Tat export system required for secretion of cofactor containing proteins with twin arginine residues in the signal peptide (Santini et al., 1998, Sargent et al., 1998, Weiner et al., 1998). To determine if *tatA* alone could also restore extracellular signal production, a fragment containing only *tatA* was generated by PCR and cloned into pBluescript SK (-) to yield pPM.Tat_{Pm}. The control vector and pPM.Tat_{Pm} were electroporated into *P. stuartii* XD37.A (Δ*aarA, cma37::lacZ*) and conditioned medium was prepared from each strain and examined for signal production. As seen in Figure A1.1, conditioned medium from XD37.A (Δ*aarA*) cells containing

pPM.Tat$_{Pm}$ resulted in a 40-fold activation of *cma37::lacZ* expression, relative to a 4-fold activation in conditioned medium from XD37.A (Δ*aarA*) cells that contained the control vector pSK. The expression of pPM.Tat$_{Pm}$ in the wild-type background did not increase signal production above the normal levels (Figure A1.1).

The identification of *tatA* from *P. mirabilis* as a multicopy suppressor of an *aarA* mutation was not unique. In a previous study, we reported the identification of *E. coli* genes that restored pigment production to *aarA* mutants (Clemmer et al., 2006). One of the genes identified was *glpG*, encoding a rhomboid protein functionally similar to AarA. The other genes were *tatA* or *tatE*. The TatA and TatE proteins of *E. coli* are highly similar and functionally redundant (Sargent et al., 1998).

AarA mutants are defective in Tat function and rescued by *tatA* in multicopy

The identification of *tatA* as a high-copy suppressor of the *aarA*-dependent production of an extracellular signal suggested a close relationship between the AarA rhomboid and the Tat system. Tat-dependent phenotypes that have been observed in *E. coli* include; cell chaining, detergent sensitivity, and the inability to use trimethylamine-N-oxide (TMAO) as an electron acceptor during anaerobic growth with glycerol as a carbon source (Bogsch et al., 1998, Stanley et al., 2001). *P. stuartii aarA* mutants exhibit a prominent chaining phenotype (Rather & Orosz, 1994) and are similar in this respect to *E. coli tat* mutants (Stanley et al., 2001). A *P. stuartii aarA* mutant (XD37.A) was tested for the additional Tat-dependent phenotypes and was unable to grow anaerobically on glycerol TMAO plates or aerobically on MacConkey agar plates (Table 2). The presence of pSK.aarA rescued all of these phenotypes indicating that loss of *aarA* was responsible for these

phenotypes (Table 2). In addition, the presence of *tatA* from *P. mirabilis* (pPM.Tat$_{Pm}$) rescued the ability of a *P. stuartii aarA* mutant to grow on MacConkey agar, restored anaerobic growth on TMAO glycerol agar plates and restored normal cell morphology (Table 2).

Isolation and expression of the *P. stuartii tat* operon

To understand how overexpression of heterologous TatA proteins could suppress an *aarA* mutation, we isolated the native *tat* locus from *P. stuartii.* Despite repeated attempts, we were unable to isolate plasmids with the *P. stuartii tat* locus by restored pigment production, a strategy used to identify *tatA* from *P. mirabilis* and *E. coli.* Complementation based on restoring growth of an *aarA* mutant on MacConkey plates also failed. Therefore, an alternative strategy was used based on the location of the *tat* operon adjacent to the *ubiB* (formerly *yigR*) gene in both *E. coli* and *P. mirabilis.* Plasmids from a *P. stuartii* genomic library were isolated that complement the *ubiB* mutation (see Experimental Procedures) and sequence analysis of several clones indicated that genes corresponding to *tatABC* were present.

Unexpectedly, a clone containing the entire *tat* operon from *P. stuartii* was unable to rescue the *aarA*-dependent phenotypes. To determine if the *tatA* gene alone from *P. stuartii* could rescue the *aarA*-dependent phenotypes in a manner seen with *tatA* from *P. mirabilis* or *E. coli,* we subcloned only the *tatA* gene resulting in pBC.TatA$_{Ps}$. However, when pBC.TatA$_{Ps}$ was introduced into the *P. stuartii aarA* mutant XD37.A, there was no rescue of pigment production, and cells were still unable to grow on MacConkey agar. In addition, these cells still exhibited the chaining morphology (Table 2). However, the

ability of XD37.A/pBC.TatA$_{Ps}$ to grow anaerobically on glycerol TMAO plates was partially rescued and cells formed pinpoint colonies. To test if the *P. stuartii* TatA protein was functional in a heterologous organism, we introduced this plasmid into a *tatA/tatE* double mutant of *E. coli*. The *P. stuartii tatA* protein was functional in *E. coli,* correcting both the cell chaining phenotype and the ability to grow on glycerol TMAO plates (Table 2). Therefore, the *P. stuartii* TatA protein functions in *E. coli,* but very poorly in a *P. stuartii aarA* mutant. Although this was unexpected based on the ability of *tatA* from *P. mirabilis* and *E. coli* to rescue *aarA* mutants, it explained our inability to isolate this locus in previous experiments using complementation of an *aarA* mutant.

Expression of the *tat* operon is not dependent on *aarA*

To determine if the basis of suppression of an *aarA* mutation by increased TatA was due to lowered levels of *tat* operon expression in an *aarA* mutant, we examined the accumulation of the *tat* operon by Northern blot analysis of RNA prepared from wild-type PR50 or PR51 Δ*aarA*. However, the accumulation of *tat* operon mRNA was similar in both backgrounds (data not shown).

The *P. stuartii* TatA protein is processed by AarA

Examination of the TatA$_{Ps}$ protein revealed an unusual feature when compared to the *P. mirabilis* TatA (TatA$_{Pm}$) and *E. coli* (TatA$_{Ec}$) proteins. The TatA$_{Ps}$ was seven amino acids longer at the amino terminus (Figure A1.2A). In fact, examination of TatA proteins from other organisms in the GenBank database revealed that *P. stuartii* was one of a few organisms containing a TatA protein with this seven amino acid extension. This peculiar

feature of $TatA_{Ps}$, combined with the inability of the native $TatA_{Ps}$ to rescue the *aarA* mutation, prompted us to test the possibility that the $TatA_{Ps}$ required cleavage by the AarA protease to remove this amino terminal extension in order to function properly. We hypothesized that the $TatA_{Pm}$ and $TatA_{Ec}$ proteins function in an AarA-independent manner because they are naturally missing this N-terminal extension. To test this possibility, a His_6 epitope tag was placed at the C-terminus of $TatA_{Ps}$ and a pACYC184 derivative encoding the $TatA_{Ps}$-His_6 protein (pAC.Tat3) was introduced into wild-type *P. stuartii* PR50 and the isogenic *aarA* mutant PR51. Cell extracts from the PR50 expressing $TatA_{Ps}$-His_6 and PR51 expressing $TatA_{Ps}$-His_6 were analyzed by Western blot analysis using an antibody to the His_6 tag (Figure A1.2B). The predicted molecular mass of the mature $TatA_{Ps}$-His_6 protein is 11,250 Da. In PR51 Δ*aarA,* the size of the $TatA_{Ps}$-His_6 hybrid protein was approximately 0.5 kDa larger than in wild-type PR50 (Figure A1.2B, lane 1 and 3). Introduction of the *aarA* gene on a compatible pBluescript.SK plasmid into PR51 Δ*aarA* resulted in a $TatA_{Ps}$-His_6 protein that was approximately 0.5 kDa smaller and identical in size to that seen in wild-type cells (Figure A1.2B, lane 4). To determine the basis of the reduced size in wild-type cells, the processed and unprocessed forms of $TatA_{Ps}$-His_6 protein were purified by nickel affinity chromatography. Analysis of the N-terminus by Edman degradation revealed that the N-terminal sequence of the smaller processed form of TatA was AFGSPWQLI, an exact match to amino acids 9-17 of TatA (Figure A1.2A). The N-terminal sequence of the unprocessed TatA protein yielded the expected sequence of MESTIATA. Therefore, the TatA protein is processed in an AarA-dependent manner by removal of the N-terminal 8 amino acids.

To determine if cleavage of $TatA_{Ps}$ by AarA required a functional Tat system, cleavage was examined in a *tatC::Sm*^{R} background. The AarA-dependent processing of TatA appeared to be normal in the *tatC::Sm*^{R} background (Figure A1.2A, lane 6).

Purified AarA cleaves TatA in-*vitro*

To determine if the AarA-dependent cleavage of TatA was direct, the ability of purified AarA to process TatA was investigated in-*vitro*. We expressed C-terminal His-tagged forms of wild-type AarA and a predicted inactive mutant in which the active site serine (Ser 150) was mutated to alanine in *E. coli* (Figure A1.3A)(Urban et al., 2001, Lemberg et al., 2005). The isolated, detergent-solubilized cellular membranes containing AarA showed robust proteolytic activity against the in vitro translated model substrate derived from *Drosophila* Gurken while, as predicted, no activity was detected for its S150A active site mutant (Figure A1.3B). Next, the detergent-solubilised AarA was purified to homogeneity (Figure A1.3A.) by nickel affinity chromatography. This purified enzyme maintaned its proteolytic activity against Gurken-derived peptide (Figure A1.3B). Purified AarA, but not its active-site mutant, caused a minor increase in electrophoretic mobility of the in-*vitro* translated TatA, consistent with the proteolytic removal of the small N-terminal extension (Figure A1.3B). To enhance the change in mobility of the processed TatA, we added an artificial N-terminal $(SerGly)_4$ extension to the in-*vitro* translated TatA. As expected, the cleaved product of this extended TatA was more easily distinguished from the uncleaved starting material. Notably, in Figure A1.3C, incubation of substoichiometric amounts of purified AarA with purified full-length $TatA_{Ps}$-His_6 led to its complete conversion to the faster migrating species. Chemical

protein sequencing confirmed that the cleavage was identical to that in-*vivo*: the N-terminus of the TatA cleavage product started with amino acids AFGSPWQLI, while incubation of $TatA_{Ps}$-His_6 with buffer only or AarA S150A did not lead to any change of the original N-terminus (MESTIATA). This demonstration that the AarA rhomboid protease is necessary and sufficient for TatA proteolytic maturation proves that TatA is a direct substrate of AarA.

A truncated form of TatA, missing seven N-terminal amino acids, is able to restore Tat function in an AarA-independent manner

The requirement for AarA in processing the $TatA_{Ps}$ protein suggested that the dependence for AarA in Tat function could be bypassed by expressing a $TatA_{Ps}$ that was artificially truncated from amino acids 2-8 (Figure A1.2A). A plasmid containing this truncated TatAD2-8_{Ps} was introduced into the *aarA* mutant XD.37.A along with the wild-type $TatA_{Ps}$ and the vector control. The presence of TatAD2-8_{Ps} now restored all the *aarA*-dependent phenotypes back to wild-type, including pigment production, cell morphology, growth on MacConkey plates and anaerobic growth on glycerol/TMAO plates (Table 2). In addition, production of the extracellular activating signal was restored by the presence of TatAD2-8_{Ps} (Figure A1.4A).

Role of the *P. stuartii* Tat system in extracellular signal production

The original observation that AarA was required for signal production, coupled with the data presented in this study that AarA is required for Tat function, suggested that AarA works through the Tat export system for production of this extracellular signal. To

test this possibility, a *tatC::Sm*^{R} mutation was created as described in the Experimental Procedures. Strain PR50.C (*tatC::Sm*^{R}) exhibited the same phenotypes as the *aarA* mutant PR51 (Table A1.1). Conditioned medium prepared from the *tatC::Sm*^{R} mutant exhibited a markedly reduced ability to activate the *cma37::lacZ* fusion (2-fold), relative to conditioned medium from wild-type PR50 (29-fold) (Figure A1.4B). The level of activation by conditioned medium from the *tatC::Sm*^{R} mutant was the same as observed with conditioned medium from the *aarA* mutant PR51 (2-fold) and from the *tatC::Sm*^{R}, *aarA* double mutant PR51.C (3-fold).

Discussion

Previously, we identified a role for AarA, a rhomboid-type protease, in production of an extracellular signaling molecule in *P. stuartii* (Rather et al., 1999). However, *aarA* mutants also possess many of the phenotypes associated with loss of Tat function in *E. coli* (Stanley et al., 2001, Ize et al., 2004), including cell chaining, detergent sensitivity and the inability to grow anaerobically with trimethylamine-N-oxide (TMAO) as an electron acceptor (19, Table 2). This information together with the finding that overexpression of TatA can suppress the loss of signal production in *aarA* mutants of *P. stuartii* suggested a close relationship between AarA and the Tat system in *P. stuartii.* The work reported here demonstrates that this relationship is based on the requirement for a functional AarA rhomboid to process TatA between the eighth and ninth amino acid from the N-terminus. This processing is required for TatA function, because an artificially truncated form of TatA missing amino acids 2-8 (TatAD2-8) restored Tat function in an AarA-independent manner (Table 2 and Figure A1.4). This explains why TatA from *P. mirabilis* or *E. coli*, which are both naturally missing this N-terminal extension, restored Tat function in an AarA –independent manner in *P. stuartii* (Table 2 and Figure A1.1).

The ability of purified AarA to direct TatA cleavage in-vitro strongly suggests that AarA is directly responsible for TatA processing in-*vivo.* This represents the first rhomboid protease/substrate pair identified in a prokaryotic system. At the current time, it is unclear why TatA processing is required for function of the Tat system in *P. stuartii.* Studies in *E. coli* have demonstrated that TatA can exist in complexes with TatB, with TatBC and as a separate TatA homo-oligomer (Bolhuis et al., 2001, Sargent et al., 2001,

de Leeuw et al., 2002, Porcelli et al., 2002, Bolhuis et al., 2000). In addition, the TatA protein itself may form the actual pore for protein secretion (Gohlke *et al.*, 2005). Based on this information, the simplest explanation is that the unprocessed form of TatA is unable to interact with one or more of the Tat proteins, possibly due to an unfavorable conformation. Alternatively, the unprocessed N-terminus may inhibit either TatA insertion or correct localization in the cytoplasmic membrane.

An additional aspect of this study is the finding that the Tat system is required for production/activity of an extracellular signaling molecule. This signaling molecule was originally found to be AarA-dependent, however, the requirement for AarA in Tat function indicates that the involvement of AarA is indirect. How the Tat system is required for signal production is unknown. One intriguing possibility that we have likely ruled out in this study is that the peptide released by TatA cleavage is the signaling molecule. This is based on the observation that in a *tatC* mutant, there is no signal activity (Figure A1.5), yet TatA is still processed normally (Figure A1.3). Therefore, the production or activity of signal may require the Tat-dependent transport of an enzyme that is involved in signal production. Alternatively, the signal itself may be transported by the Tat system. Identification of the gene product directly involved in signal production will be required to help distinguish these possibilities.

Materials and Methods

Bacterial growth conditions

Growth media was Luria Broth (LB) containing 10 g of tryptone, 5 g of yeast extract, and 5 g of NaCl per liter. Antibiotics were used at the following concentrations for *P. stuartii*: chloramphenicol 100 mg/ml. Antibiotics were used at the following concentrations for *P. mirabilis*: chloramphenicol 100 mg/ml; tetracycline 15 mg/ml.

Preparation of conditioned media (CM)

Conditioned media for cell assays was prepared by inoculation of 30 ml of LB with antibiotics as needed with either 3 ml of an overnight culture or a single colony followed by shaking (250 rpm) at 37ºC. Cultures were harvested at an OD_{600} of 0.9. The cells were pelleted at 4300 x *g* for 10 minutes and supernatants were harvested. To control for pH changes and nutrient deprivation of the conditioned media, the pH was adjusted to 7.5 and tryptone-yeast extract was added to a final concentration of 0.5x relative to the concentration present in LB. CM was filter sterilized with a 0.22m syringe filter. The first 3 ml of CM that passed through the filter was discarded and the rest was stored at -80 ºC until use. To assay CM, a dilute suspension of the cells to be tested was inoculated at a 1:1000 dilution and grown to an $O.D._{600}$ of 0.35. ß-Galactosidase assays were done on sodium dodecyl sulfate-chloroform-treated cells grown to mid-log phase by the method of Miller.

Isolation of *tatA* from *P. mirabilis*

A genomic library consisting of partial *Sau*3A fragments of *P. mirabilis* cloned into the *Bam*HI site of pACYC184 (Chang & Cohen, 1978) was introduced into *P. stuartii* XD37.A by electroporation. Plasmids that complemented *aarA* were first identified as colonies with restored production of a diffusible yellow pigment absent in *aarA* mutants. Plasmids with apparent complementation of *aarA* were sequenced by subcloning a 1.8 kb *Eco*RV fragment into pBluescript SK- and using the T3 and T7 primers. The *tatA* coding region was amplified from *P. mirabilis* 7002 by PCR using the primers 5'-ACATGTCTAGAAAAGTATAACTCCTAAAATAG-3' and 5'-TAGGCTACTCGAGCTAAAACCAATGTCAAACAC-3' and cloned into pBluescript SK- using the *Xba*I and *Xho*I sites.

Isolation of the *P. stuartii tatA* region

The *tat* region *of P. stuartii* was cloned based on linkage to the *ubiB* gene (formerly *yigR*). A genomic *P. stuartii* library of partially digested *Sau*3A fragments in pACYC184 was introduced into *E. coli* DM123 *ubiB::Km*^{R} by standard electroporation. Plasmids that complemented the *ubiB* mutation were identified as normal sized colonies in the background of microcolonies formed by the *ubiB* mutant. Plasmids from large colonies were retransformed to verify the plasmid restored normal growth. Restriction mapping and DNA sequencing using sets of primers used to "walk" along the sequence was used to identify the *tatABC* operon immediately adjacent to *ubiB*. The sequence of the *tat* region from *P. stuartii* has been deposited to GenBank under accession number DQ989793.

Northern blot analysis

The accumulation of *tat* operon mRNA from *P. stuartii* was assayed by Northern blot analysis. Cell samples were collected at an OD of 0.6 and RNA was extracted using the MasterPure RNA Purification Kit (Epicentre). RNA was separated on a formaldehyde agarose (1.2%) gel and blotted onto a nitrocellulose membrane. A digoxigenin-labeled *tatABC* probe was synthesized by PCR using the primers 5'-GTTGAGAGCAAAAATAAAGAG-3' and 5'-ATGCGGATCCCCATCCCTAAATAGAAAAGG-3'.

Construction of pET.TatA-His

Polymerase chain reaction was used to amplify the *tatA* gene from *P. stuartii* and engineer a His_6 tag at the C-terminus using the primers 5'-GGTACAGGATCCGTTAAACCTTGTTTTGCGAC-3' and 5'-CGTACGGGATCCCACGGTTTAATGATGATGATGATGATGACCCTGCTCTT TATTTTTGC-3'. These primers engineered *Bam*H1 restriction sites at both ends of the PCR product. In addition, the TatA-His_6 is missing its native *tat* promoter, but contains the native ribosome-binding site. This fragment was cloned into *Bam*H1 digested pACYC184 (Chang & Cohen, 1978) resulting in plasmid pAC.Tat3. To construct pET.TatA-His, the *Bam*H1 fragment from pAC.Tat3 was cloned into pET21a such that TatA-His_6 was transcribed from the T7 promoter. The pET.TatA-His_6 plasmid was electroporated into PR50 or PR51 which contained p184.T7, and encoded an IPTG

inducible T7 RNA polymerase gene (Macinga *et al.*, 1995). Transformants were selected on LB agar plates containing ampicillin 400 μg/ml and chloramphenicol 100 μg/ml.

Western blot analysis

Bacterial cultures were grown with shaking at 37° in LB and appropriate antibiotics. Cells were pelleted at an OD_{600} of 0.6 and resuspended in Tricine Sample Buffer (BioRad). Bacterial pellets were lysed in Tricine sample buffer by heating at 95°C for 10 min. Protein samples were heated at 95°C for 10 min and subjected to SDS-PAGE electrophoresis using the BioRad ReadyGel system and 16.5% Tris-Tricine/Peptide gels. Gels were electrotransferred to nitrocellulose and probed at 1:1,700 with a Tetra-His HRP Conjugate antibody (Qiagen). Detection was carried out using ECL Western Blotting Detection Reagents in accordance with standard procedures (Amersham Biosciences).

Purification of TatA-His_6 and N-terminal sequencing

An overnight culture of PR50 pET.TatA-His_6/p184.T7 was diluted 1:100 in 200ml LB with ampicillin (400 μg/ml) and chloramphenicol (100 μg/ml) and grown with shaking at 37°. IPTG was added (1mM) at an OD_{600} of 0.6 and cells were grown for an additional 90 minutes before pelleting. The pellet was resuspended in 10ml lysis buffer (200mM Tris pH8.0, 1% Triton X-100) and incubated at room temperature for 15 minutes prior to filtering through a 0.22uM filter. A Talon resin column (BD Biosciences) was pre-washed 3X in wash buffer (50mM NaH_2PO_4, 300mM NaCl pH7.0) prior to addition of the lysate and washed 3X prior to elution in 3ml elution buffer (50mM NaH_2PO_4,

300mM NaCl pH7.0, 150mM Imidazole pH7.0). Samples were concentrated using a Microcon YM-3 centrifugal filter device (Amicon) prior to running on a 16.5 % Tris Tricine/Peptide gel (BioRad Ready Gel). A prominent band was seen at ~20kD. For sequencing, an unstained gel was blotted into PVDF membrane and stained with Amido Black Staining Solution (Sigma). N-terminal sequencing was done using a PE-Biosystems 491A Pulsed-Liquid Sequencer with a PE-Biosystems 140S PTH Analyzer (Procise-HT).

Expression and Purification of AarA

Wild type hexahistidine-tagged AarA (Lemberg et al., 2005) was expressed from the pET25+ based construct (pET.AarA.His_6). The predicted catalytically inactive AarA mutant S150A was constructed by QuikChange (Stratagene) mutagenesis using pET.AarA.His_6 as a template and primers

5'- ACTATCGGTGTTGGGGCTGCAGGCGCGATTATGGG-3' and

5'-CCCATAATCGCGCCTGCAGCCCCAACACCGATAGT-3' to generate pET25.AarAS150A.His_6. Mutation was verified by DNA sequencing. Both AarA-His_6 and AarAS150A-His_6 were overexpressed in *E. coli* BL21(DE3) Gold and total membrane fractions were isolated as described (Lemberg et al., 2005). The whole purification procedure was carried out at 4°C. Membrane pellets originating from 1L of bacterial culture were resuspended in 4 mL of buffer B (20 mM HEPES-NaOH, pH 7.4, 10%(v/v) glycerol, 0.3 M NaCl and 10 mM imidazole) and total protein concentration was determined using the DC protein assay (Bio-Rad). If necessary, protein concentration was adjusted to 5 mg/mL and 20% (w/v) n-dodecyl-b-D-maltoside (DDM) was added to

1.5% final concentration to solubilise membrane proteins. The resulting solutions were rocked at 4°C for 1 hour and then they were centrifuged at 100,000g for 30 min at 4°C. The supernatants were loaded onto 0.5 mL Ni-NTA agarose gravity-flow columns (Qiagen) pre-equilibrated in buffer B containing 0.05% DDM. Columns were washed with 4 mL of buffer B+0.05% DDM, then 4 mL of buffer B with 0.05% DDM and 50 mM imidazole. Purified proteins were eluted by 1.5 mL of buffer B with 0.05% DDM and 250 mM imidazole, dialyzed into buffer B containing 0.05% DDM, flash-frozen into liquid nitrogen and stored at -80°C. Protein concentration was determined with BCA protein assay (Pierce).

In-vitro rhomboid cleavage assays

Radiolabelled TatA variants were generated by cell-free in vitro translation using wheat-germ extract (Promega) and [^{35}S]-Met (GE Healthcare) as described (Lemberg & Martoglio, 2003). Templates for *in vitro* transcription were generated from pET.TatA-His_6 using forward primers

5'- CGATTAGGTGACACTATAGAATACCATGGAATCAACTATTGCAACGG -3' (to produce full-length TatA) or

5'-CGATTAGGTGACACTATAGAATACCATGTCAGGTTCAGGTTCAGGTTCAG-GGAATCAACTATTGCAACGGCC -3' (to add $(SG)_4$ peptide to the N-terminus of TatA) and reverse primer 5'- AAGAAGCTACATCATCATACCCTGCTCTTTAT TTTTGCTCTC -3' (adding -MMM to the C-terminus of TatA). Rhomboid cleavage assays were conducted in 50 mM HEPES-NaOH pH 7.4, 10% glycerol and 50 mM EDTA at 30°C. Typically, 1-4 uL of translation mixtures and the indicated amount of

enzyme (Figure A1.5B) were used in 40 uL reaction volume. Cleavage reactions were stopped by protein precipitation with 10% (w/v) trichloroacetic acid and samples were prepared for SDS electrophoresis, electrophoresed and autoradiographed as described (Lemberg & Martoglio, 2003).

Site directed mutagenesis of *tatA*$_{Ps}$.

Amino acids 2-8 were deleted from pBC.TatA$_{Ps}$ by oligonucleotide-directed mutagenesis using the QuickChange kit (Stratagene). The primers used for this deletion were 5'-GAGGTATAAACAATGGCTTTTGGTAGCCCTTGG-3' and 5'-CCAAGGGCTACCAAAAGC CATTGTTTATACCTC-3'

Construction of *tatC* null allele

An internal sequence of the *tatA* gene from *P. stuartii* was amplified by PCR using the primers 5'-ATGCTCTAGAATTGAACTCAGGAAGCGGCTG-3' and 5'-ATGCGGATCCCCATCCCTAAATAGAAAAGG-3'. The PCR product was cloned into the suicide vector pKNG101 (Kaniga *et al.*, 1991) using *Xba*I and *Bam*HI sites engineered in the primers resulting in plasmid pPTA1. The pPTA plasmid was introduced into the chromosome of *P. stuartii* PR50 or PR51 Δ*aarA* by a plate mating with *E. coli* SM10/pPTA. *P. stuartii* exconjugants were selected on LB media containing tetracycline (15 mg/ml) and streptomycin (35 mg/ml). All streptomycin resistant colonies were sucrose sensitive and Southern blot analysis indicated they contained pPTA inserted into the *tatC* gene.

Acknowledgements

This work was supported by NSF award 0406047 to P.N.R and a Merit Review award from the Department of Veterans Affairs. P.N.R. is supported by a Research Career Scientist award from the Department of Veterans Affairs. K.S. is a recipient of Marie Curie Intraeuropean Fellowship.

Table A1.1

Strain	MacConkey agar	TMAO agar	Morphology
XD37 wild-type	+	+	Rods
XD37.A Δ*aarA*	-	-	Chains
XD37.A Δ*aarA*/pSK	-	-	Chains
XD37.A Δ*aarA*/pSK.aarA	+	+	Rods
XD37.A Δ*aarA*/pSK.TatA$_{Pm}$	+	+	Rods
XD37.A Δ*aarA*/ pBC.SK	-	-	Chains
XD37.A Δ*aarA*/ pBC.TatA$_{Ps}$	-	+/-	Chains
XD37.A Δ*aarA*/ pBC.TatAD2-8$_{Ps}$	+	+	Rods
PR50 wild-type	+	+	Rods
PR51 Δ*aarA*	-	-	Chains
PR50.C *tatC*::Sm^R	-	-	Chains
PR51.C Δ*aarA*, *tatC*::Sm^R	-	-	Chains

Table A1.1 – Tat phenotypes of various mutants

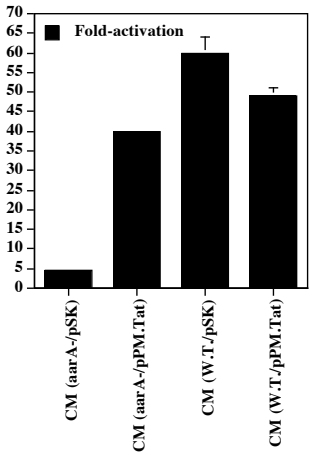
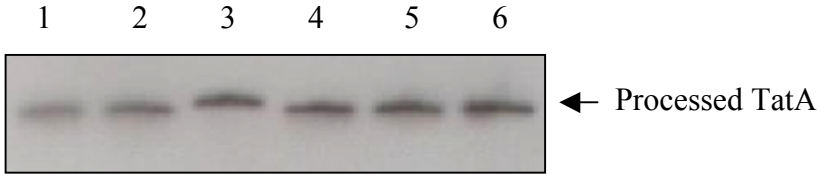


Figure A1.1. Overexpression of *tatA* from *P. mirabilis* restores signal production to *P. stuartii aarA* mutants

Cultures of *P. mirabilis* XD37 (wild-type) or XD37.A (Δ*aarA*) containing pBluescript SK (vector) or pPM.tatA containing the *tatA* gene were grown in LB with ampicillin (200 mg/ml) and conditioned medium was harvested at an optical density A_{600} = 1.2. Signal activity was tested using *P. stuartii* XD37 *cma37-lacZ* as a signal biosensor. The values represent fold-activation relative to the same cells grown in control LB and were determined from quadruplicate samples from two independent experiments.

A.

B.



1) PR50/pAC.Tat3, pSK
2) PR50/pAC.Tat3, pSK.aarA
3) PR51 Δ*aarA*/pAC.Tat3, pSK
4) PR51 Δ*aarA*/pAC.Tat3, pSK.aarA
5) PR50/pAC.Tat3
6) PR50.C *tatC::Sm*^{R}/pAC.Tat3

Figure A1.2. TatA processing in wild-type and an *aarA* mutant

In panel A, the TatA proteins from *E. coli, P. mirabilis* and *P. stuartii* are aligned. The arrowhead designated the site of TatA processing in wild-type *P. stuartii.* Also shown is the sequence of the truncated form of TatA (TatAD2-8) that was constructed by site-directed mutagenesis. In panel B, total cell protein from the indicated strains were separated on a 16.5%Tris-Tricine SDS-PAGE gene and transferred to nitrocellulose. For the Western blot analysis of TatA, filters were probed with an alkaline phosphatase conjugated Penta-His antibody.

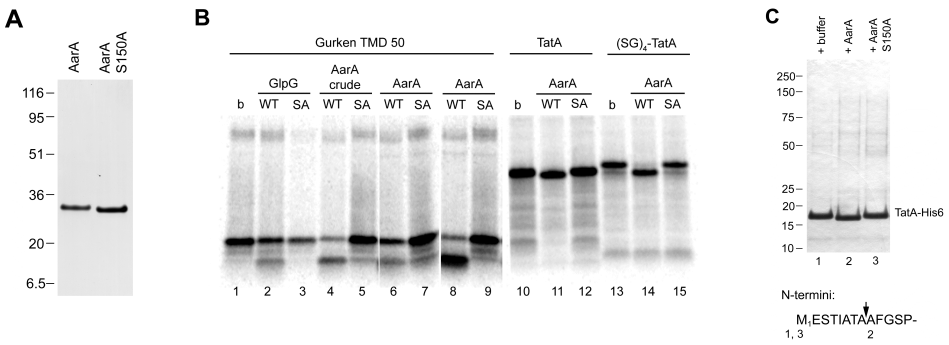


Figure A1.3. In-vitro cleavage of TatA by purified AarA protease

Panel A, a Coomassie-stained SDS-PAGE of NiNTA agarose-purified wild type AarA-His_6 (1 µg) and its active-site mutant S150A (1.5 µg) is shown. Panel B, the model Drosophila Gurken-derived substrate (Lemberg et al., 2005), full-length *P. stuartii* TatA and its variant, N-terminally extended by four SerGly repeats ($(SG)_4$-TatA) were in vitro translated and labeled with [^{35}S]-Met. Peptides were incubated with the indicated enzymes for 60 min at 30°C, separated by SDS PAGE and autoradiographed. Purified *E. coli* rhomboid GlpG is shown as a positive control. Reactions shown in lanes 2, 3, 6 – 9 contained 1.5 µg and those shown in lanes 11, 12, 14 and 15 contained 3.8 µg enzyme per 40 µL reaction. (B, buffer only; WT, wild type; SA, GlpG-S201A or AarA-S150A). Panel C, Nickel affinity chromatography-purified TatA-His_6 (190 µg/mL) was incubated in the presence of buffer only (b), purified AarA or its active-site mutant S150A (both at 15 µg/mL) at 30°C for 2.5 hrs. Mixtures were then separated by SDS PAGE and stained

by Coomassie brilliant blue. Sequences of the N-termini of the resulting TatA bands were determined by automated Edman sequencing. The AarA cleavage site in *P. stuartii* TatA is indicated by an arrow.

A. B.

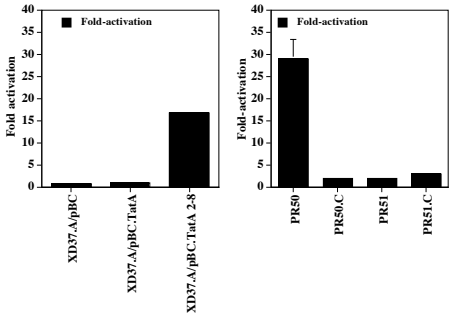


Figure A1.4. Role of the Tat transport system in extracellular signal production

In panels A and B, the ability of conditioned medium from the indicated strains to activate the *cma37::lacZ* fusion in XD37 is shown. For these experiments, conditioned medium was harvested an O.D. of 0.9. The values represent fold-activation relative to the same cells grown in control LB and were determined from quadruplicate samples from two independent experiments.

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Additional Publication (#2)

Pathogenic Bacteria Induce Colonic PepT1 Expression: An Implication in Host Defense Response ∇

Hang Thi Thu Nguyen*[1], Guillaume Dalmasso*[1], Kimberly Robin Powell[2], Yutao Yan[1], Shantanu Bhatt[2], Daniel Kalman[2], Shanthi V Sitaraman[1], Didier Merlin[1]

[1] Department of Medicine, Division of Digestive Diseases, and

[2] Department of Pathology, Emory University School of Medicine, Atlanta, GA 30322

Address correspondence:

Hang Thi Thu Nguyen, Ph.D, Department of Medicine, Emory University,

615 Michael Street, Atlanta, GA 30322

Tel: 404-727-6246; Fax: 404-727-5767; e-mail: hnguye9@emory.edu

*Both authors contributed equally to this work.

This chapter is part of the manuscript that has been cited as -

Nguyen, H.T.T., G. Dalmasso, K.R. Powell, Y. Yan, S. Bhatt, D. Kalman, S. V. Sitaraman & D. Merlin, (2009) Pathogenic bacteria induce colonic PepT1 expression: An implication in Host Defence Response. *Gastroenterology* **137**: 1435-1447.

∇ Shantanu Bhatt assisted with the immunofluorescence microscopy.

Abstract

Background and Aims: Although expression of the di/tripeptide transporter PepT1 has been observed in colon under inflammatory conditions, the inducing factors and underlying mechanisms remain unknown. Here, we addressed the role of pathogenic bacteria in PepT1 expression/function.

Methods: Colonic HT29-Cl.19A cells were infected with enteropathogenic *E. coli* (EPEC). PepT1 promoter activity and PepT1 expression/activity were analyzed using the luciferase assay, RT-PCR, nuclear run-on assay, immunoblotting, immunofluorescence staining and uptake experiments. Cdx2-PepT1 promoter binding was assessed by gel-shift and chromatin-immunoprecipitation assay. Colonic tissues from wild-type and PepT1 over-expressing mice were infected with *Citrobacter rodentium*. Interleukin (IL)-8 and keratinocyte-derived chemokine (KC) expression levels were examined by real-time RT-PCR and ELISA.

Results: EPEC transcriptionally induced PepT1 expression/activity in HT29-Cl.19A cells. PepT1 was expressed in Cdx2-overexpressing HT29-Cl.19A cells, indicating the importance of Cdx2 in PepT1 expression. Importantly, EPEC induced PepT1 expression in lipid rafts (LRs). PepT1 expression required intimate adherence of EPEC to host cells through LRs. Remarkably, the presence of PepT1 in LRs delayed EPEC-LR binding as monitored in real time by an electric cell-substrate impedance-sensing technique. Furthermore, PepT1 overexpression in HT29-Cl.19A cells reduced EPEC-triggered NF-kB and MAP kinase activation and IL-8 production. *C. rodentium* increased PepT1 mRNA and protein expressions in mouse colonic tissues. In agreement with *in vitro* data,

overexpression of PepT1 in the mouse colon reduced *C. rodentium* adherence and KC production.

Conclusions: We demonstrate that EPEC induces functional PepT1 expression in LRs of colonocytes via Cdx2, and that PepT1 has a role in bacterial-epithelial interactions and intestinal inflammation.

Introduction

The di/tripeptide transporter PepT1 is normally expressed in brush border membranes of enterocytes in the small intestine, the proximal tubular cells of the kidney, bile duct epithelial cells and immune cells (Charrier *et al.*, 2007). PepT1 expression is induced in colonic epithelial cells under inflammatory condition (Merlin *et al.*, 2001, Ziegler *et al.*, 2002). Although the mechanism of PepT1 expression under pathological conditions remains unknown, it has been suggested that PepT1 expression is likely induced at the transcriptional level and that specific transcriptional regulation by signaling pathway(s) may be activated. Recently, we have demonstrated that PepT1 is localized in lipid rafts (LRs) from mouse intestinal brush border membranes, polarized intestinal epithelial cells (IECs) and immune cells (Nguyen *et al.*, 2007). LRs have been proposed to compartmentalize proteins and lipids to regulate many functions in cellular signal transduction and trafficking (Simons & Toomre, 2000). Increasing evidences have recently explored the role of LRs as docking sites for pathogens to attack host cells (Lafont & van der Goot, 2005, Manes *et al.*, 2003).

The non-invasive enteropathogenic *Escherichia coli* (EPEC) is a food-borne pathogen that has been implicated in the pathophysiology of infantile diarrhea (Nataro & Kaper, 1998). EPEC intimately attaches to host IECs through the binding of the translocated receptor Tir, secreting through the type III secretion system, and its ligand intimin (Nougayrede *et al.*, 2003). EPEC adherence consequently causes epithelial microvilli destruction and promotes formation of actin-rich pedestal at the binding site (Knutton *et al.*, 1989, Moon *et al.*, 1983). It has been shown that EPEC induces inflammatory responses in IECs characterized by activation of the transcription factor NF-kB (Savkovic

et al., 1997) and MAP kinases (Savkovic *et al.*, 2001, Czerucka *et al.*, 2001), leading to production of pro-inflammatory cytokines such as interleukin (IL)-8 (Savkovic et al., 2001, Czerucka et al., 2001). EPEC has been reported to modulate epithelial membrane transport activities, such as butyrate uptake(Borthakur *et al.*, 2006) and Na^+/H^+ (Hecht *et al.*, 2004) and Cl^-/OH^- (Gill *et al.*, 2007) exchanges. Although the underlying mechanisms have not yet been fully characterized, these activity changes are suggested to involve the redistribution of surface proteins from the apical membrane into the intracellular compartment (Gill et al., 2007, Borthakur et al., 2006). The regulation of membrane transport by EPEC at a transcriptional level has not yet been investigated.

In the present study, we addressed i) the role of EPEC in the induction of PepT1 expression and function in colonic epithelial cells, ii) the molecular mechanisms underlying EPEC-induced colonic PepT1 expression, and iii) the role of colonic PepT1 in bacterial-epithelial interactions and EPEC-induced intestinal inflammation. *In vitro* studies were validated by *ex vivo* experiments using *Citrobacter rodentium* as an EPEC homologue in mouse.

Materials and Methods

Cell culture

HT29-Cl.19A cell line was grown in DMEM (Invitrogen) supplemented 10% FBS (Invitrogen) and 1.5mg/ml plasmocin (Invivogen).

Bacteria growth and infection

Wild-type (WT) EPEC E2348/69, EPEC mutants Δ*eae* and Δ*tir* strains were grown in Luria-Bertani (LB) broth. For infection, EPEC was transferred to serum-free and antibiotic-free DMEM, and a multiplicity of infection (MOI) of 10 of bacteria was added to confluent monolayers. After infection, cells were washed with PBS to remove non-adherent EPEC. For adhesion assay, cells were lysed in water containing 0.1% BSA, and appropriate dilutions were plated in duplicate in LB plates(Dahan *et al.*, 2003).

Plasmid construction and transfection

cDNA encoding Cdx2 or PepT1 was cloned as previously described(Nduati *et al.*, 2007, Nguyen et al., 2007). HT29-Cl.19A cells were transfected using Lipofectin (Invitrogen) and stably selected in culture medium containing 1.2 mg/ml geneticin (Sigma).

Lipid raft isolation from HT29-Cl.19A cells

Isolation of lipid rafts (LRs) was performed as previously described(Nguyen *et al.*, 2006). Briefly, HT29-Cl.19A cells were lysed with 1% TX-100 in TNE buffer (25 mM Tris-HCl pH 7.4, 150 mM NaCl, 5 mM EDTA). Cell lysate (2 mg protein) was adjusted

to 40% sucrose in TNE buffer, overlaid with 2 vol of 30% sucrose and 1 vol of 5% sucrose, and then centrifuged at 40,000 rpm for 18 h at 4°C in a Sorval SW 41 Ti rotor (Beckman). Twelve fractions (1-ml each) were collected from the top of the gradient. The floating membrane fraction was defined as LRs.

In some experiments, cells were treated with 10 mM methyl-β-cyclodextrin (mβCD) for 30 min and replenished with 2 mM water-soluble cholesterol for 1 h as previously described(Nguyen et al., 2007).

Dual-luciferase reporter assay

HT29-Cl.19A cells were transfected with 5 ng of a construct encoding *Renilla* luciferase (Promega) and 2 μg of PepT1 promoter construct previously cloned(Nduati et al., 2007) using lipofectin. Luciferase activity was measured and normalized based on the control *Renilla* luciferase activity.

RNA extraction and RT-PCR

Total RNA was extracted using TRIzol (Invitrogen) and reverse transcribed using cDNA Synthesis kit (Fermentas). RT-PCR was performed using GeneJET Fast PCR kit (Fermentas) and specific primers.

Real-time RT-PCR

Real-time RT-PCR was performed using an iCycler (Bio-Rad). Briefly, cDNA was amplified by 40 cycles of 95°C-15 s and 60°C-1 min, using the iQ SYBR Green Supermix (Biorad) and specific primers (Supplementary data). 18S and 36B4 were used

as housekeeping genes. Fold-induction was calculated using the Ct method: ΔΔCT = (Ct_{Target}-$Ct_{housekeeping}$)infected - (Ct_{Target}-$Ct_{housekeeping}$)uninfected, and the final data were derived from $2^{-\Delta\Delta CT}$. The specific primers used were:

IL-8 sense 5'-GTG CAG TTT TGC CAA GGA GT-3'; IL-8 antisense 5'-AAA TTT GGG GTG GAA AGG TT-3'.

18S sense 5'-CCC CTC GAT GAC TTT AGC TGA GTG T-3'; 18S antisense 5'-CGC CGG TCC AAG AAT TTC ACC TCT-3'.

Mouse 36B4 sense 5'-TCC AGG CTT TGG GCA TCA-3'; mouse 36B4 antisense 5'-CTT TAT CAG CTG CAC ATC ACT CAG A-3'.

Mouse PepT1 sense 5'-CGT GCA AGT AGC ACT GTC CAT-3'; mouse PepT1 antisense 5'-GGC TTG ATT CCT CCT GTA CCA-3'.

Cloning of full-length cDNA encoding PepT1 expressed in EPEC-infected HT29-Cl.19A cells

Total RNA was isolated from EPEC-infected HT29-Cl.19A cells and reverse transcribed using cDNA Synthesis kit (Fermentas). RT-PCR was performed using Platininum Taq DNA Polymerase (Invitrogen) and specific primers (sense 5'-CGC CAT GGG AAT GTC CAA ATC-3', antisense 3'-CCC CGG TTA AGT GTC TTT GTC TAC-5'). After an initial denaturation at 94°C for 5 min, PCR was performed for 35 cycles (denaturation 94°C-1 min, annealing 60°C-30 sec and extension 72°C-3 min), followed by a final extension at 72°C for 10 min. The resulting PCR product (2117 bp) was cloned into pGEM-T Easy Vector (Promega). Plasmids were grown and purified using the Qiagen Maxiplasmid kit (Qiagen), and the insert was sequenced (Lark Technologies).

Immunofluerescence staining

HT29-Cl.19A cells grown on glass cover slip were stained for human PepT1 expression using Alexa Fluor 488-conjugated anti-rabbit antibody (Molecular Probes) as previously described (Nguyen et al., 2007).

Electrophoretic mobility shift assay (EMSA)

Cdx2-DNA binding was analyzed in cellular extracts prepared in totex buffer (20 mM HEPES pH 7.9, 350 mM NaCl, 20% glycerol, 1% NP-40, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.1 mM EGTA). Samples (5 μg) were incubated for 20 min at room temperature with a biotin-labeled double-stranded oligonucleotide encoding PepT1 promoter containing $Cdx2^{-579}$ binding site. Complexes were resolved by electrophoresis on 5% TBE gels. Gels were transferred to Biodyne B Nylon Membranes (Pierce) and complexes were visualized using the Chemiluminescent Nucleic Acid Detection System (Pierce). Specificity of the complexes was analyzed by incubation with a 200-fold excess of unlabeled oligonucleotides. Supershift assay was performed using 2 μg of Cdx2 antibody (Zymed Laboratories).

Chromatin immunoprecipitation assay (ChIP)

ChIP was performed using a ChIP kit (Upstate). Briefly, after protein-DNA cross-linking, cells were lysed, sonicated and the supernatant was pre-cleared with protein A-agarose/salmon sperm DNA to reduce the nonspecific background. The samples were then immunoprecipitated using Cdx2 antibody. The complexes were collected in protein

A-agarose/salmon sperm DNA slurry and washed. The immunoprecipitated chromatin was eluted from the protein A and the cross-linked protein-DNA complexes were reversed. The DNA was purified by incubation with proteinase K, followed by phenol/chloroform extraction and ethanol precipitation. Cdx2 promoter elements were detected by PCR using specific primers (Supplementary data).

Nuclear run-on assay

5 x 10^7 nuclei isolated as previously described (Cuff *et al.*, 2002) were incubated at 30°C for 20 min in 300 ml transcription buffer (5 mM Tris-HCl pH 8.0, 2.5 mM $MgCl_2$, 150 mM KCl, 0.25 mM each of ATP, GTP, CTP, and UTP). The reaction was stopped by addition of 30 µl of 10 mM $CaCl_2$ and 20 µl of 8.5 mg/ml DNase I for 15 min at 37°C. The mixture was deproteinized with 20 µg/ml proteinase K for 45 min at 37°C. After RNA precipitation, cDNA was synthesized and used for RT-PCR using specific primers (Supplementary data).

Protein extraction, Western blot and dot blot

Total and membrane proteins were extracted as previously described (Nduati et al., 2007). Total cell lysates or gradient fractions extracted from HT29-Cl.19A were resolved on 10% polyacrylamide gels and transferred to nitrocellulose membranes (Bio-Rad). Membranes were probed with relevant antibodies followed by incubation with appropriate HRP-conjugated secondary antibodies (Amersham Biosciences). Immunoreactive proteins were detected with the enhanced chemiluminescence detection kit (Amersham Biosciences).

To detect ganglioside GM1 in the gradient fractions, 2 μg of protein from each fraction was spotted on nitrocellulose membranes. Membranes were blocked, washed as described above for Western blot analysis, and then incubated with HRP-conjugated cholera toxin B (CTB; Sigma, St. Louis, MI). Blots were developed using ECL detection kit.

Uptake experiments

PepT1-mediated [^{3}H]KPV uptake in HT29-Cl.19A cells was performed as previously described (Dalmasso *et al.*, 2008a). Briefly, confluent monolayers were washed and stabilized in HBSS supplemented with 10 mM HEPES pH 7.4 for 15 min at 37°C. Cells were incubated for 15 min at room temperature with 20 nM [^{3}H]KPV ± 20 mM glycine-leucine in the same buffer. Cells were washed with PBS. Radioactivity was counted in a b-counter (Beckman).

Measurement of EPEC attachment to LRs

Attachment of EPEC was monitored using the electric cell-substrate impedance sensing (ECIS) 1600R device (Applied BioPhysics). EPEC were seeded in ECIS electrodes pre-coated with LR fractions at a final total protein concentration of 20 mg/ml. Capacitance was measured at 40 kHz, 1 V. The time necessary for EPEC to cover half of the available electrode ($t_{1/2}$) was calculated for each electrode as previously described (Wegener *et al.*, 2000).

ELISA

IL-8 and KC concentrations in culture media were determined using the Quantikine Immunoassay (R&D System).

Ex vivo experiments

Proximal and distal colons were removed from WT and PepT1$^{+/+}$ transgenic FVB male mice (6-8 week-old) (Dalmasso et al., Abstract FASEB). After incubation with 10 mg/ml gentamicin for 1 h, tissues were washed and organ culture *Citrobacter rodentium* infections were performed as described previously for 6 h (Hicks *et al.*, 1998). After infections, specimens were washed extensively with PBS to remove non-adherent bacteria and then processed for RNA and protein extraction or adhesion assay.

Statistical analysis

Values are expressed as means ± S.E.M. Statistical analysis was performed using unpaired two-tailed Student's *t*-test by InStat v3.06 (GraphPad) software. $P < 0.05$ was considered statistically significant.

Results

EPEC induces PepT1 promoter activity and PepT1 transcription

Although there are evidences of PepT1 expression in epithelial cells of inflamed colon(Merlin et al., 2001, Ziegler et al., 2002), the inducing factor has not yet been identified. Starting from the hypothesis that pathogenic bacteria could be inducers of PepT1 expression, we initially investigated the possible involvement of EPEC in PepT1 expression in human colonic HT29-Cl.19A cells. We found that EPEC induced increases in PepT1 promoter activity and mRNA expression in HT29-Cl.19A cells in a time-dependent manner (Table 1A,B). The resulting full-length PepT1 cDNA was successfully cloned (GeneBank accession number ACB71122), further demonstrating that EPEC induces PepT1 mRNA expression. In addition, run-on assay results showed that EPEC strongly enhanced PepT1 gene transcription *in vitro* (Table A2.1C). Together, these data demonstrate that EPEC transcriptionally induces PepT1 expression in HT29-Cl.19A cells.

EPEC induces PepT1 protein expression and transport activity

Further examination by Western blot analysis showed that EPEC induced a time-dependent increase in PepT1 protein expression in HT29-Cl.19A cells (Table A2.1D). Confocal microscopy images showed PepT1 staining in the membranes of EPEC-infected cells (Table A2.1E). In contrast, no PepT1 signal was evident in uninfected cells (Table A2.1E). Importantly, PepT1-mediated specific uptake of the tripeptide KPV was increased in infected cells (Table A2.1F), indicating that the expressed PepT1 was functionally active.

EPEC induces PepT1 expression in lipid rafts

As we have previously showed the localization of PepT1 in LRs of intestinal epithelial Caco2-BBE cells (Nguyen et al., 2007), it was of interest to investigate if PepT1 expressed in HT29-Cl.19A cells upon EPEC infection is targeted to LRs. Immunoblot and dot blot analyses of sucrose-gradient fractions prepared from EPEC-infected cells revealed that PepT1 and the LR marker GM1 levels were increased in the LR fraction (fraction 4; Table A2.2A). Furthermore, GM1 co-immnoprecipitated with PepT1, but not with the control IgG_1, in the membranes of HT29-Cl.19A cells (Table A2.2B). Treatment of infected cells with the cholesterol-disrupting agent methyl-b-cyclodextrin (mbCD) caused most PepT1 and GM1 to move from LRs to high-density soluble fractions (Table A2.2A). This change was accompanied by a 70% decrease in EPEC-induced PepT1 activity, which was recovered by replenishing cell membranes with cholesterol (Table A2.2C). Given from our previous report that association of PepT1 with LRs can modulate its activity (Nguyen et al., 2007), this finding further supports the localization of PepT1 in LRs. Together, these results indicate that EPEC induces functional PepT1 expression in cholesterol-enriched LRs of HT29-Cl.19A cells.

The transcription factor Cdx2 is important for EPEC-induced PepT1 expression

Our previous studies have pointed out the importance of Cdx2 in the regulation of PepT1 expression in IECs (Nduati et al., 2007, Dalmasso *et al.*, 2008b). We therefore investigated the possible involvement of Cdx2 in EPEC-induced PepT1 expression. We found that EPEC markedly increased Cdx2 mRNA and protein expressions in HT29-

Cl.19A cells (Table A2.3A,B) and, importantly, that Cdx2 bound to the PepT1 promoter at the Cdx2^{-579} site, as shown by EMSA (Table A2.3C). The loss of the retarded protein-DNA complexes upon addition of an excess of unlabeled oligonucleotide competitor (cold probe) and their shift in the presence of Cdx2 antibody indicated that the binding of Cdx2 to the PepT1 promoter was sequence-specific (Table A2.3C). The Cdx2-PepT1 promoter interaction was confirmed by chromatin immunoprecipitation analysis. RT-PCR analysis of the Cdx2 precipitate from infected cells identified a sequence specific to the Cdx2^{-579} binding site, indicating that Cdx2 binds to the PepT1 promoter at the Cdx2^{-579} site (Table A2.3D).

Having shown that EPEC increases Cdx2 expression and that Cdx2 binds to the PepT1 promoter, we hypothesized that Cdx2 might play a crucial role in EPEC-induced PepT1 expression. We found that PepT1 mRNA and protein expressions were induced in HT29-Cl.19A cells over-expressing Cdx2 (Table A2.3E). In contrast, PepT1 expression was not observed in wild-type HT29-Cl.19A cells or HT29-Cl.19A cells transfected with the empty vector, which exhibited low Cdx2 levels (Table A2.3E). Accordingly, silencing of Cdx2 in HT29-Cl.19A cells by Cdx2 siRNA markedly reduced EPEC-induced PepT1 expression (Table A2.3F). Together, these results indicate the importance of Cdx2 in EPEC-induced PepT1 transcription.

EPEC-induced PepT1 expression is dependent on intimate adherence of EPEC to host cells through LRs

We next investigated if EPEC-induced PepT1 expression requires intimate attachment of EPEC to host cells. As Tir and intimin are known to be the virulence factors necessary

for EPEC intimate attachment (Nougayrede et al., 2003), we examined the effects of the EPEC mutants *eae*- or *tir*-, which are deficient in intimin and tir expression, respectively, on PepT1 expression. We found that deficiency in tir or intimin abrogated the ability of wild-type EPEC to induce PepT1 expression and transport activity (Table A2.4A,B). These data indicate that EPEC needs to intimately attach to host cell to induce PepT1 expression.

LRs have been shown to be important for the adherence of EPEC and the translocation of Tir(Allen-Vercoe *et al.*, 2006). We therefore investigated if EPEC-induced PepT1 expression is LR-dependent. Western blot and RT-PCR analyses showed that treatment of HT29-Cl.19A cells with mbCD prior to infection markedly reduced PepT1 mRNA and protein expressions (Table A2.4C,D). In addition, EPEC failed to induce PepT1 transport activity in mbCD-treated cells (Table A2.4E). The suppression of PepT1 expression and activity by mbCD treatment was effectively recovered by membrane cholesterol replenishment (Table A2.4C,D,E). These data suggest that EPEC induces PepT1 expression via cholesterol-enriched membrane microdomains.

PepT1 plays a role in EPEC adherence and EPEC-induced inflammation

Having shown that EPEC induces PepT1 expression in LRs of HT29-Cl.19A cells, we next addressed the role of PepT1 associated with LRs in bacterial-epithelial interactions by examining EPEC adherence to HT29-Cl.19A cells over-expressing PepT1 (HT29-Cl.19A/PepT1) or HT29-Cl.19A cells containing the empty vector (HT29-Cl.19A/vector). Interestingly, we observed a significant decrease in EPEC adherence to HT29-Cl.19A/PepT1 compared to HT29-Cl.19A/vector (Figure A1.5A), suggesting a

role for PepT1 in EPEC adherence. To verify the involvement of PepT1 in EPEC attachment, we prepared LR fractions from HT29-Cl.19A/PepT1 and HT29-Cl.19A/vector and monitored EPEC adhesion on these LR surfaces in real-time using the electric cell-substrate impedance-sensing (ECIS) technique. Western blot analysis showed the enrichment of PepT1 in LRs prepared from HT29-Cl.19A/PepT1 and its absence in the gradient fractions extracted from HT29-Cl.19A/vector (Figure A1.5B). Interestingly, the presence of PepT1 in LRs delayed EPEC attachment (Table A2.5C). The time necessary for EPEC to cover half of the available electrode surface ($t_{1/2}$) confirmed this observation ($t_{1/2}$ = 4.1 ± 0.38 h on LRs from HT29-Cl.19A/PepT1 *vs* $t_{1/2}$ = 5.8 ± 0.25 h on LRs from HT29-Cl.19A/vector; Table A2.5C). Together, these data demonstrate the role of PepT1 in EPEC adherence.

EPEC has been reported to induce inflammatory responses in IECs characterized by activation of the transcription factor NF-kB and the MAP kinases (Savkovic et al., 1997, Savkovic et al., 2001), which consequently leads to IL-8 secretion (Czerucka et al., 2001, Savkovic et al., 2001). We found that EPEC induced faster and stronger IkB-a degradation in HT29-Cl.19A/vector compared with HT29-Cl.19A/PepT1 (Table A2.6A). Furthermore, EPEC-induced phosphorylation of ERK1/2 and p38 kinases was delayed in HT29-Cl.19A/PepT1 compared with HT29-Cl.19A/vector (Table A2.6A). In accordance with these results, we observed lower levels of IL-8 mRNA and protein in HT29-Cl.19A/PepT1 compared with HT29-Cl.19A/vector upon EPEC infection (Table A2.6B,C). Together, these findings suggest a role for PepT1 in EPEC-induced inflammation.

PepT1 has a role in *Citrobacter rodentium* adherence and intestinal inflammation in mouse colon

We next investigated whether *C. rodentium*, a mouse-pathogenic attaching and effacing bacterium (Luperchio *et al.*, 2000, Schauer & Falkow, 1993), can induce PepT1 expression in the mouse colon. We found that *C. rodentium* significantly increased PepT1 mRNA and protein expression levels in both distal and proximal colonic tissues (Table A2.7A,B). These results, which are in agreement with *in vitro* data, support the role of pathogenic bacteria in induction of PepT1 expression.

The role of PepT1 in *C. rodentium* adherence and pro-inflammatory cytokine production was assessed by using a transgenic mouse model that over-expresses PepT1 (Dalmasso et al., Abstract FASEB). Table A2.7C shows a significant decrease in *C. rodentium* adherence to the colon from transgenic mice compared with that from wild-type mice. Furthermore, *C. rodentium* induced lower mRNA and protein levels of keratinocyte-derived chemokine (KC), a functional homologue of IL-8, in the distal and proximal colon from transgenic mice compared with wild-type mice (Table A2.7D,E).

Together, these findings support the *in vitro* results and highlight the importance of PepT1 in the modulation of bacterial adherence and bacteria-induced inflammation.

Discussion

In the present study, we demonstrate that EPEC transcriptionally induces PepT1 expression and activity in colonocytes. EPEC, a human intestinal food-borne pathogen, has been shown to modulate membrane transport activities in IECs, such as butyrate uptake via MCT-1(Borthakur et al., 2006), Na^+ transport via Na^+/H^+ exchange (NHE) isoforms(Hecht et al., 2004) and Cl^-/OH^- exchange (Gill et al., 2007). These activity changes have been suggested to be due to the redistribution of surface proteins from the apical membrane into the intracellular compartment upon EPEC infection (Gill et al., 2007, Borthakur et al., 2006), and not due to changes in transcriptional regulation. Regarding pathogenic effects on PepT1 expression, to date only one study has reported the transcriptional up-regulation of PepT1 in the rat small intestine in response to *Cryptosporidium parvum* infection (Barbot *et al.*, 2003). PepT1 expression in colonic epithelial cells has been shown exclusively during chronical inflammation of the colon (Merlin et al., 2001, Ziegler et al., 2002). However, the molecular mechanisms of PepT1 expression under pathological conditions have not yet been investigated. In an effort to identify a causal factor of colonic PepT1 expression, we found that EPEC transcriptionally induces PepT1 expression and transport activity in human colonic epithelial HT29-Cl.19A cells. One of our important findings is that the transcription factor Cdx2 is crucial for EPEC-induced PepT1 expression in colonocytes. We have previously shown the importance of Cdx2 in the regulation of PepT1 expression in IECs (Dalmasso et al., 2008b, Nduati et al., 2007). In addition, PepT1 expression was detected in the intestinal metaplastic mucosa developed from the gastric mucosa of transgenic Cdx2 over-expressing mice (Mutoh *et al.*, 2005). Together with these observations, our

finding inducates that Cdx2 might be the main transcription factor regulating PepT1 expression.

We have previously shown that PepT1 is localized in membrane LR microdomains, which are known to provide specialized lipid environments to regulate the organization and function of many plasma membrane proteins (Simons & Toomre, 2000). Here, we show that EPEC induces functional PepT1 expression in the LRs of HT29-Cl.19A cells, and that the association of PepT1 with LRs can modulate its transport activity. The latter observation, which has previously been demonstrated in IECs (Nguyen et al., 2007), is of physiological importance, suggesting that PepT1 expressed in EPEC-infected HT29-Cl.19A cells is settled in its physiological organization to be functional.

Increasing lines of evidences have recently revealed the role of LRs as portals exploited by pathogens to attack host cells (Lafont & van der Goot, 2005, Manes et al., 2003). It has been proposed that in response to bacterial binding, all the required signaling molecules gather at membrane LR platforms, thereby participating in bacterial adherence and invasion (Manes et al., 2003, Lafont & van der Goot, 2005). The interaction of LRs with EPEC has been made evident from the findings that several LR-associated molecules, such as CD44 (Goosney *et al.*, 2001), annexin II, cholesterol and glycosyl phosphatidylinositol-anchored proteins (Zobiack *et al.*, 2002), are clustered at EPEC adherence site. Here, we demonstrate that EPEC-induced PepT1 expression requires LR integrity and intimate attachment of EPEC via binding of the translocated receptor Tir to its ligand intimin. It is therefore tempting to speculate that EPEC induces PepT1 expression by intimately attaching to host cell membrane through LRs.

The findings that EPEC-induced PepT1 expression is dependent on LRs and that PepT1 expressed in EPEC-infected colonocytes is targeted to LRs drove us to investigate the role of PepT1 present in LRs in the virulence of this pathogen. Strikingly, we found that over-expression of PepT1 in HT29-Cl.19A cells markedly reduced EPEC adherence. Furthermore, PepT1 interfered the binding of EPEC to LRs monitored in real-time using the ECIS technique. Given that LRs are important for bacterial adherence to host IECs, these data strongly suggest that LR-associated PepT1 is involved in bacterial-epithelial interactions. Another intriguing finding is that PepT1 attenuates EPEC-triggered pro-inflammatory responses in IECs, characterized by activation of NF-kB and MAP kinases and IL-8 production. Since EPEC-induced IL-8 production in host epithelia has been shown to be dependent on intimate adherence of EPEC (Czerucka et al., 2001), the decrease in EPEC-induced inflammation might be a consequence of the reduction in bacterial adherence. The role of PepT1 in bacterial adherence and bacterial-induced inflammation found here might be a host mechanism to modulate inflammatory responses to pathogenic attack.

Importantly, the *in vitro* data were supported by the results obtained from *ex vivo* studies in which mouse colon was infected with *C. rodentium*. We showed that *C. rodentium* strongly enhanced PepT1 mRNA and protein expression levels in both distal and proximal mouse colon. We further demonstrated that *C. rodentium* adherence and KC production level in the colon from PepT1-overexpressing transgenic mice were reduced compared with wild-type mice, confirming the role of PepT1 in modulating bacterial-epithelial interactions and bacterial-induced inflammation.

In conclusion, our study demonstrates that: i) EPEC transcriptionally induces functional PepT1 expression in LRs of colonocytes by intimately attaching to host cell membranes through LRs, ii) the transcription factor Cdx2 is crucial for the EPEC-induced PepT1 expresison, and iii) PepT1 associated with LRs has an important role in bacterial-epithelial interactions and intestinal inflammation. Our findings not only reveal a novel mechanism underlying the regulation of colonic epithelial PepT1 expression/function under pathological conditions, but also point out the potential contribution of this transporter to host defense mechanisms in response to pathogenic attack.

Acknowledgments

This work was supported by National Institutes of Health of Diabetes and Digestive and Kidney Diseases under RO1-DK061941-02 grant (to D.M.). G.D. and Y.Y. are recipients of a research fellowship award from the Crohn's and Colitis Foundation of America. We thank Dr. Jan Michael Klapproth (Department of Medicine, Division of Digestive Diseases, Emory University, Atlanta, GA) for kindly providing bacterial strains and Maiko Sasaki for the helpful comments on the manuscript.

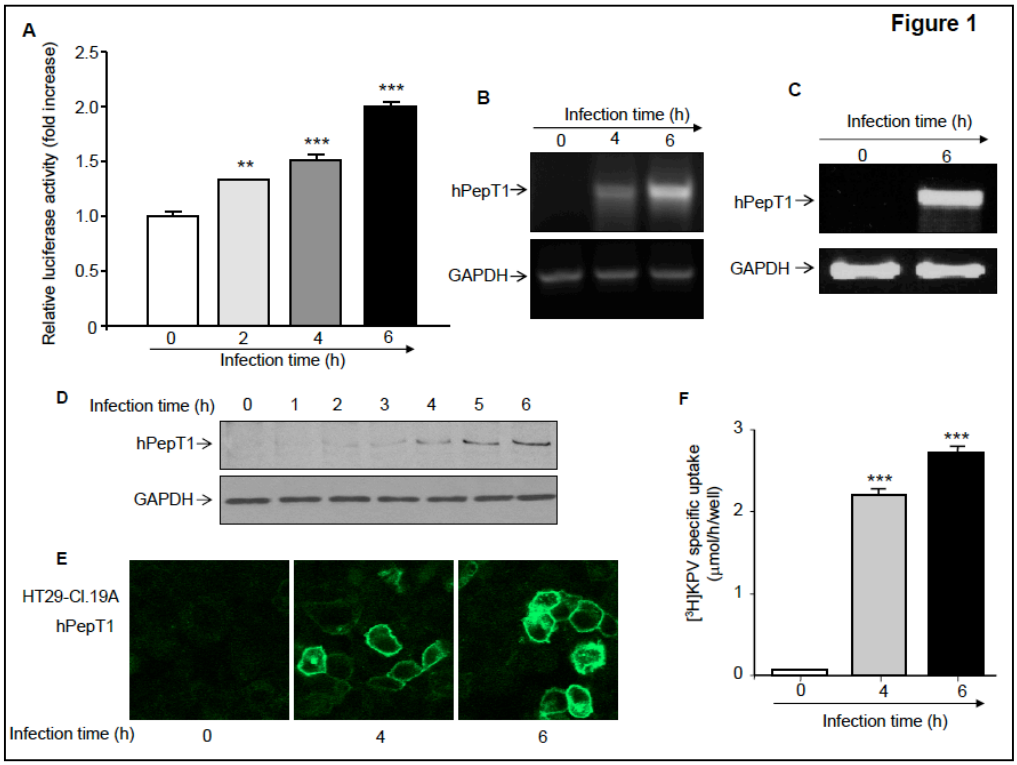

Figure A2.1: EPEC transcriptionally induces PepT1 expression and transport activity in HT29-Cl.19A cells

A) Cells were transfected with full-length PepT1 promoter construct and infected with EPEC. PepT1 promoter activity was assessed by measuring luciferase activity. Data were normalized to *Renilla* luciferase activity and expressed as fold increases compared with data for uninfected cells. B) RT-PCR analysis of PepT1 mRNA expression. C) *In vitro* transcription run-on assay was performed on nuclei isolated from uninfected and infected cells. D) Western blot analysis of PepT1 protein expression. E) Representative images of immunofluorescence staining for PepT1 of uninfected and EPEC-infected cells. F) PepT1-mediated specific uptake of [^{3}H]KPV in HT29-Cl.19A monolayers. Data are means ± S.E.M of three independent determinations. (**$P < 0.05$; ***$P < 0.001$) *vs* uninfected cells.

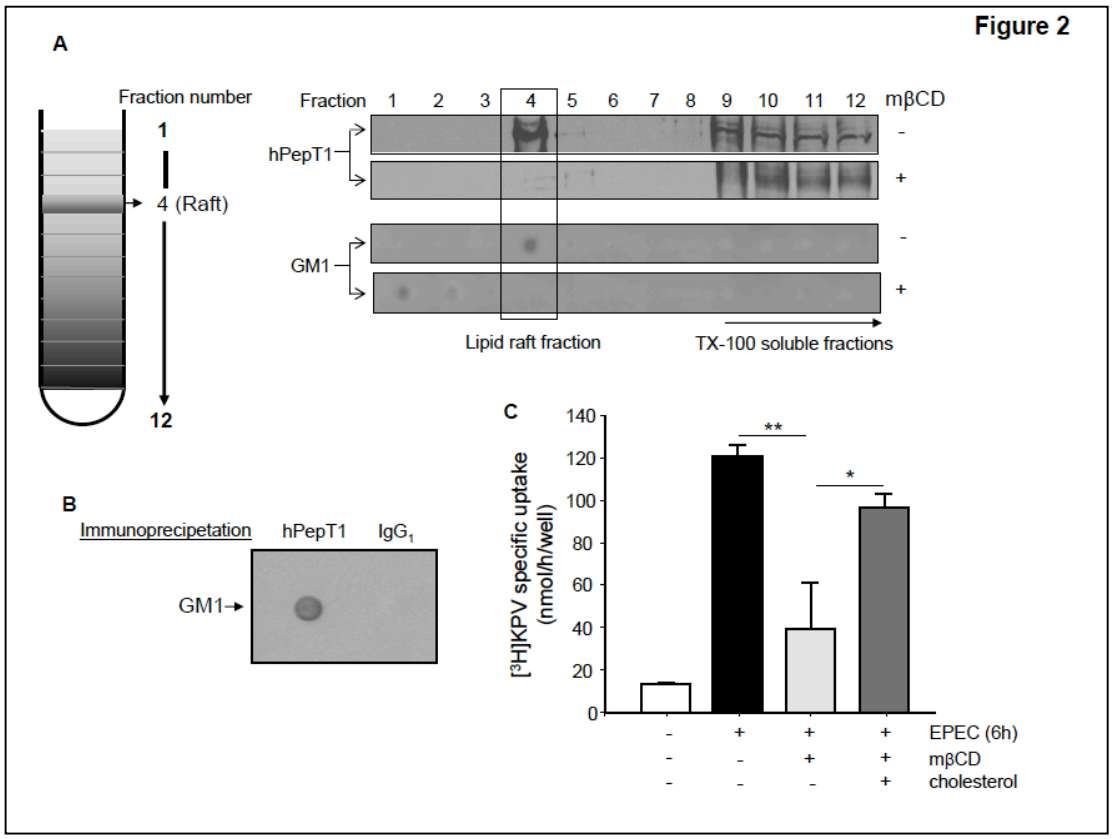

Figure A2.2: EPEC induces PepT1 expression in lipid rafts of HT29-Cl.19A cells

A) Cells were infected with EPEC for 6 h, treated with (+) or without (-) methyl-b-cyclodextrin (mbCD), lysed, and subjected to sucrose-gradient fractionation. Equal amounts of total protein from the gradient fractions were analyzed for PepT1 or GM1 distribution by Western blot or dot blot, respectively. B) EPEC-infected cell lysates were immunoprecipitated using PepT1 antibody or the isotype IgG_1. Immunoprecipitates were analyzed by dot blot to detect GM1. C) EPEC-infected cells were treated with mbCD and then replenished or not with cholesterol, and PepT1-mediated specific uptake of [^{3}H]KPV was measured. Values are means ± S.E.M of three independent determinations. $^{**}P < 0.005$; NS, not significant.

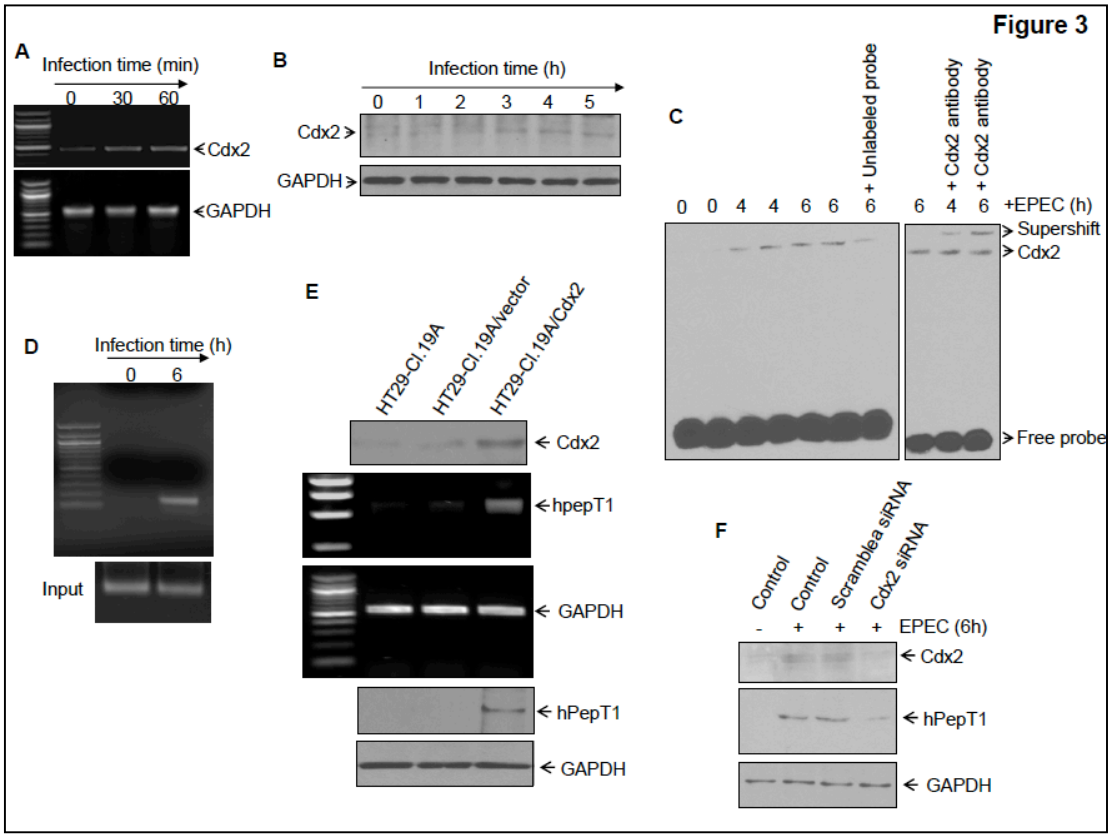

Figure A2.3: Cdx2 is important for EPEC-induced PepT1 expression

HT29-Cl.19A cells were infected with EPEC. Cdx2 expression was analyzed by RT-PCR (A) and Western blot (B). Binding of Cdx2 to the PepT1 promoter was analyzed by EMSA (C) and chromatin immunoprecipitation analysis (D). C) EMSA was performed using a Cdx2^{-579}-specific probe. Specificity of complexes was verified using 200-fold excess of unlabeled probe (left panel) and by supershift experiment using Cdx2 antibody (right panel). D) Soluble chromatin was prepared from infected and uninfected cells. Protein-bound DNA complexes were immunoprecipitated using Cdx2 antibody. After cross-link reversal, the purified DNA was amplified by RT-PCR using Cdx2^{-579}-specific primers. E) Cells were transfected with the empty vector (HT29-Cl.19A/vector) or Cdx2

(HT29-Cl.19A/Cdx2) and analyzed for Cdx2 expression by Western blot or PepT1 expression by RT-PCR and Western blot. F) HT29-Cl.19A cells were transfected with scramble or Cdx2 siRNA for 24 h, and infected with EPEC for 6 h. Cdx2 and PepT1 expression was assessed by Western blot.

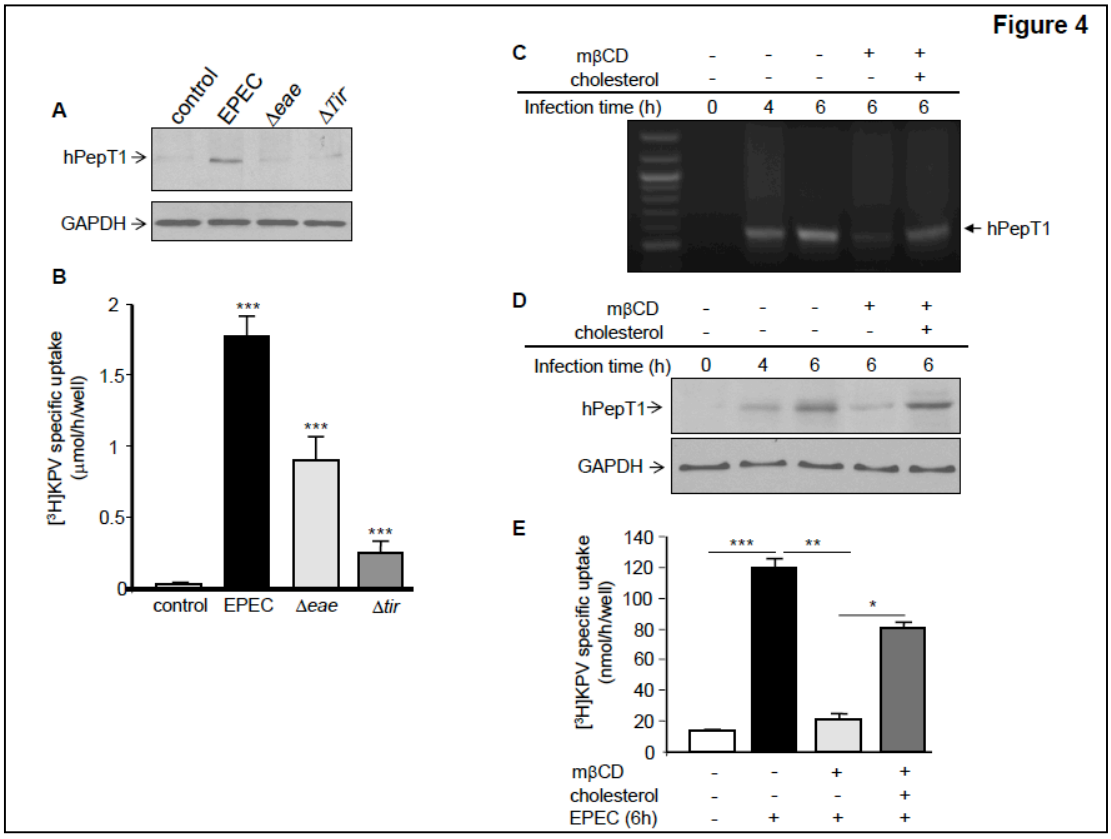

Figure A2.4: EPEC-induced PepT1 expression requires Tir, intimin and intact host lipid rafts

A, B) HT29-Cl.19A cells were infected with wild-type EPEC or EPEC mutants deficient in Tir (Δ*tir*) or intimin (Δ*eae*) for 6 h. Western blot analysis of PepT1 expression (A) and PepT1-mediated specific uptake of [^{3}H]KPV (B). C, D, E) Cells were treated with 10 mM mbCD and replenished or not with 2 mM cholesterol prior to EPEC infection. PepT1 expression and activity were assessed by RT-PCR (C), Western blot (D) and [^{3}H]KPV specific uptake (E). Values are means ± S.E.M of three independent determinations. **P* < 0.05; ***P* < 0.005; ****P* < 0.001.

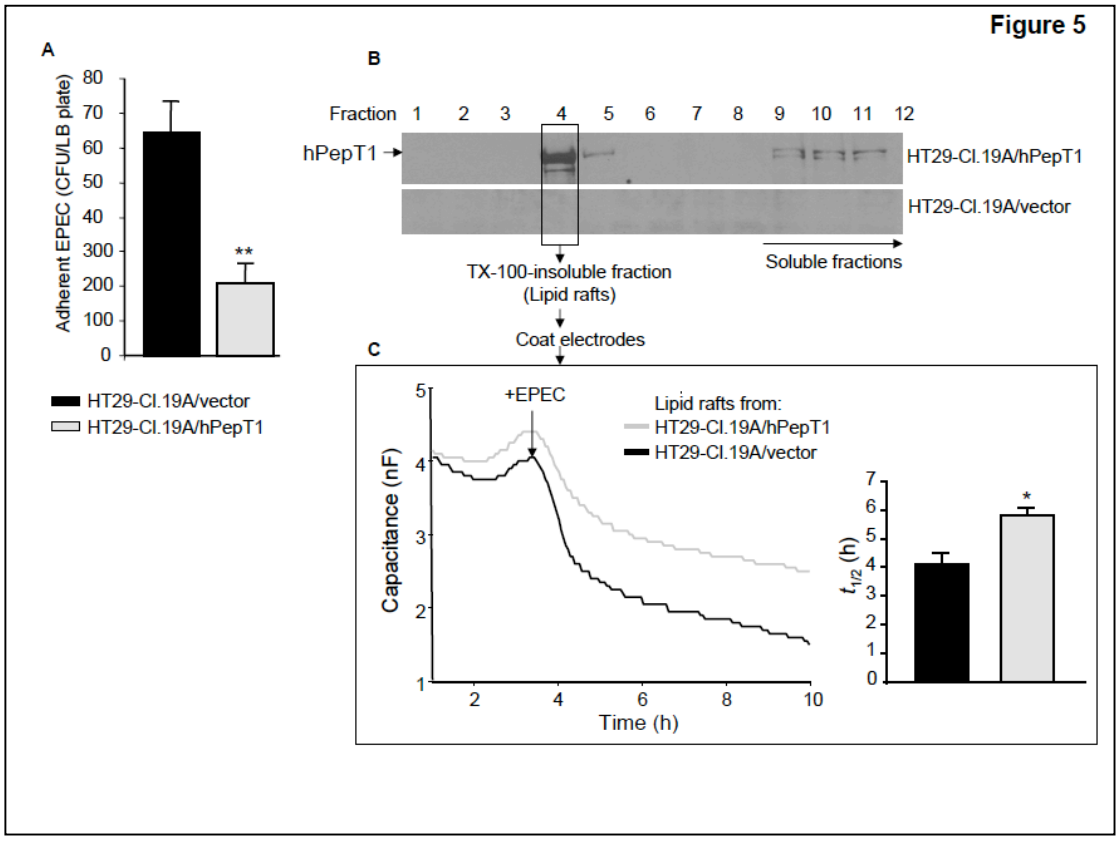

Figure A2.5: PepT1 associated with lipid rafts modulates EPEC adherence to HT29-Cl.19A cells

A) EPEC adherence to HT29-Cl.19A cells stably transfected with PepT1 (HT29-Cl.19A/PepT1) or the empty vector (HT29-Cl.19A/vector) were determined after 4 h of infection. B) Western blot analysis for PepT1 expression in the sucrose-gradient fractions obtained from HT29-Cl.19A/PepT1 and HT29-Cl.19A/vector. C) Electrodes were coated with lipid raft fractions extracted from HT29-Cl.19A/PepT1 and HT29-Cl.19A/vector, and EPEC adherence was monitored in real-time using the electric cell-substrate impedance sensing (ECIS) technique. The time required for EPEC to cover half of the available electrode ($t_{1/2}$) was accordingly determined. Values are means ± S.E.M of three independent determinations. *$P < 0.05$.

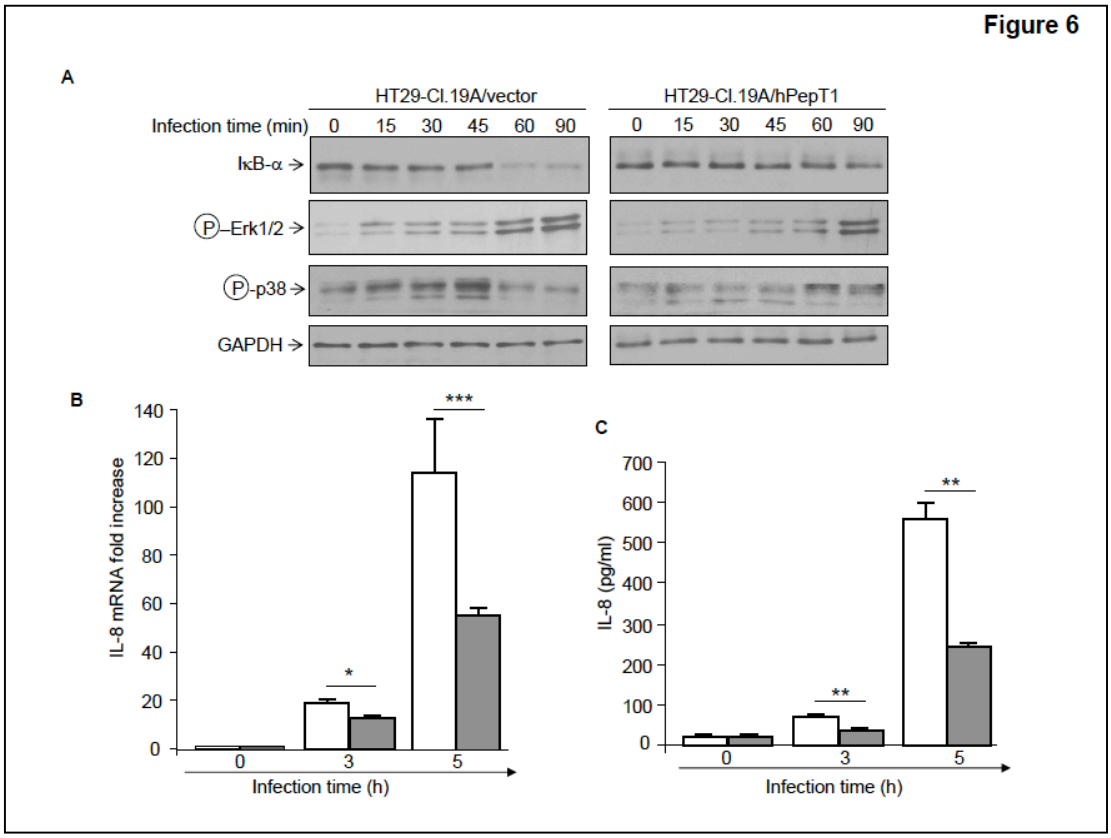

Table A2.6: Over-expression of PepT1 in HT29-Cl.19A cells has a role in EPEC-induced inflammation

A) Degradation of IkB-a and phosphorylation of ERK1/2 and p38 were assessed by Western blot. IL-8 mRNA and protein expression levels were analyzed by real-time RT-PCR (A) and ELISA (B). Values are means ± S.E.M of three independent determinations. *$P < 0.05$; **$P < 0.005$; ***$P < 0.001$.

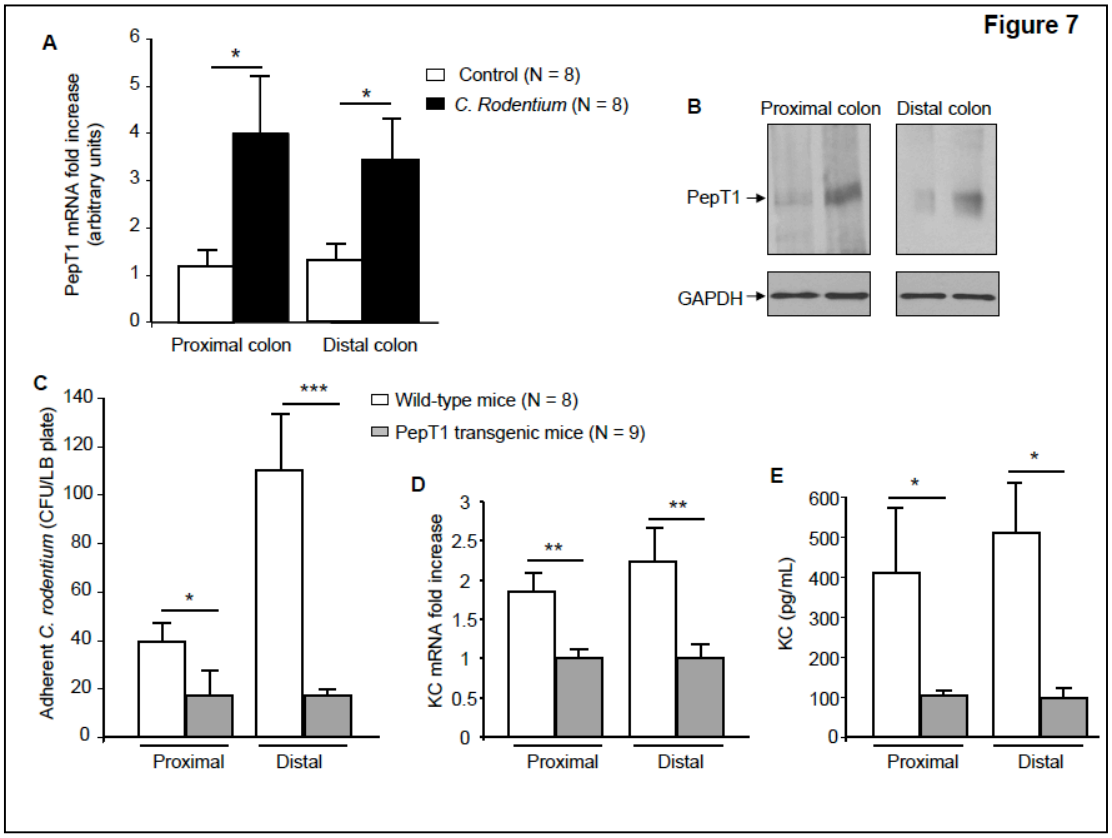

Table A2.7: *Citrobacter rodentium* induces PepT1 expression in mouse colon. Role of PepT1 in *C. rodentium* adherence and *C. rodentium*-induced keratinocyte-derived chemokine production in mouse colon

Distal and proximal mouse colonic tissues were treated with gentamicin for 1 h and infected *ex vivo* with *C. rodentium* for 6 h. Real-time RT-PCR (A) and Western blot (B) analyses of PepT1 expression in colonic tissues from wild-type mice. *C. rodentium* adherence to colonic tissues from wild-type and PepT1-transgenic mice (C). Real-time RT-PCR (D) and ELISA (E) analyses of keratynocyte-derived chemokine (KC) expression. Values represent means ± S.E.M of three independent determinations. *$P <$ 0.05; **$P < 0.005$; ***$P < 0.001$.

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