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The conserved mechanism of IcsA polar targeting among proteobacteria, characterization of the omptin family, and the roles and regulation of IcsP in Shigella flexneri

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THE CONSERVED MECHANISM OF ICSA POLAR TARGETING AMONG PROTEOBACTERIA, CHARACTERIZATION OF THE OMPTIN FAMILY, AND THE ROLES AND REGULATION OF ICSP IN *SHIGELLA FLEXNERI*

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ABSTRACT

The Conserved Mechanism of IcsA Polar Targeting Among Proteobacteria, Characterization of the Omptin Family, and the Roles and Regulation of IcsP in *Shigella flexneri*

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Shigella flexneri is a gram-negative intracellular pathogen that is a global health problem, causing severe bacillary dysentery. In the past 30 years, research has enabled us to have a better understanding of the molecular basis of *Shigella* pathogenesis. In this study, we further expand our knowledge of key determinants at molecular and cellular levels. This work is composed of four separate investigations.

Firstly, we examined whether the elusive mechanism of protein targeting found in *Shigella* is conserved in three closely related γ-proteobacterial families by using GFP fusions and microscopy. Our results indicate that the mechanism of protein localization to the bacterial pole was conserved in the species investigated. Secondly, we further characterized omptin proteins found in three pathogenic organisms: *Shigella* (IcsP), *E. coli* (OmpT), and *Salmonella* (PgtE). We addressed whether the LPS environment and/or the inherent amino acid differences in the surface loops and LPS binding motif affect the cleavage specificity of these proteases by introducing a known substrate into each of these strains and comparing cleavage patterns by western blot analysis. Our results

indicate that the cleavage specificity of these omptins are likely due to the inherent amino acid differences in the surface loops and/or LPS binding region. Thirdly, we examined whether IcsP, an outer membrane protease in *Shigella,* promotes resistance to a cationic antimicrobial peptide, LL-37 by using minimum inhibitory concentration assays. Our results suggest that IcsP does not promote resistance to LL-37. Finally, we examined whether *icsP* was regulated by RyhB, a small regulatory RNA, by western blot analysis and β-galactosidase assays. Our results suggest that RyhB does not down-regulate the expression of *icsP* in *S. flexneri*.

Taken together, this study has improved our understanding of *Shigella* pathogenesis and omptin family proteases. Ultimately, the relevance of this study has potential use for the development of prevention and intervention strategies against specific bacterial infections and disease.

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ABBREVIATIONS

Å	angstrom
A	adenine
Ala	alanine
Amp	ampicillin
Arg	arginine
Asp	aspartic acid
bp	base pair
C	cytosine
Caco-2	human colonic adenocarcinoma cells
CAMP	cationic antimicrobial peptides
CDC	Centers for Disease Control and Prevention
Cm	chloramphenicol
DNA	deoxyribonucleic acid
ECM	extracellular matrix
g	gravity
G	guanine
GFP	green fluorescent protein
Gm	gentamycin
Glu	glutamic acid
His	histidine
IgG	immunoglobulin G
IL	interleukin
Ile	isoleucine
IPTG	isopropyl-β-D-galactopyranoside
Kan	kanamycin
kb	kilobase
kDa	kiloDalton
LB	Luria-Bertani broth
LD_{50}	median lethal dose
Leu	leucine
LPS	lipopolysaccharide
Lys	lysine
M	molar
M cell	microfold cell
Met	methionine
MIC	minimum inhibitory concentration
MOI	multiplicity of infection
Oligo	oligonucleotide
ONPG	*o*-nitrophenyl-β-D-galactopyranoside

PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFU	phage forming units
PMN	polymorphonuclear cells
PVDF	polyvinylidene fluoride
RNA	ribonucleic acid
RNase	ribonuclease
S-D	Shine-Dalgarno
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
Ser	serine
SP	signal peptide
Spec	spectinomycin
T	thymine
Tet	tetracycline
Thr	threonine
Tris	Tris (hydroxymethyl) aminoethane
TSB	tryptic soy broth
Tween-20	polyoxyethylene (20) sorbitan monolaurate
Tyr	tyrosine
UPEC	uropathogenic *Escherichia coli*

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CHAPTER 1

GENERAL INTRODUCTION

1.1 The genus *Shigella*

Shigella was first discovered by the Japanese scientist Shiga in the 1890s and was adopted as a genus in the 1950s. The genus *Shigella* is part of the Enterobacteriaceae, a family that includes intestinal bacteria, which contains other genera such as *Escherichia*, *Salmonella*, and *Yersinia*. Historically, *Shigella* was first described as *Bacillus dysenteriae,* the causative agent of bacillary dysentery. At the time, it was understood that *Shigella* was closely related to *Bacillus* (now *Escherichia*) *coli* but was categorized into its own genus because *B. coli* was considered to be a commensal organism (Pupo *et al.*, 2000). *Shigellae* can be categorized based on the results of a few biochemical assays. Some of the determining factors for differentiating *Shigella* from *Escherichia* are the inability of *Shigella* to produce gas from glucose fermentation and inability to ferment lactose. Generally, compared to *Escherichia*, *Shigella* is less active in its utilization of carbohydrates (Lan *et al.*, 2002). Additionally, *Shigella* is non-motile while most strains of *Escherichia* are motile by peritrichous flagella. Tests for DNA homology show that strains of *Shigella* have 70% or higher genomic homology with *Escherichia coli* (Brenner *et al.*, 1972). Consequently, in today's classification system, *Shigella* spp. would be considered *Escherichia.* However, the conventional nomenclature for *Shigella* spp., justified on historical and clinical grounds will be used throughout this thesis.

In the genus *Shigella,* there are four different species that are differentiated based on their O-antigen of their lipopolysaccharide. The four species include: *S. dysenteriae* (13 serotypes), *S. boydii* (18 serotypes), *S. sonnei* (1 serotype), and *S. flexneri* (6 serotypes) (Sansonetti, 2001). Relatively few biochemical properties are used to separate the species. However, mannitol fermentation, indole production, and ornithine decarboxylation can be used to differentiate some of the species. *S. sonnei* is grouped by being mannitol and ornithine positive, while *S. dysenteriae* is negative for both. *S. flexneri* and *S. boydii* are both positive for mannitol fermentation but negative for decarboxylation of ornithine. To differentiate *S. flexneri* from *S. boydii*, O serotyping is essential (Lan *et al.*, 2002).

1.2 Epidemiology of *Shigella* spp.

Shigella spp. cause one of the most communicable of bacterial dysenteries, shigellosis. *Shigella* is highly infectious, with ingestion of as few as 10 to 100 cells resulting in disease (DuPont *et al.*, 1989). The transmission of *Shigella* occurs via the fecal-oral route and commonly occurs in developing nations where poor hygiene and limited access to clean drinking water promote the spread of enteric diseases. It is estimated that about 164 million people worldwide contract shigellosis annually and 1.1 million cases result in death from the disease, mostly in children under 5 years of age (Kotloff *et al.*, 1999). In the United States alone, the Centers for Disease Control estimates that approximately 448,200 total cases occur annually. Because of malnutrition and the lack of proper medical attention in young children and the elderly infected with *Shigella*, mortality rates are high. The emergence of multi-drug resistant *Shigella* strains

contributes partly to the continuous high disease incidence rates (Sansonetti, 2006). Consequently, shigellosis remains an unresolved global health problem.

Deadly epidemics in developing nations are generally attributed to *S. dysenteriae,* while *S. flexneri* and *S. sonnei* are primarily responsible for endemic disease in developed nations. *S. boydii* remains a problem in areas restricted to the Indian subcontinent. In this work, studies focus primarily on *S. flexneri.*

1.3 Pathogenesis of *Shigella flexneri*

Shigella flexneri is a gram-negative facultative intracellular pathogen. The pathogenesis of *S. flexneri* is a multi-step process that is based on the ability of the bacterium to invade and replicate within host colonic epithelial cells, causing severe destruction of host cells and inflammation. Following ingestion via the fecal-oral route, *Shigella* survives as it passes through the acidic environment of the stomach because *Shigella* possesses effective acid resistance systems (Gorden *et al.*, 1993). Subsequently, *Shigella* reaches the colon and rectum, where it accesses the intestinal mucosa. *Shigella* does not invade the apical side of the colonic epithelium but exploits microfold cells (M cells), the specialized epithelial cells in the follicular associated epithelium that overlie lymphoid tissue (Wassef *et al.*, 1989) (Figure 1). These specialized cells continuously sample particles and microorganisms from the gut lumen and deliver them to the underlying mucosal lymphoid tissue (Neutra *et al.*, 1992; Man *et al.*, 2004).

As *Shigella* is released into the intraepithelial area, it encounters resident macrophages that function to engulf and degrade foreign material. Macrophages engulf *Shigella,* but instead of successfully destroying the bacterium, *Shigella* rapidly induces

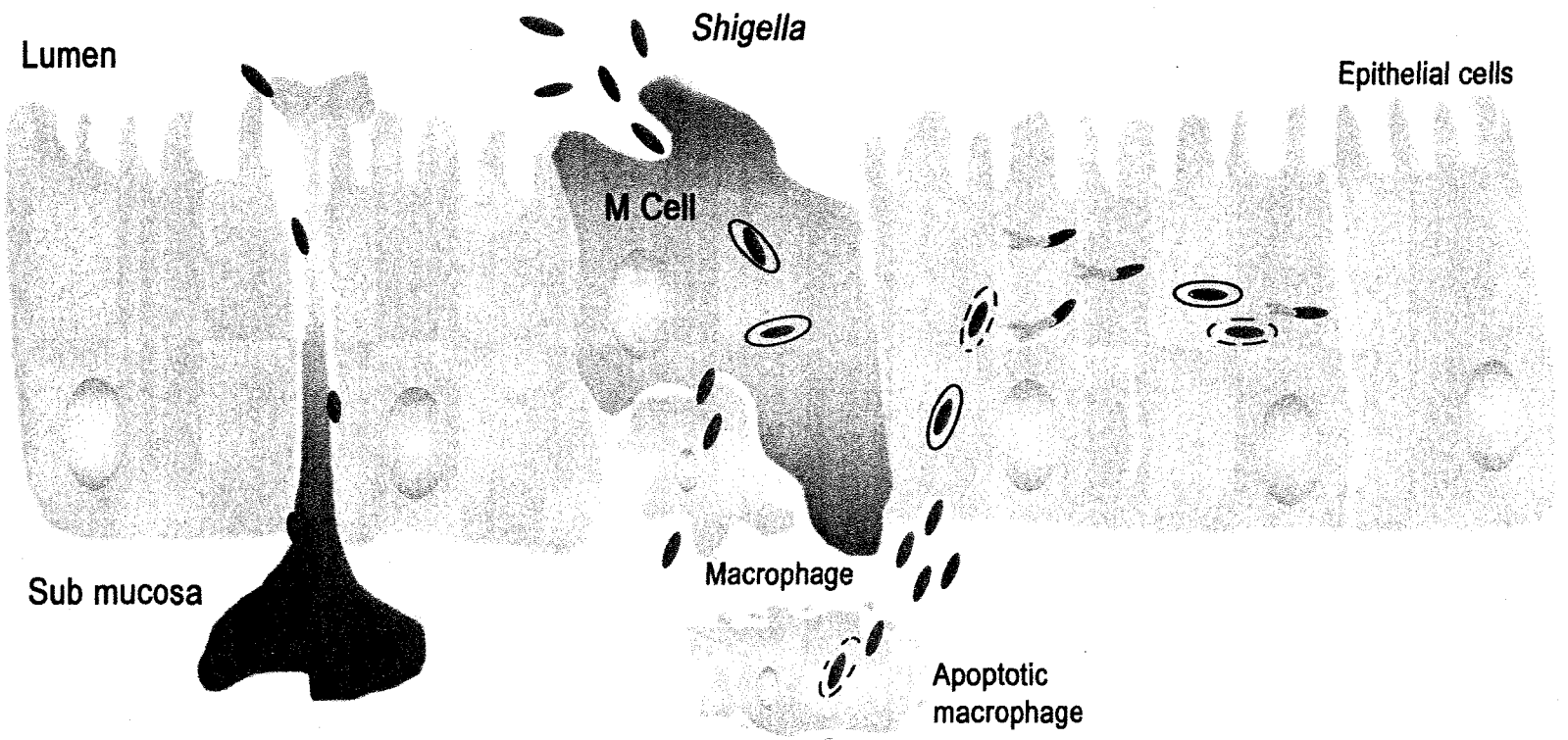

Figure 1.1: Cellular pathogenesis of *Shigella*
S. flexneri is translocated across the colonic epithelial barrier through M cells and encounters resident macrophages. The bacteria evade degradation in macrophages by inducing macrophage apoptosis. The influx of PMNs disrupts the colonic epithelial cell lining, which facilitates the invasion of more bacteria. Free bacteria invade the basolateral surface of the epithelial cells by inducing rearrangement of host actin. Once in the cytosol, *Shigella* recruits host actin to the bacterial pole, which propels *Shigella* through the cytoplasm and into adjacent cells and is essential for intercellular and intracellular spread.

apoptosis of the macrophage (Zychlinsky *et al.*, 1992). IpaB, a secreted effector molecule encoded on the *Shigella* virulence plasmid, is sufficient to induce macrophage apoptosis by binding and activating caspase-1 (Chen *et al.*, 1996). Caspases are intracellular cysteine proteases that induce mammalian cell apoptosis (Miura *et al.*, 1993). Caspase-1 dependent apoptosis is not an immunologically silent death, because once caspase-1 is activated, it is able to cleave and activate its specific substrates, interleukin-1β (IL-1β) and IL-18, into their biologically active form (Chen *et al.*, 1996; Dinarello,1998; Hilbi *et al.*, 1998).

During macrophage cell death, proinflammatory cytokines, IL-1β and IL-18 are released. Both of these cytokines are crucial mediators involved in the massive inflammatory response induced by *S. flexneri* (Zychlinsky *et al.*, 1994; Sansonetti *et al.*, 2000). The massive inflammatory response involves the recruitment of polymorphonuclear cells (PMNs) that transmigrate through the epithelial lining to reach the bacteria (Beatty *et al.*, 1997). As a result of transmigration, this infiltration of PMNs towards the infected site destabilizes the epithelium. The influx of PMNs across the epithelial layer, in response to the presence of *Shigella* disrupts the integrity of the epithelium allowing lumenal bacteria to traverse into the sub epithelial space and ultimately permit the organisms to access the basolateral surface of the epithelial cells (Perdomo *et al.*, 1994). Ironically, PMN-mediated interruption of the barrier function contributes to the local spread of *Shigella,* although the objectives of PMNs are to restrict the infection and prevent systemic dissemination.

In order to invade the intestinal mucosa, bacterial pathogens must find a site of entry. Once released from the macrophage, *S. flexneri* is able to invade the basolateral

surface of colonic epithelial cells. It has been proposed that the basolateral membrane of epithelial cells displays cellular components that are utilized by *Shigella* as cell adhesion receptors (Jennison *et al.*, 2004). Once attached, effector molecules are secreted into the host cell through the type III secretion apparatus. These effector molecules induce the rearrangement of the host cell cytoskeleton at the site of bacterial-host cell interaction (Blocker *et al.*, 1999). This mechanism, similar to macrophage and polymorphonuclear leukocyte phagocytosis, entails localized and transient actin polymerization and the accumulation of myosin at the site of entry for the bacteria (Clerc *et al.*, 1987a). This leads to the formation of large membrane protrusions within the colonic epithelial cell, which forms a macropinocytic pocket enclosing the bacterium (Tran Van Nhieu *et al.*, 1999).

After triggering its uptake into host cells, *Shigella* is encased in an endocytic vacuole called the phagosome. *Shigella* can escape by lysing the surrounding membranes of the phagosome and as a result, is released into the cytosol, where it grows and divides (Clerc *et al.*, 1987b). Lysis of the membrane is dependent on the expression of the *mxi/spa* and *ipa* genes to produce the type III secretion apparatus and the Ipa secretory effectors, specifically IpaC (Finlay *et al.*, 1988). Of the Ipa proteins, IpaC is the significant factor for mediating escape from the phagosome. IpaC disrupts the integrity of phospholipid vesicles, thereby mediating membrane lysis (Harrington *et al.*, 2006). Once in the cytosol, the bacteria are contained within the epithelial cell, and are consequently protected from immune cells. This intracellular residence of *Shigella* ultimately provides a favorable environment for replication.

After bacteria escape the phagosome, *Shigella* quickly recruits filamentous actin to the bacterial pole (Bernardini *et al.*, 1989). Recruitment of actin at the bacterial pole, mediated by the outer membrane protein IcsA (Bernardini *et al.*, 1989; Goldberg *et al.*, 1993; Goldberg *et al.*, 1995), results in actin tail formation on the surface of *Shigella*. This process propels *Shigella* through the cytoplasm and into adjacent cells and is essential for intercellular and intracellular spread (Makino *et al.*, 1986).

1.4 Signs, symptoms, and treatment of shigellosis

Shigellosis is concentrated in the colonic mucosal layer and results in severe tissue damage and ulceration. Destruction of this layer leads to the symptoms of shigellosis, which can vary from mild watery diarrhea to severe inflammatory dysentery. These symptoms can be described as severe abdominal cramps, fever, and blood present in mucoid stools. Usually shigellosis is self-limiting, however in immuno-compromised patients, if left untreated, patients can develop critical complications such as septicemia and hemolytic uremic syndrome (Bennish, 1991). Successful treatment of patients with shigellosis incorporates an intensive regimen of antibiotics and hydration. Fluoroquinolones, broad-spectrum antibiotics, are highly effective against *Shigella*. Therefore, fluoroquinolones have become the preferred agent for the treatment of shigellosis (Hooper *et al.*, 1985). Fluoroquinolones kill bacteria by binding to DNA gyrase, a type II topoisomerase, and thereby inhibiting DNA replication. Currently, there is no vaccine against *Shigella*, but candidate shigellosis vaccines, both killed and live attenuated are under development and are being tested in different clinical phases (World Health Organization, 2008).

1.5 Objectives of this study

The aim of this work is to improve our understanding of the molecular basis of *Shigella* pathogenesis and the characterization of the omptin family of proteases, which is present in several pathogenic organisms. This thesis is divided into two main chapters (chapter 2 and 3) encompassing four objectives.

Objective 1: To determine whether the mechanism of polar targeting found in *Shigella* is conserved in three closely related families in the γ-proteobacteria. This objective is addressed in chapter 2.

Objective 2: To further characterize the omptin proteins found in three pathogenic organisms, *Shigella flexneri* (IcsP), *Escherichia coli* (OmpT), and *Salmonella typhimurium* (PgtE). The beginning of chapter 3 expands on this objective.

Objective 3: To determine whether IcsP, an outer membrane protease, plays an additional role in the pathogenesis of *S. flexneri* by promoting resistance to a cationic antimicrobial peptide, LL-37. This objective is addressed in chapter 3.

Objective 4: To determine whether *icsP* is regulated by a small regulatory RNA, RyhB. This objective is addressed in the end of chapter 3.

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CHAPTER 2

THE CONSERVED MECHANISM OF ICSA TARGETING TO THE BACTERIAL POLE IN PROTEOBACTERIA

2.1 Introduction

This chapter addresses whether a mechanism of protein targeting, found in *Shigella* is conserved among other γ-proteobacteria. In this study we examine whether the mechanism of polar targeting, used by the polar protein IcsA found in *Shigella*, is conserved in species of the Enterobacteriaceae, Vibrionaceae, and Pseudomonadaceae families.

Asymmetric localization of proteins is essential for many biological functions in bacteria. Bacteria must indulge in efficient, economical metabolic processes if they are to survive. Consequently, they have evolved their own strategies to seek out nutrients and to survive a variety of environments. These strategies may encompass chemotaxis, cell division, motility, adhesion, and pathogenesis. Although bacterial cells are small in size and lack discrete cellular compartments such as organelles, they have an elaborate internal structure of organization that is important for many of these fundamental processes to occur (Callaway, 2008). This internal organization requires specific proteins to localize to discrete locations within the bacterial cell. Thus, many bacterial proteins have sub-cellular addresses.

Bacteria are capable of sorting and delivering proteins to specific sites within the cell with exquisite precision, yet bacterial cells lack transport vesicles, a golgi apparatus and an endoplasmic reticulum, which comprise the transport machinery in eukaryotic cells. How proteins reach their sub-cellular destination within bacterial cells remains unclear.

Examples of localized proteins can be found in *Caulobacter crescentus, Pseudomonas aeruginosa, Myxococcus xanthus, and Shigella flexneri.* In bacteria, the essential cell division protein FtsZ localizes to the cell midpoint early in cytokinesis where it assembles a cytokinetic ring (Donachie, 1993). Without the proper positioning of FtsZ, normal septation cannot occur (Ma *et al.*, 1996). *Caulobacter crescentus* expresses many chemosensory receptor proteins that localize to the flagellated cell pole (Alley *et al.*, 1992). Proper positioning of these proteins is necessary for chemotaxis (Liu *et al.*, 1997). Another example of protein targeting can be found in *Pseudomonas aeruginosa,* which has a polar flagellum. For proper motility to occur, all flagellar proteins need to be localized to the pole for flagellar assembly. *Myxococcus xanthus* utilizes polar type IV pili and adhesion complexes for gliding motility (Wall *et al.*, 1999). During vegetative growth, cells swarm to find prey bacteria or to gather nutrients in the environment utilizing these polar proteins for motility. Consequently, the localization and coordination of this polar motility apparatus is essential for bacteria to show directed motility (Mauriello *et al.*, 2007). All these observations establish that protein localization plays a critical cellular role and that bacterial organization is highly complex.

The *S. flexneri* asymmetrically located outer membrane protein, IcsA, nucleates actin tail assembly in the host cytoplasm. Actin tail formation allows *Shigella* to move

throughout the host cytoplasm and into adjacent cells. IcsA is classified as an autotransporter protein, which utilizes the type V secretion pathway (Brandon *et al.*, 2003).

Autotransporters are the largest family of secreted virulence-associated proteins of gram-negative bacteria and are secreted in a type V secretion manner. These large proteins share a common domain organization that includes an amino-terminal signal peptide, a conserved carboxy-terminal translocation domain that forms a β-barrel in the outer membrane, and a divergent functional domain that is exposed on the bacterial surface (Oomen *et al.*, 2004). Secretion of autotransporter proteins by the type V secretion pathway involves export through the cytoplasmic membrane via the Sec machinery. Once through the inner membrane, the signal sequence is cleaved and the β-domain inserts into the outer membrane as a β-barrel structure that forms a pore in the outer membrane. After formation of the β-barrel, the passenger domain is translocated onto the bacterial cell surface (Henderson *et al.*, 2004).

Once embedded in the outer membrane at the bacterial pole, IcsA recruits host actin. Proper positioning of IcsA to the old pole is necessary for the accumulation of short actin filaments into an F-actin comet tail (Bernardini *et al.*, 1989). Consequently, accurate placement of IcsA is necessary for *Shigella* virulence and contributes to its intracellular lifestyle.

Maintenance of IcsA at the bacterial pole is significant for *Shigella* virulence. IcsA has been shown to diffuse laterally along the sides of *Shigella* away from the old pole (Steinhauer *et al.*, 1999). To maintain IcsA at the pole, an outer membrane protease called IcsP cleaves IcsA by proteolytically cleaving and releasing the amino terminal

domain of IcsA into the extracellular environment. The released amino terminal domain contains the determinant for actin tail formation. In mutants that lack IcsP, IcsA is observed to exist in small amounts over the entire bacterial surface (Egile *et al.*, 1997; Shere *et al.*, 1997). Additionally, in a strain expressing a non-cleavable form of IcsA, localization of IcsA occurs over the entire bacterial surface and leads to the formation of abnormal F-actin comet tails in infected cells (d'Hauteville *et al.*, 1996). Therefore, cleavage of IcsA by IcsP appears to be important in the maintenance of an IcsA polar cap.

The mechanism of polar targeting of IcsA to the bacterial pole is not fully understood. However, there are several proposed mechanisms of IcsA polar targeting in *Shigella*. In the first proposed mechanism, targeted insertion, IcsA is directly and selectively inserted into the membrane at the site where it will ultimately reside. The second proposed mechanism, selective degradation, includes the random insertion of IcsA all over the membrane followed by the degradation of IcsA from locations other than its correct destination by proteases that are spatially restricted within the cell. In the third proposed mechanism, diffusion and capture, IcsA is inserted randomly into all membranes and becomes localized by diffusion to the site where it ultimately resides. Of these proposed mechanisms, targeted insertion has been shown to be the mechanism by which the outer membrane protein, IcsA achieves polar localization (Steinhauer *et al.*, 1999; Charles *et al.*, 2001).

Charles *et al.* (2001) have shown that IcsA is localized to the bacterial pole and is targeted to the old pole within the cytoplasm of *Shigella*. Translational fusions between portions of IcsA and GFP were used to determine the regions of IcsA that are necessary

for its targeting to the bacterial old pole. GFP is a cytoplasmic protein that cannot be secreted through the Sec apparatus, therefore these experiments, utilizing IcsA-GFP fusions addressed whether targeting occurred within the cytoplasm. Two sequences within IcsA, residues 1-104 and residues 507-620 are sufficient for IcsA localization to the pole in the bacterial cytoplasm. Interestingly, an IcsA-GFP fusion that lacks a signal peptide, localized to the old pole, indicating that signal peptide-mediated secretion is not required for localization to occur. IcsA-GFP fusions were also expressed in other types of bacteria to determine whether the mechanism of polar targeting to the bacterial pole is conserved. Charles *et al.* (2001) observed that the IcsA-GFP fusions target to the bacterial pole in *V. cholerae* and a variety of Enterobacteriacae, including *E. coli*, *S. typhimurium*, *Y. pseudotuberculosis*. This chapter expands on a third γ-proteobacterial family, the Pseudomonadaceae and addresses whether the mechanism of polar targeting to the bacterial pole is conserved in this family.

2.2 Materials and Methods

2.2.1 Bacterial strains, growth media, growth and expression conditions

Bacterial strains used in this study are listed in Table 2.1. *S. flexneri* strains were routinely grown in tryptic soy broth (TSB). *Pseudomonas aeruginosa*, *P. syringae*, *S. typhimurium*, and *E. coli* were routinely grown in Luria-Bertani (LB) medium. *Vibrio cholerae* was routinely grown in Lenox broth. For microscopy studies all strains were grown overnight from a single colony in rich media and back-diluted 1:50 the following day into M9 minimal media supplemented with 0.2% (w/v) casamino acids, 0.2% (v/v) glycerol, 0.5 mg mL^{-1} thiamine, 0.5 mg mL^{-1} tryptophan, 0.5 mg mL^{-1} nicotinic acid, 0.1

Table 2.1 - Bacterial Strains

Strain	Relevant Genotype	Source
E. coli		
DH10B	K-12 cloning strain	Invitrogen
DH5α	K-12 cloning strain	Invitrogen
MBG263	MC1061 Δ*ompT*::Kan^R	Goldberg & Theriot, 1995
MC1061	F$^-$ *araD139 Δ(ara-leu)7696 galE15 galK16 Δ(lac) X74 rpsL (Str^R) hsdR2 (r_k-m_k+)mcrA mcrB1*	Meissner *et al*,1987
P. aeruginosa		
PAK	Wild-type	Lu *et al*, 1997
PAK Δ*pilA* Δ*xcp* all	PAK Δ*pilA* Δ*xcpR*	Lu *et al*, 1997
PAK Δ*pscC*	PAK Δ*pscC*	Wolfgang *et al*, 2003
PAK Δ*vfr*	PAK Δ*vfr*	Wolfgang *et al*, 2003
PAK Δ*fleQ*	PAK Δ*fleQ*	Arora *et al*, 1997
P. syringae		
B728A	Wild-type	Willis *et al*, 1991
S. typhimurium		
14028s	Wild-type	American Type Culture Collection
S. flexneri		
2457T	Wild-type serotype 2a	Labrec *et al*, 1964
MBG283	2457T *icsA*::W Spec^R	Steinhauer *et al*,1999
V. cholerae		
O395	Classical Ogawa wild-type Sm^R	Mekalanos *et al*, 1983
KKV46	O395 Δ*flrA1*::Cm^R Δ*lacZ*	Klose *et al*, 1998

mM $CaCl_2$, and 0.5 mM $MgSO_4$. Antibiotic concentrations used were as follows: ampicillin, 100 mg mL^{-1}; carbenicillin, 150 mg mL^{-1}; spectinomycin, 100 mg mL^{-1}; streptomycin, 100 mg mL^{-1}; and tetracycline, 100 mg mL^{-1} for *P. aeruginosa* and 12.5 mg mL^{-1} for strains other than *P. aeruginosa.*

P. aeruginosa, *V. cholerae*, *S. typhimurium*, and *E. coli* cultures were grown at 37°C to OD_{600} 0.3-0.5 and then shifted to 25°C. At OD_{600} 0.6 expression of *icsA::gfp* was induced at 25°C for 1h by the addition of either 0.2% (w/v) L-arabinose or 500 mM IPTG (final concentration), as appropriate. *P. syringae* cultures were grown at 25°C throughout and expression was induced for 1h by the addition of either 2% (w/v) L-arabinose or 500 mM IPTG (final concentration), as appropriate.

2.2.2 Plasmid constructions and conjugation

Plasmids used in this study are listed in Table 2.2.

2.2.2.1 Construction of pEHK01, full-length IcsA, L-arabinose inducible promoter

To construct pEHK01, full-length *icsA* was cloned into a broad host-range vector, pJN105 downstream of an L-arabinose inducible promoter to be introduced into *P. aeruginosa* (PAK) as follows. A non-methylated pMGB270 was digested with *AccIII* and one of the resulting fragments contained full-length *icsA*. The *AccIII icsA* fragment was ligated to a linearized *XmaI* pJN105 fragment, thereby creating pEHK01.

2.2.2.2 Construction of pEHK10, pJN105 with XbaI and PvuI sites removed

To construct pEHK10, a *PvuI* and *XbaI* site were removed by linearizing pJN105 with *XbaI* and *PvuI* and ligating to an annealed sequence, used with oligonucleotides 28 and 29 (Table 3). These two sequences were annealed in a thermocycler with the

Table 2.2 - Plasmids

Plasmid	Relevant Genotype	Reference/Source
pACYC184	*E. coli* plasmid cloning vector Tet^R	Chang & Cohen
pBAD24-*icsA*$_{507\text{-}620}$::*gfp*	pBAD24-P_{BAD}-*icsA*$_{507\text{-}620}$::*gfp* Amp^R	Charles *et al.*, 2001
pBAD24-*icsA*$_{\Delta 507\text{-}729}$::*gfp*	pBAD24-P_{BAD}-*icsA*$_{\Delta 507\text{-}729}$::*gfp* Amp^R	Charles *et al.*, 2001
pEHK01	pJN105-P_{BAD}-*icsA* Gm^R	This work
pEHK10	pJN105, PvuI and XbaI sites removed, NheI introduced Gm^R	This work
pEHK11	pEHK10-P_{BAD}-*icsA* Gm^R	This work
pEHK13	pEHK10-P_{BAD}-*icsA* Mutated Shine-Dalgarno and abolished false start codon Gm^R	This work
pEHK18	pMMB67EH-P_{tac}-*icsA*$_{\Delta 507\text{-}729}$::*gfp* Tet^R	This work
pEHK20	pMMB67EH-P_{tac}-*icsA*$_{507\text{-}620}$::*gfp* Tet^R	This work
pJEH3	pMMB67EH-P_{tac}-*icsA*$_{507\text{-}620}$::*gfp* Amp^R	This work
pJEH7	pMMB67EH-P_{tac}-*icsA*$_{\Delta 507\text{-}729}$::*gfp* Amp^R	This work
pJN105	Broad host range vector, Gm^R	Newman *et al.*, 1999
pMBG270	pBR322-*icsA*	Magdalena & Goldberg 2002
pMMB67EH	Broad-host-range vector, Amp^R	Furste *et al.*, 1986
pRK2013	Helper plasmid for conjugal transfer, Kan^R	Furste *et al.*, 1986

following cycles; 95°C for 2 minutes, a decreasing ramp temperature from 95°C to 25°C for 45 minutes. When this sequence was ligated to a linearized pJN105, the *PvuI* and *XbaI* sites were abolished and an *NheI* site was introduced, thereby creating pEHK10.

2.2.2.3 Construction of pEHK11, full-length icsA in pEHK10

To construct pEHK11, full-length *icsA* was cloned into pEHK10 as follows. pMBG270 was digested with *AccIII* and one of the resulting fragments contained full-length *icsA*. The *AccIII icsA* insert was ligated to a linearized *XmaI* pEHK10 fragment, thereby creating pEHK11.

2.2.2.4 Construction of pEHK13, full-length IcsA, L-arabinose inducible promoter

A 114 bp megaprimer was amplified from pEHK01 with oligonucleotides 23 and 24 (Table 2.3) to create a mutated Shine-Dalgarno sequence of IcsA (Table 2.4). This megaprimer was used along with oligonucleotide 25 (Table 2.3) to amplify a fragment including: part of IcsA, the mutated Shine-Dalgarno, and mutated false start codon from pEHK01, thereby creating a 394 bp fragment. The 394 bp fragment was digested with *EcoRI* and *XbaI* and ligated to pEHK11 digested with *XbaI* and *EcoRI* thereby removing the old Shine-Dalgarno sequence and thus creating pEHK13.

2.2.2.5 Construction of pJEH3

To construct pJEH3 for expression of $IcsA_{507\text{-}620}$-GFP in *P. aeruginosa*, the coding sequence for $IcsA_{507\text{-}620}$-GFP was cloned into pMMB67EH downstream of its IPTG-inducible promoter using the following strategy. pBAD24-*icsA*$_{507\text{-}620}$::*gfp* was cut with *Nhe*I and the resulting 3'-overhangs were filled in with T4 DNA polymerase. The resulting blunt-ended linear vector was then cut with *Hind*III, liberating the coding

Table 2.3: Oligonucleotides

Oligo 23	GGGCTAGC**GAATTC**CTGCAG CCC	Megaprimer of mutated Shine-Dalgarno sequence off pEHK01, Forward
Oligo 24	GGGTCATATTACAAAAAAAT TTGTGAATTTGATTCATG**GA** CTAT**TCCTC**C	Megaprimer of mutated Shine-Dalgarno sequence off pEHK01, Reverse
Oligo 25	GCTTAGTTCTAGATGCATGA GAGGGG	Used with megaprimer to amplify part of *icsA*, the mutated Shine-Dalgarno, and mutated false start codon off pEHK01
Oligo 28	CTAGTGGGATAGCCCCTA**GC TAGC**ATCGTAACGGTAGGAA GCCAT	Annealed with Oligo 29 to remove *XbaI* and *PvuI* sites off pJN105
Oligo 29	GCCTTCCTACCGTTACGAT**G CTAGC**TAGGGGCTATCCCA	Annealed with Oligo 28 to remove *XbaI* and *PvuI* sites off pJN105
	* Bold, *NheI* restriction site was introduced	
Oligo 30	CGAATCCGATCGGGCTGCTG GAGATGGCGG	Amplify *tet* off pACYC184, Forward
Oligo 31	ATCGAATCCGATCGGCACCT GAAGTCAGCCCC	Amplify *tet* off pACYC184, Reverse

Table 2.4 - Shine-Dalgarno (S-D) sequence of *icsA*

Original S-D sequence	ACTG**ATAAT**ATAG T**G**CATG
Mutated S-D sequence	ACTG**GAGGA**ATAG T**C**CATG

The *icsA* Shine-Dalgarno sequence found in *Shigella* appeared weak. For *icsA* to be expressed in *Pseudomonas,* the Shine-Dalgarno was strengthened by mutating the A-T rich sequence to become G-C rich. Additionally, there appeared to be a false start codon upstream of the translational start site. Consequently, the codon was mutated from G to C to avoid premature translation.

sequence for IcsA$_{507-620}$-GFP. This DNA fragment was ligated into pMMB67EH, which had been linearized with *Sma*I and *Hind*III, thereby creating plasmid pJEH3.

2.2.2.6 Construction of pJEH7

In a similar manner, pJEH7, which contains the sequence coding for IcsA$_{D507-729}$-GFP, was constructed by ligating a *BamH*I-*Pst*I fragment that was derived from pBAD24-*icsA*$_{D507-729}$::*gfp* and that contains the coding sequence for IcsA$_{D507-729}$-GFP into pMMB67EH. Tetracycline-resistant derivatives of pJEH3 and pJEH7, pEHK20 and pEHK18, were generated by introducing a tetracycline resistance gene into the unique *Pvu*I site located in the *bla* gene of pJEH3 and pJEH7. In both pEHK18 and pEHK20, the introduced tetracycline resistance gene was orientated in the same direction as the interrupted *bla* gene.

2.2.2.7 Construction of pEHK18 and pEHK20

To construct pEHK18 and pEHK20 for expression of IcsA$_{507-620}$-GFP in *PAK* Δ*vfr* and PAK Δ*fleQ*, the sequence coding for IcsA$_{507-620}$-GFP was cloned into a broad host-range vector pMMB67EH downstream of its IPTG-inducible promoter as follows. pJEH3 was introduced into DM1, a dam$^-$ *E. coli* strain. The non-methylated pJEH3 was linearized with *PvuI* and ligated to a Tetracycline gene, which was amplified from pACYC184 with oligonucleotides 30 and 31 (Table 2.3), thereby creating pEHK20. In a similar manner, pEHK18, which contains the sequence coding for IcsA$_{Δ507-729}$-GFP, was constructed by ligating a *PvuI* fragment containing the tetracycline gene that was derived from pACYC184 into pJEH7. Both pEHK18 and pEHK20 were transferred from the *E. coli* cloning strain DH10B to the appropriate *P. aeruginosa* strain by conjugation using the helper plasmid pRK2013.

2.2.3 Expression of icsA::gfp (pJEH3, pJEH7, pEHK18 and pEHK20), western blot analysis

To determine whether *icsA::gfp* is expressed from pJEH3, pJEH7, pEHK18 and pEHK20 in *P. aeruginosa*, *V. cholerae*, *S. typhimurium*, and *E. coli,* cultures were grown overnight at 37°C. The following day, cultures were back-diluted (1:50) to OD_{600} 0.3-0.5 and then shifted to 25°C. At OD_{600} 0.6 expression of *icsA::gfp* was induced at 25°C for one hour by the addition of either 0.2% (w/v) L-arabinose or 500 mM IPTG (final concentration), as appropriate. *P. syringae* cultures were grown at 25°C throughout and expression was induced for 1 hour by the addition of either 2% (w/v) L-arabinose or 500 mM IPTG (final concentration), as appropriate.

Whole cell proteins were prepared from this culture by harvesting equivalent number of cells and hence protein. Samples were then centrifuged at 12,800 x *g* at 4°C for two minutes. The pellets were resuspended in 0.2 M Tris (pH 8.0) at 4°C in half of the original harvested volume. The samples were then centrifuged at 12,800 x *g* at 4°C for two minutes. The supernatants were discarded and the pellets were resuspended in 200 µl 10 mM Tris (pH 7.4) plus 50 µl 4X SDS-PAGE loading buffer containing β-mercaptoethanol.

The whole cell protein preparations were loaded onto a 12.5% (v/v) SDS-polyacrylamide gel and separated by electrophoresis at 30 mA. Proteins were then transferred to polyvinylidene fluoride (PVDF) membrane for two hours at 150 mA. PVDF membranes were blocked with 1X PBS containing 5% (w/v) dried non-fat powdered milk (PBS/5% (w/v) milk) overnight at 4°C. The following day, the PVDF membrane was probed with a rabbit GFP polyclonal antibody (1:5000) for two hours at

37°C. Then it was washed with PBS/5% (w/v) milk and PBS/Tween-20 for 15 minutes. Finally, the membrane was incubated with the secondary antibody, anti-rabbit IgG, horseradish peroxidase linked $F(ab')^2$ fragment from donkey (1:10,000) for one hour at 25°C. GFP was detected by ECL kit (Amersham) according to the manufacturer's instructions and visualized on a Typhoon scanner using ImageQuant Software.

2.2.4 Expression of IcsA, (pEHK01 and pEHK13), western blot analysis

To determine whether full-length IcsA is expressed from pEHK01, pEHK01 was induced in three *E. coli* backgrounds, DH10B (wild-type), MC1061 (wild-type) and MBG263 (MC1061 Δ*ompT)*; and in two *Pseudomonas* backgrounds, PAK (wild-type) and B728A (wild-type). DH10B pEHK01 or pEHK13 was grown overnight in LB medium supplemented with 0.02% (w/v) glucose and gentamycin, 100 μg mL^{-1}. The following day the culture was back-diluted (1:50) in LB medium containing gentamycin, 100 μg mL^{-1}. Upon reaching an OD_{600} 0.4-1.0, pEHK01 was induced with 0.2% (w/v) L-arabinose for one hour at 37°C.

Whole cell proteins were prepared as previously described in section 2.2.3. The whole cell protein preparations were loaded into a 12.5% (v/v) SDS-polyacrylamide gel and separated at 30 mA. Proteins were then transferred to polyvinylidene fluoride (PVDF) membrane for two hours at 150 mA. PVDF membranes were incubated with PBS/5% (w/v) milk overnight at 4°C. The following day, the PVDF membrane was probed with a rabbit IcsA polyclonal antibody (1:10,000) for two hours at 37°C. Then it was washed with PBS/5% (w/v) milk and PBS/Tween-20 for 15 minutes. Finally, the membrane was incubated with the secondary antibody, anti-rabbit IgG, horseradish peroxidase linked $F(ab')^2$ fragment from donkey (1:10,000) for one hour at 25°C. IcsA

was detected by ECL kit (Amersham) according to the manufacturer's instructions and visualized on a Typhoon scanner using ImageQuant Software.

2.2.5 Indirect immunofluorescence of IcsA in S. flexneri

For indirect immunofluorescence, 2457T was grown overnight in minimal medium at 37°C. The following day, cultures were back-diluted (1:100) and grown to an OD_{600} 0.4-0.6. Cells were centrifuged onto coverslips at 700 x *g* for ten minutes and fixed with 3.7% (v/v) paraformaldehyde in PBS for 20 minutes at 25°C. After fixation, cells were labeled with IcsA antiserum (1:200). Subsequently, cells were incubated with the secondary antibody, goat anti-rabbit rhodamine-conjugated IgG (1:1000) in the dark for 20 minutes at 25°C. Cells were mounted onto a glass and observed immediately. Fluorescence and phase microscopy were performed by using an Olympus BX51 microscope with Olympus filters. Images were captured digitally by using an Olympus U-CMAD3 camera and PictureFrame 2.3 Software. The color figure was assembled by capturing in the rhodamine wavelength.

2.2.6 Indirect immunofluorescence of IcsA in P. syringae

For indirect immunofluorescence, B728A pEHK13 was grown overnight in minimal medium at 25°C. The following day, cultures were back-diluted (1:100) and grown to an OD_{600} 0.4-0.6 and induced with 2% (w/v) L-arabinose for two hours at 25°C. Cells were centrifuged onto coverslips at 700 x *g* for ten minutes and fixed with 3.7% (v/v) paraformaldehyde in PBS for 20 minutes at 25°C. After fixation, cells were labeled with IcsA antiserum (1:200). Subsequently, cells were incubated with the secondary antibody, goat anti-rabbit rhodamine-conjugated IgG (1:1000) in the dark for 20 minutes

at 25°C. Cells were mounted onto a glass slide and visualized as described in section 2.2.5.

2.2.7 Microscopy and tabulation of localization patterns

Tabulation of the localization pattern of each construct was performed in each of 3-5 independent experiments on approximately 120 randomly selected cells for each strain. Cells were mounted onto a glass slide and visualized as described in section 2.2.5. Each cell was categorized as polar, non-polar or diffuse based on the distribution and location of the GFP in the bacterial cell.

2.2.8 Media

Liquid and solid media were prepared by dissolving the specified quantities of reagents in distilled water.

2.2.8.1 *Tryptic Soy Broth Medium* (TSB) (per liter): 30g tryptic soy broth, 15g Bacto agar, for *Shigella* growth, TSB contains 0.1g Congo Red

2.2.8.2 *Luria-Bertani Medium* (LB) (per liter): 10g tryptone, 5g yeast extract, 10g NaCl, 15g Bacto Agar

2.2.8.3 *10X M9 Salts* (per liter): 60g Na_2HPO_4, 30g KH_2PO_4, 10g NH_4Cl, 5g NaCl

2.2.8.4 *M9 Medium* (per liter): 100 ml 10X M9 salts, 1 ml 1 M $MgSO_4$, 10 ml 10 mM $CaCl_2$, 10 ml 50% (v/v) glycerol, 100 µl 10 mg ml^{-1} thiamine

2.3 Results and Discussion

As previously reported, $IcsA_{507-620}$-GFP localized to the pole in *S. flexneri, E. coli, S. typhimurium* and *V. cholerae* (Charles *et al.*, 2001) (Figure 2.1 B and C, and Table 2. 5). The percentage of cells containing fluorescent polar foci in *V. cholerae*, O395, expressing pBAD24-*icsA*$_{507-620}$::*gfp* was substantially lower than in each of the Enterobacteriaceae (Table 2.5 and Figure 2.1 C compared to B). The $IcsA_{507-620}$-GFP

fusion was stable in *V. cholerae*, but its level of expression was 6- to 10-fold lower than for each of the Enterobacteriaceae, as determined by western blot analysis (data not shown – HJW). For each enterobacterial strain and *V. cholerae*, the IcsA-GFP fusion that lacks the polar localization sequence, $IcsA_{\Delta 507\text{-}729}$-GFP, displayed diffuse cytoplasmic fluorescence in essentially all cells examined, consistent with the localization of $IcsA_{507\text{-}620}$-GFP to the pole of these organisms being a result of a specific targeting mechanism. Whereas *S. flexneri* lacks flagella and *Salmonella* spp. and *E. coli* have peritrichous flagella, flagella expressed by *V. cholerae* are polar, therefore it was possible that the decreased frequency of polar IcsA foci in *V. cholerae* might be a result of interference of polar flagellar structures with the proper localization of IcsA. Such interference might occur if, for example, a polar flagellar protein were binding to the same polar protein or structure to which IcsA would normally bind. However, in a non-flagellated *V. cholerae* strain in which FlrA, a master regulator of flagellar biosynthesis, was absent (Klose *et al.*, 1998), polar localization of IcsA was unaltered (strain KKV46, data not shown - HJW), indicating that the presence of flagellar structures dependent on FlrA was not responsible for the observed low efficiency of IcsA localization to the pole. Therefore, although we cannot eliminate the possibility that a flagellum-associated structure that is present in the non-flagellated $FlrA^-$ *V. cholerae* strain interferes with polar localization of IcsA, we believe that the low efficiency of polar localization of $IcsA_{507\text{-}620}$-GFP in *V. cholerae* is more likely due to the relatively low level of expression of the fusion protein in this organism (data not shown – JEH).

To explore whether the mechanism of targeting of IcsA was conserved among *Pseudomonas* species, we examined localization of $IcsA_{507\text{-}620}$-GFP in *P. aeruginosa* and

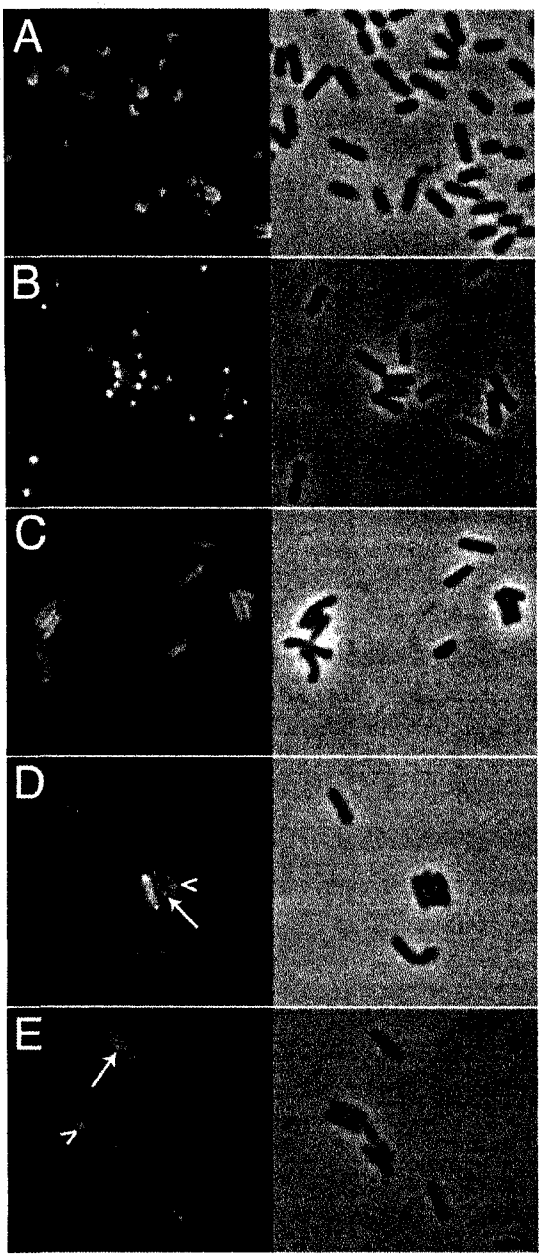

Figure 2.1: IcsA localizes to the pole of *V. cholerae* and *Pseudomonas* species.
(**A**) Distribution of IcsA on the surface of intact *S. flexneri* 2457T cells. Left, indirect immunofluorescence using anti-IcsA antiserum. Right, phase image of same microscopic field. (B through E) Localization of $IcsA_{507\text{-}620}$-GFP in *S. flexneri icsA* strain MBG283 (**B**), *V. cholerae* wild-type strain O395 (**C**), *P. aeruginosa* wild-type strain PAK (**D**), and *P. syringae* wild-type strain B728A (**E**), by direct epifluorescence microscopy. Left panels, GFP. Right panels, phase image of same microscopic field. Arrows, non-polar foci of $IcsA_{507\text{-}620}$-GFP in cells that also contain polar foci; arrowheads, non-polar foci in cells that lack polar foci. Images are representative of those obtained in three or more independent experiments. Bar, 2 μm.

Table 2.5: Distribution of $IcsA_{507-620}$-GFP in Enterobacteriaceae, *V. cholerae*, and *Pseudomonas* species

Organism	**Relevant Strain Background**	**Distribution of Fluorescent Signal (%, mean ± S.D.)**		
		Polar Foci*	**Diffuse**	**Non-polar Foci**
S. flexneri	*icsA-*	97 ± 2	1 ± 2	<1
E. coli	wild-type	94 ± 3	6 ± 3	<1
S. typhimurium	wild-type	98 ± 2	2 ± 2	<1
V. cholerae	wild-type	29 ± 14	70 ± 14	<1
P. aeruginosa	wild-type	43 ± 21	44 ± 26	14 ± 5

* - cells displaying bipolar foci, unipolar foci in the presence of additional foci, or bipolar foci in the presence of additional foci were all categorized as "polar" in this study. $IcsA_{507-620}$-GFP was expressed from either pBAD24-*icsA*$_{507-620}$::*gfp* or pJEH3. All strains carrying either pBAD24-*icsA*$_{\Delta 507-729}$::*gfp* or pJEH7, which also expresses $IcsA_{\Delta 507-729}$-GFP, displayed diffuse fluorescence in >99% of the cells examined (data not shown).

P. syringae. In both wild-type *P. aeruginosa* (PAK) and wild-type *P. syringae* (B728A), $IcsA_{507\text{-}620}$-GFP localized to the cell poles. In *P. aeruginosa* cells and *P. syringae* cells, discrete polar fluorescent foci, which were similar in morphology and pattern to those seen in the Enterobacteriaceae and *V. cholerae* (Figure 2.1 D and E; Table 2.5 and 2.6), were observed. However, a second localization phenomenon was seen in both *P. aeruginosa* and *P. syringae*, yet was rarely observed (≤ 1%) in either the Enterobacteriaceae tested or in *V. cholerae*. In *Pseudomonas* cells, non-polar fluorescent foci were observed at one or more locations within the bacteria (Table 5 & 6, "Non-polar Foci"), in either the presence or absence of polar foci (Figure 2.1 D and E, arrow or arrowhead, respectively). The non-polar foci observed in the *Pseudomonas* strains were unlikely to be the result of over-expression of $IcsA_{507\text{-}620}$-GFP, as western blot analysis showed that the level of expression of $IcsA_{507\text{-}620}$-GFP was modestly (2- to 4-fold) lower in *Pseudomonas* strains than in the Enterobacteriaceae tested. Furthermore, non-polar foci were not observed in cells that expressed $IcsA_{\Delta 507\text{-}729}$-GFP. Taken together, these data suggest that the mechanism of polar localization is conserved in *Pseudomonas* species because a sub-population of cells displays polar foci. The presence of both non-polar and polar foci in *Pseudomonas* species, but only polar foci in Enterobacteriaceae and *V. cholerae* raise the possibility that polar proteins present in the *Pseudomonas* species tested may compete with localization of IcsA to the pole, resulting in the localization of some IcsA to non-polar sites in the cytoplasm. Alternatively, these findings might reflect an alteration of IcsA recognition of the polar localization machinery in the Pseudomonads that may have occurred as a result of evolutionary divergence of these organisms.

To explore whether the presence of non-polar $IcsA_{507\text{-}620}$GFP foci in *P. aeruginosa*

Table 2.6 - Distribution of $IcsA_{507-620}$-GFP in *Pseudomonas* species.

Organism	**Relevant Strain Background**	**Distribution of Fluorescent Signal (%, mean ± S.D.)**		
		Polar Foci*	**Diffuse**	**Non-polar Foci**
P. aeruginosa	wild-type	40 ± 5	46 ± 4	13 ± 3
	vfr-	40 ± 4	44 ± 2	16 ± 4
	fleQ-	65 ± 3	32 ± 4	3 ± 1
P. syringae	wild-type	29 ± 5	56 ± 3	15 ± 5

* Cells displaying bipolar foci, unipolar foci in the presence of additional foci, or bipolar foci in the presence of additional foci were all categorized as "polar" in this study. $IcsA_{507-620}$-GFP was expressed from pEHK20. All strains carrying pEHK18, which expresses $IcsA_{D507-729}$-GFP, displayed diffuse fluorescence in >99% of the cells examined (data not shown). The distribution of fluorescent signals observed in the *P. aeruginosa fleQ* mutant differed significantly from that seen in the wild type ($P < 0.01$). There was no significant difference between the *vfr* mutant and wild type *P. aeruginosa* ($P > 0.4$).

might result from interference of certain discrete cellular structures with IcsA, we next examined whether either of two known polar structures of *P. aeruginosa*, the polar flagellum or the polar type IV pilus, might interfere with the polar localization of $IcsA_{507-620}$-GFP in *P. aeruginosa* (Lory 1998). To address this, two mutant strains, a *P. aeruginosa* strain that lacks *fleQ*, the major flagellar transcriptional regulator, which controls the expression of the entire polar flagellum proteins and is unable to synthesize polar flagella (Dasgupta *et al.*, 2000), and a *P. aeruginosa* strain that lacks *vfr*, the major virulence factor regulator, which controls the expression of many virulence proteins and specifically is unable to synthesize the polar type IV pili biogenesis machinery, were used (Frost *et al.*, 1977) (Beatson *et al.*, 2002). We hypothesized that if interference between our targeting construct and polar proteins in *P. aeruginosa* were occurring, then in the absence of these global regulators of polar proteins, we would detect an increase in polar foci. However, our results showed that the pattern of localization of $IcsA_{507-620}$-GFP in the *vfr* mutant was indistinguishable from that of the wild-type strain, with both polar and non-polar foci observed at frequencies that were comparable to those observed for the wild type (Figure 2.2 C compared to 2.2 A; Table 2.6). In contrast, in the *fleQ* mutant, an altered pattern of localization of $IcsA_{507-620}$-GFP was observed (Figure 2.2 E, Table 2.6). The number of polar foci observed in this strain background was significantly higher than that observed in wild-type PAK ($P < 0.01$, two tailed t-test, Table 2.6; Figure 2.2 E and 2.2 A) and was concomitant with a significant decrease compared to the wild-type strain in the number of cells displaying diffuse signals ($P < 0.01$, two tailed t-test, Table 2.6) and non-polar foci ($P < 0.01$, two tailed t-test, Table 2.6). As in the wild-type strain (Figure 2.2 B), the localization-deficient derivative of IcsA, $IcsA_{\Delta 507-729}$-GFP,

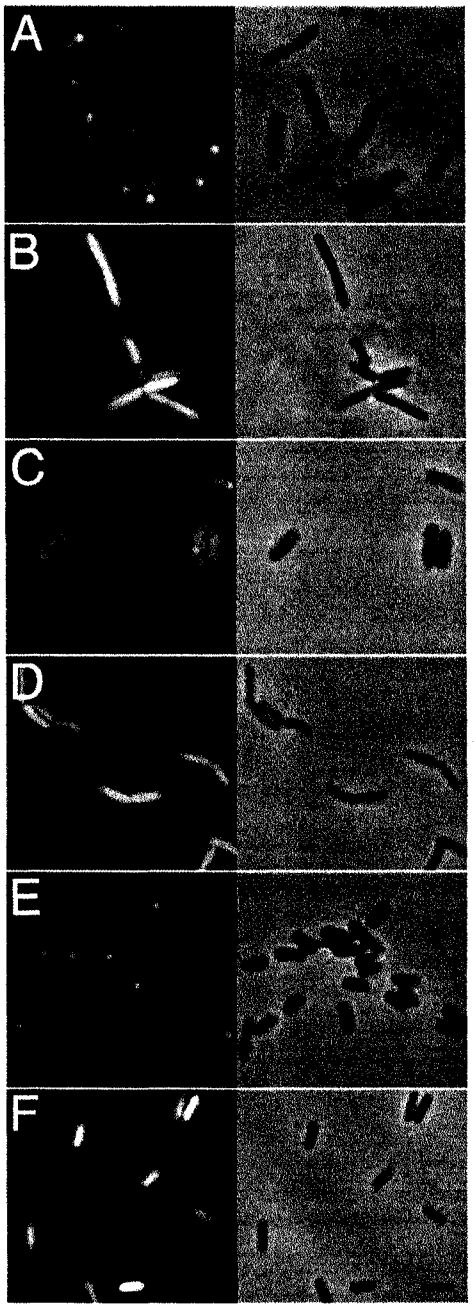

Figure 2.2: Enhanced polar localization of IcsA in *P. aeruginosa* in the absence of the polar protein FleQ.
Localization of $IcsA_{507\text{-}620}$-GFP (A, C, E) or of $IcsA_{D507\text{-}729}$-GFP (B, D, F) in wild-type *P. aeruginosa* (strain PAK) (A, B), an isogenic *vfr*⁻ mutant (C, D), and an isogenic *fleQ*⁻ mutant (E, F), by direct epifluorescence microscopy. Left panels, GFP. Right panels, phase image of same microscopic field. Images are representative of those obtained in three or more independent experiments. Bar, 2 μm.

displayed diffuse fluorescence in both the *fleQ* and the *vfr* mutant.

Taken together, these data are consistent with competition for the polar targeting machinery between FleQ-dependent proteins and $IcsA_{507-620}$-GFP. Competition between FleQ-dependent proteins and IcsA would imply that *P. aeruginosa* and *Shigella* spp. have evolved the capability for discrete polar proteins to utilize the same polar targeting machinery. Moreover, the ability of discrete proteins to use the same polar targeting machinery implies that the targeting machinery is likely to be highly conserved. However, we cannot eliminate the possibility that a FleQ-dependent protein directly or indirectly interacts with the IcsA-GFP fusion protein, thereby altering its interaction with the targeting apparatus. It also remains unclear whether pili synthesis is occurring in *P. aeruginosa* cells, which may account for the similar number of polar foci in both wild-type and *vfr* mutant cells.

We observed that the frequency of polar foci was significantly lower in *Pseudomonas* than in the *Shigella, Escherichia,* and *Salmonella* strains. Therefore, it is possible that some unidentified polar proteins in *Pseudomonas* interfere or compete for the targeting mechanism used by $IcsA_{507-620}$-GFP. Since it was unclear which *Pseudomonas* proteins were responsible for interference, we decided to conduct interference assays in which full-length IcsA and $IcsA_{507-620}$-GFP would be expressed simultaneously in the same cells to determine whether full-length IcsA interfered with the targeting of $IcsA_{507-620}$-GFP to the bacterial pole in *Pseudomonas*. We went to great lengths to express *icsA* in *P. aeruginosa*, but were never able to stably express full-length *icsA* in a *P. aeruginosa* background. Consequently, interference assays were never conducted. Our attempts to express IcsA in *Pseudomonas* are described below.

To determine whether the pJN105 derivative carrying *icsA*, pEHK01 expressed IcsA, a ~125 kDa protein, pEHK01 was induced with 0.2% (w/v) L-arabinose for one hour at 37°C in DH10B (K-12 wild-type). It was determined that IcsA was expressed from this plasmid and resulted in a ~95 kDa band, as judged by western blot analysis (Figure 2.3). A ~95 kDa resulted because OmpT, an outer membrane protease found in *E. coli* is able to cleave IcsA. Therefore, pEHK01 was then introduced into a different *E. coli* background that lacked *ompT*, an outer membrane protease that is capable of cleaving IcsA. IcsA was expressed in MC1061 and MBG263 (MC1061-Δ*ompT*) which resulted in a ~95 kDa band and ~125 kDa band, respectively, as judged by western blot analysis (Figure 2.4).

Subsequently, *icsA* from pEHK01 was expressed in a *P. aeruginosa*, PAK and induced with 2% L-arabinose for one hour at 37°C. However, IcsA could not be detected in PAK, as judged by western blot analysis (Figure 2.5). Therefore, the Shine-Dalgarno (S-D) sequence of *icsA* was examined and was found to be weak. As a result, the S-D sequence of *icsA* was improved from being ATAAT to GAGGA (Table 2.4). Furthermore, upon close inspection, there appeared to be an alternative translational start that if used would result in translation of the wrong frame. Therefore, this sequence GTG was changed to GTC (Table 2.4). The resulting plasmid, pEHK13, containing full-length *icsA* with a strengthened S-D sequence and an obliterated false start codon was introduced in an *E. coli* background, DH10B. IcsA was expressed from pEHK13 in DH10B and as a result of these changes, levels of IcsA expression were significantly higher that those observed previously (Figure 2.6).

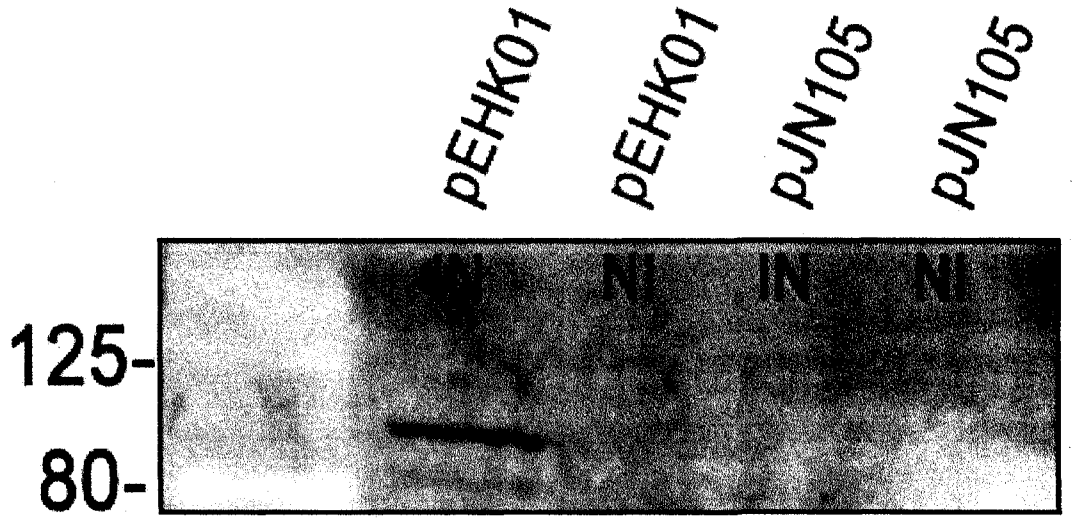

Figure 2.3: IcsA is expressed from pEHK01 in *E. coli*
pEHK01 (pJN105-*icsA*) and pJN105 (empty vector) was induced for one hour at 37°C with 0.2% (w/v) L-arabinose in an *E. coli* background, DH10B. Samples were normalized to total protein, separated by SDS-PAGE, and transferred to a PVDF membrane. IcsA was detected by immunoblotting with an anti-IcsA antibody, induced with 0.2% (w/v) L-arabinose, one hour at 37°C. NI, not induced. A ~95 kDa band is seen because of the presence of OmpT in *E. coli*, which is able to cleave ~125 kDa IcsA.

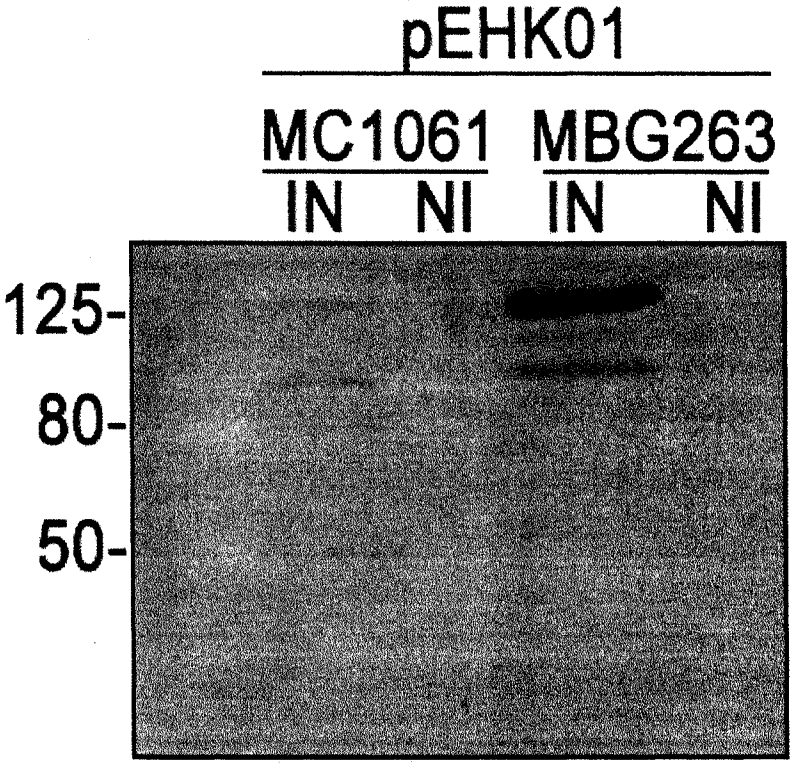

Figure 2.4: IcsA is expressed from pEHK01 in two *E. coli* strains, MBG263 and MC1061.
pEHK01 (pJN105-*icsA*) was induced for one hour at 37°C with 0.2% (w/v) L-arabinose in MC1061 and MBG263 (MC1061-Δ*ompT*). Samples were normalized to total protein, separated by SDS-PAGE, and transferred to a PVDF membrane. IcsA was detected by immunoblotting with an anti-IcsA antibody. OmpT cleaves IcsA and results in a ~95 kDa band.

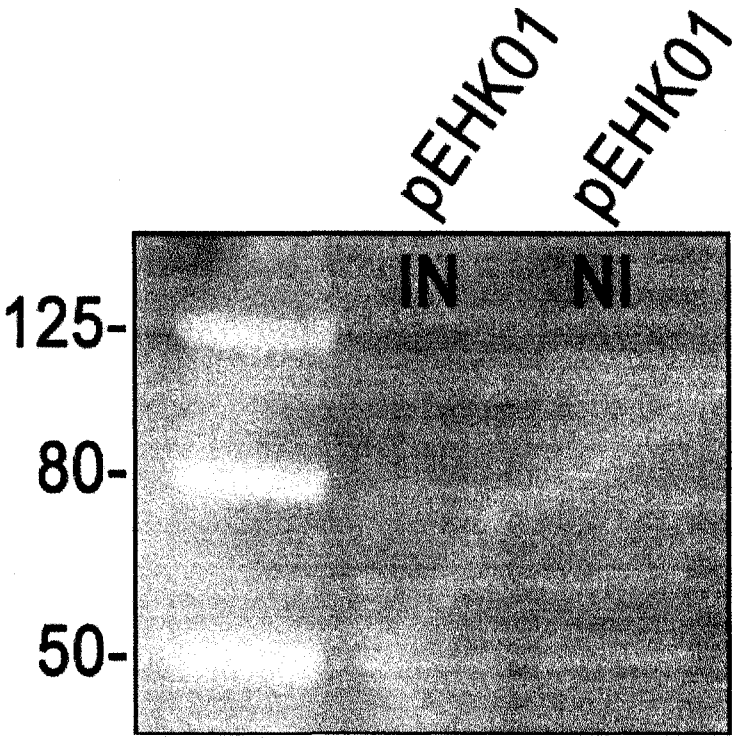

Figure 2.5: PAK does not express IcsA from pEHK01
Samples were normalized to total protein, separated by SDS-PAGE, and transferred to a PVDF membrane. IcsA was detected by immunoblotting with an anti-IcsA antibody, induced with 0.2% (w/v) L-arabinose, one hour at 37°C. NI, not induced. IcsA cannot be detected in *PAK*.

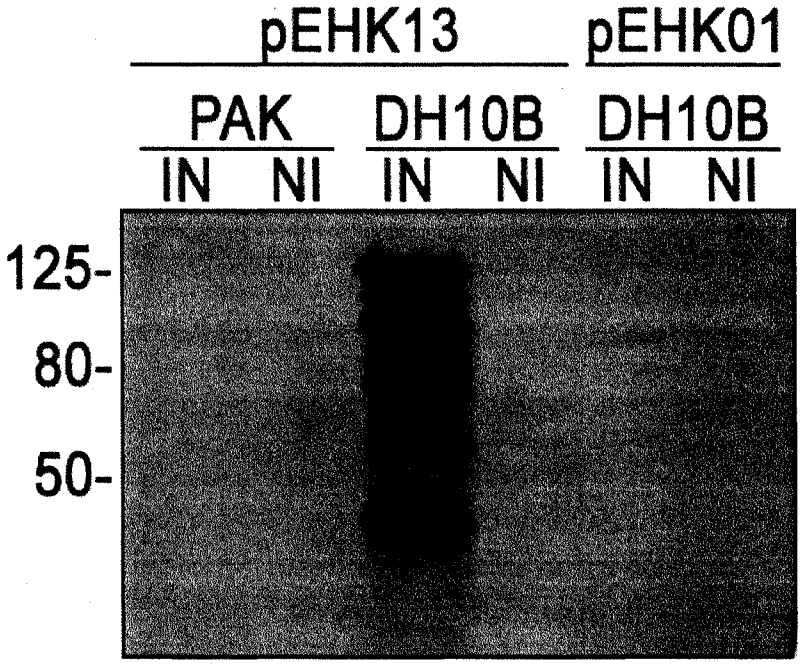

Figure 2.6: DH10B expresses IcsA from pEHK01 and pEHK13. PAK does not express IcsA from pEHK13

Samples were normalized to total protein, separated by SDS-PAGE, and transferred to a PVDF membrane. IcsA was detected by immunoblotting with an anti-IcsA antibody. IN, induced with 0.2% (w/v) L-arabinose in *E. coli* strains and 2% (w/v) L-arabinose in PAK, one hour at 37°C. NI, not induced. PAK does not express IcsA from pEHK13.

Next, to determine whether IcsA could be expressed in *P. aeruginosa,* pEHK13 was introduced into PAK and induced with different concentrations of L-arabinose for one hour at 37°C (Figure 2.7). However, IcsA, expressed from pEHK13 could not be detected in *P. aeruginosa* (Figure 2.7).

Because IcsA could not be detected in *P. aeruginosa*, we attempted to express this construct in another *Pseudomonas* species, *P. syringae*, B728A. pEHK01 and pEHK13 were both introduced into B728A and induced with L-arabinose at 25°C for one hour. IcsA was expressed in B728A from pEHK13 and not from pEHK01 (Figure 2.8).

We next needed to determine whether IcsA was localized to the bacterial pole on the surface of *P. syringae*. Surface labeling of IcsA in B728A, using indirect immunofluorescence was done in order to conduct the interference assays. However, from indirect immunofluorescence, IcsA was not found to be polar (Figure 2.9). Consequently, interference assays with *P. syringae* were not conducted.

2.4 Conclusion

In summary, our data indicate that the positional information specifying polar localization of *Shigella* IcsA is conserved in *P. aeruginosa* and *P. syringae* because in each of the strains examined, a sub-population of cells contained polar foci. Moreover, the data are consistent with interference by FleQ-dependent proteins in *P. aeruginosa* with polar localization of IcsA, suggesting that FleQ-dependent proteins and IcsA may use the same molecular machinery to localize to the cell pole. Based on these and earlier results, it seems possible that positional machinery recognized by at least some polar proteins may be conserved widely among γ-proteobacteria. The extent of conservation of

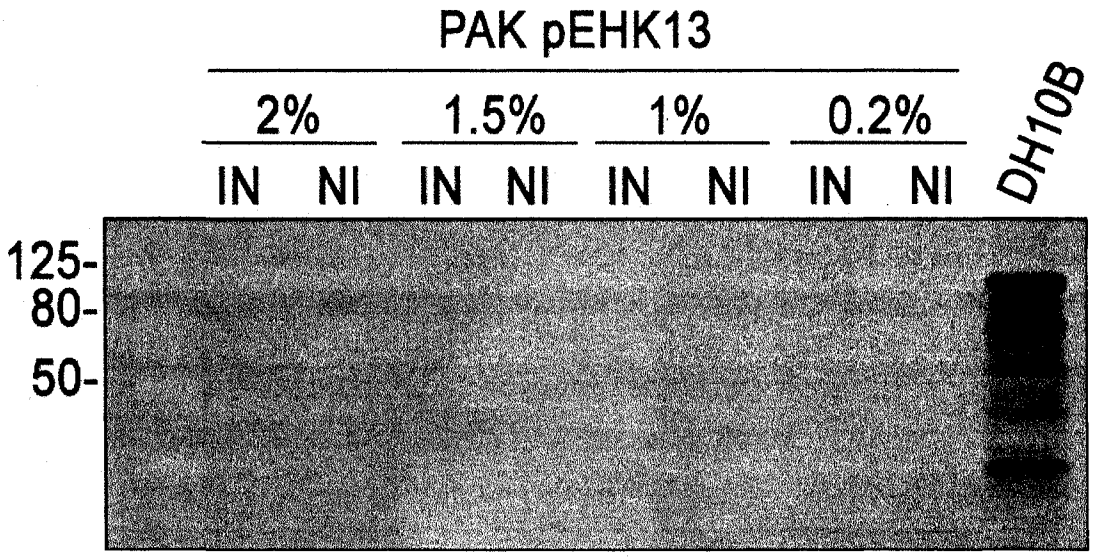

Figure 2.7: PAK does not express IcsA from pEHK13
Samples were normalized to total protein, separated by SDS-PAGE, and transferred to a PVDF membrane. IcsA was detected by immunoblotting with an anti-IcsA antibody. IN, induced with different concentrations of L-arabinose, 1 hour at 37°C. NI, not induced. IcsA, expressed in pEHK13 can be detected in *E. coli*, but not in *Pseudomonas aeruginosa*.

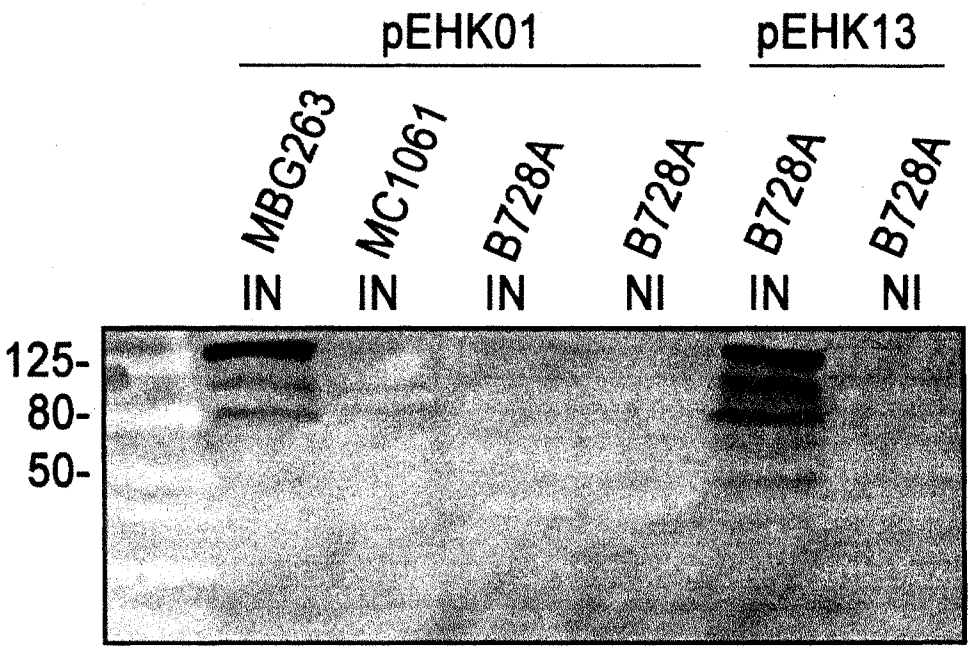

Figure 2.8: B728A expresses IcsA from pEHK13
Samples were normalized to total protein, separated by SDS-PAGE, and transferred to a PVDF membrane. IcsA was detected by immunoblotting with an anti-IcsA antibody. IN, induced with 0.2% (w/v) L-arabinose, 1 hour at 37°C in *E. coli* strains. IN, induced with 2% (w/v) L-arabinose, one hour at 25°C in *P. syringae* (B728A). NI, not induced. IcsA, expressed from pEHK01 cannot be detected in B728A. However, IcsA, expressed from pEHK13, can be detected in B728A.

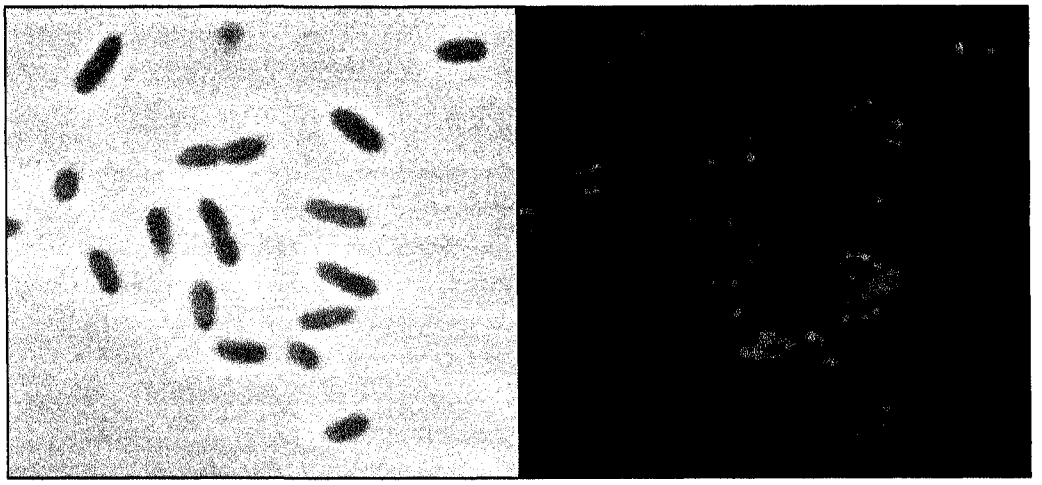

Figure 2.9: IcsA is distributed all over the bacterial surface in *P. syringae*.
Secretion of IcsA in a *P. syringae* (B728A) background. IcsA detected on the surface of intact bacteria that carry pEHK13. Indirect immunofluorescence (right panel) and phase (left panel).

this positional machinery suggests that it is utilized widely for the proper positioning of proteins in many γ-proteobacterial genera and/or that it is required for fundamental cellular processes (Shapiro *et al.*, 2002; Janakiraman *et al.*, 2004).

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CHAPTER 3

CHARACTERIZATION OF THE OMPTIN FAMILY AND THE ROLES AND REGULATION OF THE OUTER MEMBRANE PROTEASE, ICSP IN *SHIGELLA FLEXNERI*

3.1 Introduction

IcsP, an outer membrane protease, belongs to the omptin family of proteins, which are highly homologous. These omptins have been identified in several gram-negative bacteria that are pathogenic to humans (Table 3.1). Omptin-expressing bacterial species cause a variety of infectious diseases ranging from highly fatal plague to beneficial commensalism. The omptin family includes OmpT and OmpP of *E. coli*, PgtE of *Salmonella typhimurium,* Pla of *Yersinia pestis* and IcsP of *Shigella flexneri.* Omptin proteases are highly related in structure and share approximately 50% sequence identity, have major forms of 290-301 amino acids and lack or have a low cysteine content (Table 1) (Kukkonen *et al.*, 2004).

3.1.1 Structure of OmpT

The structure of omptin proteins can be predicted from the best-characterized member of the omptin family, OmpT of *E. coli.* The crystal structure of OmpT reveals a vase-shaped β-barrel with 10 antiparallel β-strands connected by four short periplasmic turns, of unknown function and five extracellular loops (Cowan *et al.*, 1992; Vandeputte-

Table 3.1: The Omptin Family of Proteases

Bacterium	**Omptin**	**Identity to OmpT (%)**	**Identity to IcsP (%)**
Escherichia coli	OmpT	100	58
Salmonella typhimurium	PgtE	50	39
Shigella flexneri	IcsP	60	100
Yersinia pestis	Pla	47	41

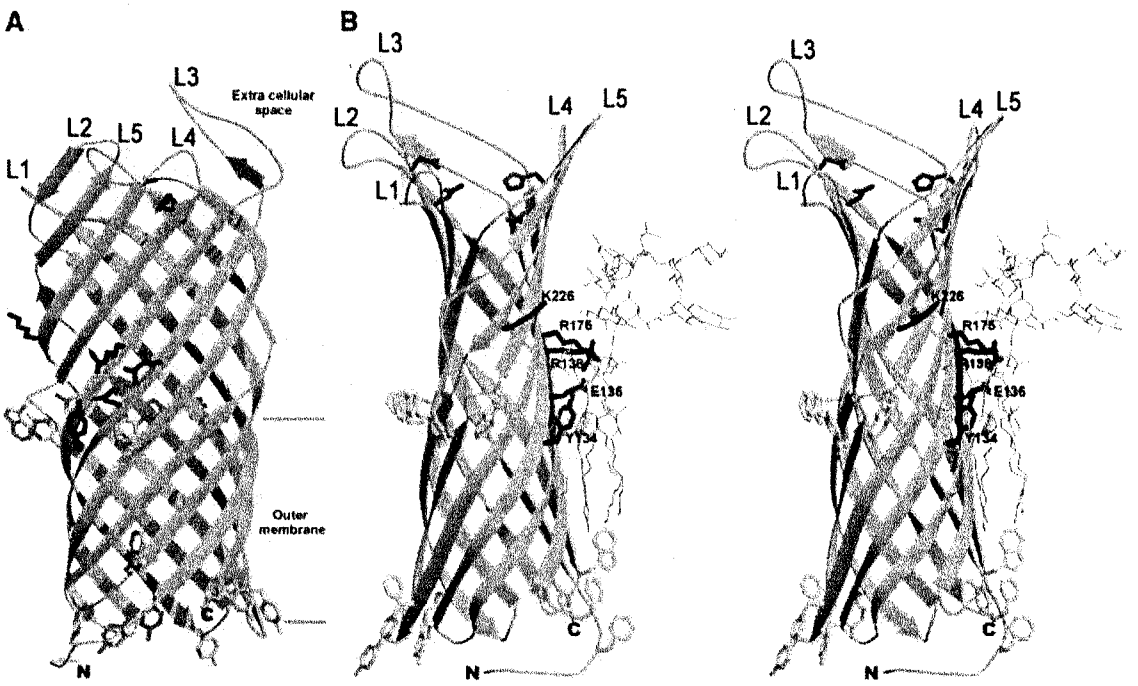

Figure 3.1: Overall structure of OmpT (Vandeputte-Rutten *et al.*, 2001)
(**A**) Ribbon representation of OmpT. The extracellular space is at the top of the figure and the periplasm is at the bottom. The extracellular loops are labeled L1 through L5. Aromatic residues that are located at the boundary of the hydrophobic and hydrophilic area on the molecular surface are colored yellow. The proposed catalytic residues are depicted in red and the purple colored areas are the putative LPS-binding sites. **(B)** Stereo representation of a modeled LPS molecule at the putative binding site. The orientation of the OmpT molecule is rotated 90° along the barrel axis, with respect to (**A**). This figure was prepared by (Vandeputte-Rutten *et al.*, 2001).

Rutten *et al.*, 2001) (Figure 3.1). The extracellular loops are predicted to be responsible for the protein's activity and specificity (Kukkonen *et al.*, 2001). The barrel extends approximately 40 Å from the lipid bilayer with the outermost mobile loops, surrounding the active site, located just above the edge of the lipopolysaccharide core region. A hydrophobic band is located in the outer membrane, and like other trans membrane proteins, OmpT has two rings of aromatic residues at the water-lipid interface. Within the membrane region, the barrel is hollow and carries an internal positive charge.

3.1.2 Proteolytic characteristics of OmpT

OmpT cleaves its substrates between two consecutive basic residues. A consensus sequence, (Arg/Lys) (Arg/Lys)-Ala has been identified (Dekker *et al.*, 2001). The active site of OmpT is located at the top of the protein placing it on the bacterial surface. It is located within a deep negatively charged groove, formed by loops L4 and L5 on one side and L1, L2, and L3 on the other side (Figure 3.1 B) (Vandeputte-Rutten *et al.*, 2001). The catalytic residues in this proposed active site are conserved among all members of the omptin family and are comprised of four residues; 3 Asp and 1 His (Hritonenko *et al.*, 2007). It has been proposed that a water molecule positioned between Asp83 and His212 is activated by the His212-Asp210 dyad and subsequently performs the nucleophilic attack on the scissile peptide bond (Vandeputte-Rutten *et al.*, 2001). Sequence alignment analysis of omptin proteases shows that the catalytic amino acids are completely conserved among the omptins (Table 3.2). Furthermore, it has been shown that amino acid residues vary in the surface loops surrounding the active site (Table 3.2). It has been speculated that minor sequence variations in the surface loops of OmpT may have profound effects on the target specificities of the individual omptins. In this regard

Table 3.2: Amino acids among the omptin proteins

	OmpT	IcsP	Pla	PgtE
Catalytic amino acids				
Asp83	+	+	+	+
Asp 85	+	+	+	+
Asp 210	+	+	+	+
His 212	+	+	+	+
Surface loop amino acids				
Glu 27	+	+	+	+
Ser 40	+	+	+	+
Leu 42	+	+	+	+
Met 81	+	+	+	+
Asp 97	+	+	+	+
Ser 99	+	+	+	+
His 101	+	+	+	+
Ala 143	+	+	+	+
Tyr 150	+	+	+	+
Ile 170	+	+	+	+
Asp 208	+	+	+	+
Ser 223	+	Asp	Glu	Glu
Thr 263	+	+	+	+
Ala 280	+	+	+	+
Ile 282	+	+	+	+
LPS binding region				
Tyr 134	+	+	+	+
Glu 136	+	+	+	+
Arg 138	+	+	+	+
Arg 175	+	Leu	+	+
Lys 226	+	+	Ser	Glu

most studies have focused on the omptin protease, OmpT of *E. coli*. Previous work has shown that a change of the amino acid from Ser to Arg at position 223 within the surface loops of OmpT, significantly alters its cleavage specificity from Arg-Arg to Ala-Arg (Varadarajan *et al.*, 2005). As yet, it remains unclear whether subtle amino acid differences within the extracellular loops of the other omptins confer different cleavage specificities.

3.1.3 Omptins functions are dependent on LPS

LPS, a molecule present in the outer leaflet of the gram-negative envelope, affects the assembly and proper folding of several outer membrane proteins, including the omptins (Kramer *et al.*, 2000; Kramer *et al.*, 2002). Previous work has shown that OmpT displays enzymatic activity only in the presence of lipopolysaccharide (LPS) (Kramer *et al.*, 2000). Kramer *et al.* (2002) hypothesized that LPS induces a subtle conformational change in the omptin protein that is required for native active site geometry. However, it is still not entirely clear how LPS contributes to OmpT stability and activity. *Ferguson et al.* (1998) identified a structural motif for LPS binding consisting of four basic amino acids in the crystal structure FhuA-LPS. A structure-based search in the Protein Data Bank revealed that a subset of four amino acids involved in the FhuA-LPS interactions is conserved in several LPS-binding prokaryotic proteins (Ferguson *et al.*, 2000). When OmpT is compared to FhuA-LPS complex, three of the four residues (shown in blue in Figure 3.1) of the consensus LPS-binding motif are similarly positioned (Vandeputte-Rutten *et al.*, 2001).

Some omptins have been found to be sterically inhibited by the O-side chain of smooth LPS. For instance, recent studies have shown that O-antigen in the outer core of

LPS sterically inhibits Pla and PgtE proteins from cleaving larger substrates (Kukkonen *et al.*, 2004; Pouillot *et al.*, 2005). *S. typhimurium* reduces the length of its O-antigen and regulates the expression of *pgtE* during its intracellular growth in macrophages (Lahteenmaki *et al.*, 2005). In *E. coli,* OmpT targets short peptides that are small enough to avoid steric hindrance from O-antigen. In *Y. pestis*, the O-antigen is not made due to mutations in genes encoding for the biosynthesis of the O-antigen pathway (Kukkonen *et al.*, 2004). Finally, in *S. flexneri,* it remains unclear whether *Shigella* modulates its LPS or whether LPS affects the proteolytic activity of IcsP.

3.1.4 Roles of omptins in bacterial pathogenesis

Omptin proteins are a family of enterobacterial proteases that share high sequence identity. They have a variety of roles that are predominantly involved in bacterial virulence and function as proteases, adhesins, or invasins (Stathopoulos,1998; Kukkonen *et al.*, 2004). Intriguingly, the omptins have a wide array of functions, yet the catalytic amino acids are completely conserved (Table 3.2). The expansive pathogenic roles and functions may be attributed to the minor sequence variations within the extracellular loops and/or the cellular background in which the omptin resides. This section highlights the known roles of omptins and the functions they serve in host environments.

3.1.4.1 OmpT of Escherichia coli

OmpT, encoded by a cryptic lambdoid phage on the chromosome, is an outer membrane protease found in *E. coli*. It is detected in both nonpathogenic (e.g., K12) and human uropathogenic *E. coli* strains (UPEC). In the UPEC strains, OmpT is reported to cleave protamine P1, an antimicrobial peptide that is secreted by epithelial cells of the urinary tract (Marrs *et al.*, 2002). Host cells secrete protamine in order to kill invading

bacterial cells. Protamine acts on the bacterial cytoplasmic membrane and causes membrane permeabilization, ultimately creating a large pore in the bacterial membrane (Aspedon *et al.*, 1996; Johansen *et al.*, 1997). It is thought that the cleavage of protamine by OmpT results in shorter peptide fragments that are physically unable to destabilize the bacterial membrane. Consequently, cleavage of protamine by OmpT protects *E. coli* from the bactericidal effects of this antimicrobial.

3.1.4.2 Pla of Yersinia pestis

Pla is a surface protease found in *Y. pestis*. Bacteria that express Pla are highly virulent yielding an LD_{50} increase of 10^6 fold in the absence of Pla (Sodeinde *et al.*, 1992; Sebbane *et al.*, 2006). Sebbane *et al.* (2006) have shown that the expression of Pla is essential for establishment of pneumonic and bubonic plague. One of the major roles of Pla is to enhance bacterial migration through tissue barriers, ultimately leading to the bacterial spread from a subcutaneous infection site (Kukkonen *et al.*, 2004). This process is described in Figure 3.2. Pla proteolytically cleaves an abundant circulating zymogen, plasminogen and consequently activates it into plasmin. The function of plasmin in host cells is to degrade extracellular matrices and fibrin clots that form barriers that hinder bacterial migration. Pla also inactivates the antiprotease, α_2-antiplasmin (Lahteenmaki *et al.*, 1998; Kukkonen *et al.*, 2004) (Figure 3.2). α_2-antiplasmin functions to tightly control the amount of plasmin in the host by deactivating free plasmin. Thus, the overall effect of Pla in the host is to degrade tissue barriers, which facilitates bacterial dissemination through host tissue.

Pla has been shown to cleave C3 protein of the complement system on the bacterial surface (Sodeinde *et al.*, 1992). Since C3 is one of the significant players in the

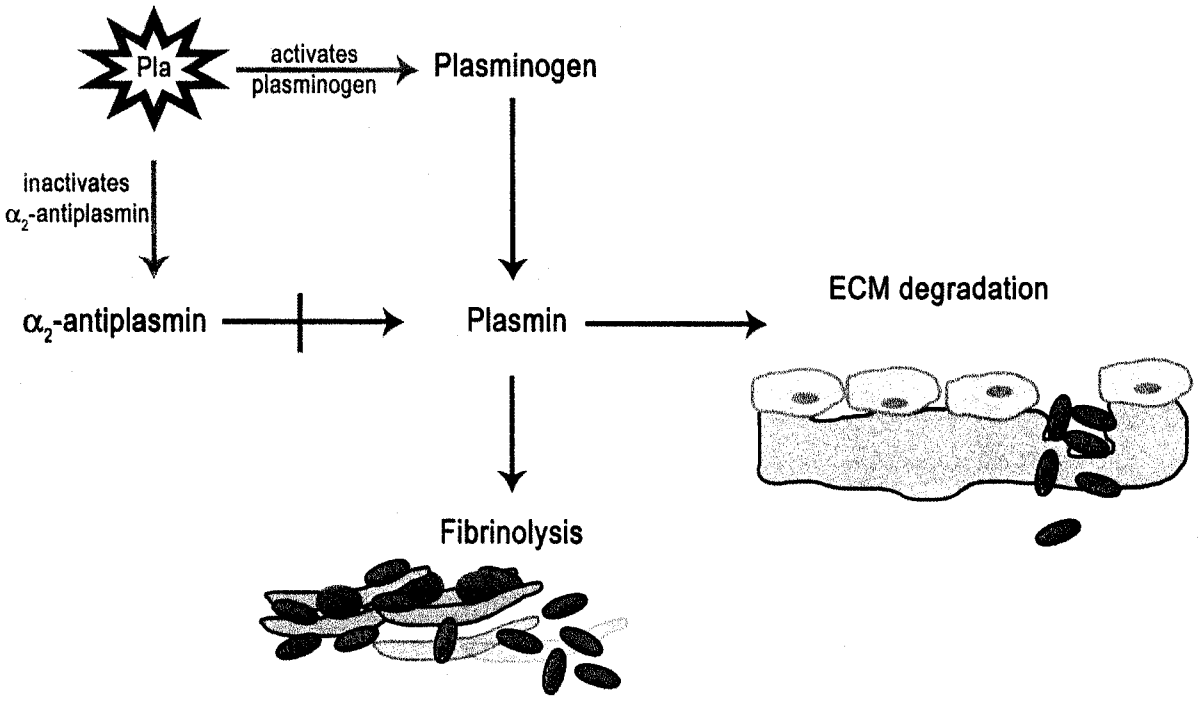

Figure 3.2: Overview of mammalian plasminogen system and the effects of *Y. pestis*
Plasminogen is an abundant circulating precursor that is proteolytically activated into plasmin. Plasmin is a serine protease that degrades fibrin clots (fibrinolysis) and extracellular matrices (ECM) that form barriers for cellular migration. Pla of *Y. pestis* intervenes with the system (in red) by activating plasminogen into plasmin and inactivating α_2-antiplasmin. The overall effect is bacterial migration through tissue barriers.

complement activation cascade, its deactivation leads to the reduced production of chemoattractants and subsequent suppression of local inflammation. Another function of Pla, independent of its proteolytic activity, is its ability to adhere to basement membrane preparations, extracellular matrix and human epithelial cells (Kukkonen *et al.*, 2004; Hritonenko *et al.*, 2007). Bacterial cell attachment to host tissues contributes to a persisting bacterial infection.

3.1.4.3 PgtE of Salmonella typhimurium

PgtE is a chromosomally encoded outer membrane protease of *S. typhimurium*. PgtE, like Pla can activate plasminogen to plasmin, which degrades fibrin clots and extracellular matrices of eukaryotic tissues and allows for bacterial dissemination (Guina *et al.*, 2000; Navarre *et al.*, 2005). Furthermore, previous work has shown that *Salmonella* PgtE expression promotes resistance to α-helical cationic antimicrobial peptides (CAMPs) (Guina *et al.*, 2000). CAMPs are a major component of the innate immune response and are part of the phagocytic vacuole of macrophages and neutrophils (Zasloff, 1992). These peptides are involved in a complex interplay between mammalian host cells and microorganisms at epithelial surfaces. The role of cationic antimicrobial peptides is to kill bacteria by permeabilizing their membrane. However, *Salmonella* uses the activity of PgtE to circumvent the immune defenses by cleaving CAMPs (Guina *et al.*, 2000), preventing the formation of large pores within the bacterial membrane and therefore protecting *Salmonella* against these antimicrobial compounds.

3.1.4.4 IcsP of Shigella flexneri

IcsP, an outer membrane protease, is encoded by a monocistronic gene on the 230 kb virulence plasmid in *S. flexneri*. The only known function of IcsP is to cleave a

virulence determinant, IcsA, between Arg_{758} and Arg_{759} and release a ~95 kDa fragment of IcsA from the bacterial surface (Egile *et al.*, 1997) (Figure 3.3). Substitution of either Arg_{759} or both Arg_{758} and Arg_{759} results in loss of cleavage (Fukuda *et al.*, 1995). Experiments indicate that IcsP functions to maintain IcsA exclusively at the bacterial pole, where IcsA recruits host actin and allows for intracellular movement (Steinhauer *et al.*, 1999). In the absence of IcsP, there is an alteration in the distribution of surface IcsA. In these *icsP* mutants, the typical tight polar cap of IcsA is not maintained. Thus, it has been proposed that IcsP functions to prevent diffusion of IcsA in the outer membrane (Steinhauer *et al.*, 1999). Egile *et al.* (1997) characterized the phenotype of *Shigella icsP* mutants by analyzing the formation of plaques on confluent monolayers of Caco-2 cells. Cell monolayers were infected with wild-type *Shigella* and an *icsP* mutant. Results showed that the *icsP* mutant formed as many plaques as the wild-type strain but plaque sizes were smaller. To date, studies on IcsP have focused primarily on its role in modulating the amount and distribution of IcsA and consequently actin-based motility. Since IcsP is regulated by known virulence gene activators and has high similarity to other omptins, we hypothesize that there are additional functions of IcsP.

3.1.5 Transcriptional activators, VirF and VirB regulate the expression of icsP

The pathogenicity of *Shigella* is a complex phenomenon, which requires the coordinated expression of several genes located both on the virulence plasmid and on the chromosome. The virulence plasmid contains a 31 kb region termed the entry region, which encodes proteins required for invasion of host colonic epithelial cells, intra- and intercellular spread, and secretion of the invasion proteins via a type III secretion apparatus. Located on the virulence plasmid are two regulatory genes, *virF* and *virB*.

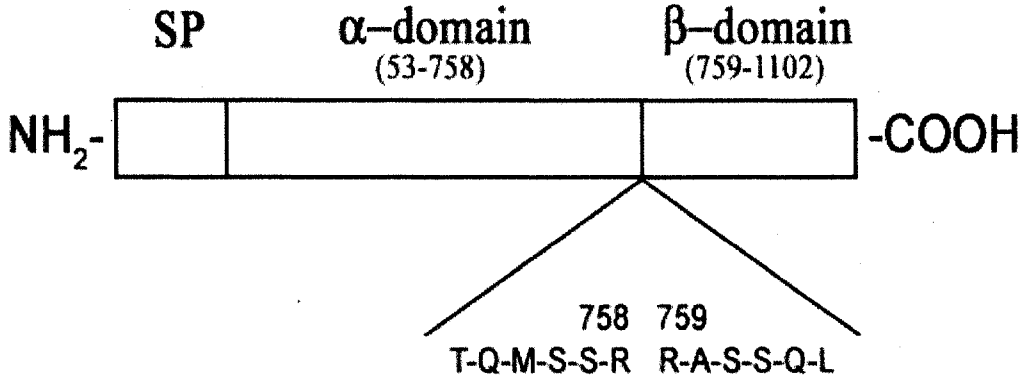

Figure 3.3: Schematic representation of IcsA and the IcsP cleavage site.
The letters under the cleavage site bar indicate the amino acid sequences containing the IcsP cleavage sites near the bacterial surface. IcsP cleavage of IcsA occurs between Arg_{758}-Arg_{759}. SP, signal peptide.

VirF, a member of the AraC family of transcriptional activators, is required for transcription of *virB* (Sakai *et al.*, 1986; Tobe *et al.*, 1993). VirB, a member of the ParB family of partitioning proteins is necessary for transcription of many genes clustered in operons including *ipa*, *mxi/spa,* and *icsP* (Buysse *et al.*, 1987; Andrews *et al.*, 1991; Wing *et al.*, 2004). Expression of genes on the virulence plasmid, such as VirF and VirB has been shown to be thermoregulated (Maurelli *et al.*, 1984). H-NS, a global regulator, has been shown to direct the temperature-regulated expression of virulence genes by repressing their transcription during growth at 30°C (Dorman *et al.*, 1990). The current model for temperature-dependent regulation suggests that at temperatures lower than 37°C, H-NS represses transcription by preventing the binding of the positive regulator, VirF to target promoters. At 37°C, changes in DNA conformation at the promoters of *virF* and *virB* lead to an increased transcription of *virF* and to the activation of the *virB* promoter by VirF (Dorman *et al.*, 2001). Subsequently, production of VirB leads to activation of the *icsP* promoter (Wing *et al.*, 2004) and the promoters of entry region of virulence plasmid genes (Adler *et al.*, 1989; Beloin *et al.*, 2002; Beloin *et al.*, 2003).

Previous work has shown that IcsP expression is undetectable in mutants lacking either of two transcriptional activators, VirF and VirB (Wing *et al.*, 2004). Expression of *virB* was found to be entirely dependent on VirF (Tobe *et al.*, 1993). Wing *et al.* (2004) showed that VirB significantly enhanced *icsP* transcription, even in the absence of VirF, suggesting VirB or a VirB-regulated gene product enhances transcription of the *icsP* gene.

3.1.6 A small RNA, RyhB suppresses transcriptional activator VirB in S. dysenteriae

Iron is one of the most essential metals in the metabolism of bacteria because it serves as a cofactor in multiple enzymatic reactions (Masse *et al.*, 2007). Consequently, bacteria have evolved mechanisms to acquire iron for these essential metabolic processes. One mechanism for iron acquisition involves the inactivation of a global iron-responsive transcriptional repressor, the ferric uptake repressor protein (Fur). Fur binds to specific DNA sequences called Fur boxes, usually located in the promoter region of a target gene (Gruer *et al.*, 1994). The binding of Fur to Fur boxes is iron dependent and this in turn blocks access of the RNA polymerase to the promoter of many iron acquisition genes. Conversely, the inactivation of Fur in the absence of iron results in the derepression of iron acquisition genes and ultimately, the uptake of iron (Figure 3.4 A).

Fur also represses RyhB. RyhB, a non-coding RNA first identified in *E. coli,* is a small regulatory RNA molecule that down regulates a set of iron-storage and iron-using proteins when iron is limiting (Masse *et al.*, 2002). In high iron conditions, Fur is activated and results in the repression of *ryhB*, which allows for the activation of iron storage genes. In low iron conditions, Fur does not bind to *ryhB* and consequently, RyhB is able to repress iron storage genes.

RyhB has been shown to decrease the stability of specific transcripts when it binds to the complementary nucleic acid sequence within the target mRNA molecule (Vecerek *et al.*, 2003). Once RyhB is bound to the target mRNA, this complex is degraded, which is mediated by RNase E and RNase III (Masse *et al.*, 2003). Previous work has shown that suppression of *S. dysenteriae* virulence occurs via RyhB-dependent repression of the transcriptional activator, VirB (Murphy *et al.*, 2007) (Figure 3.4 B).

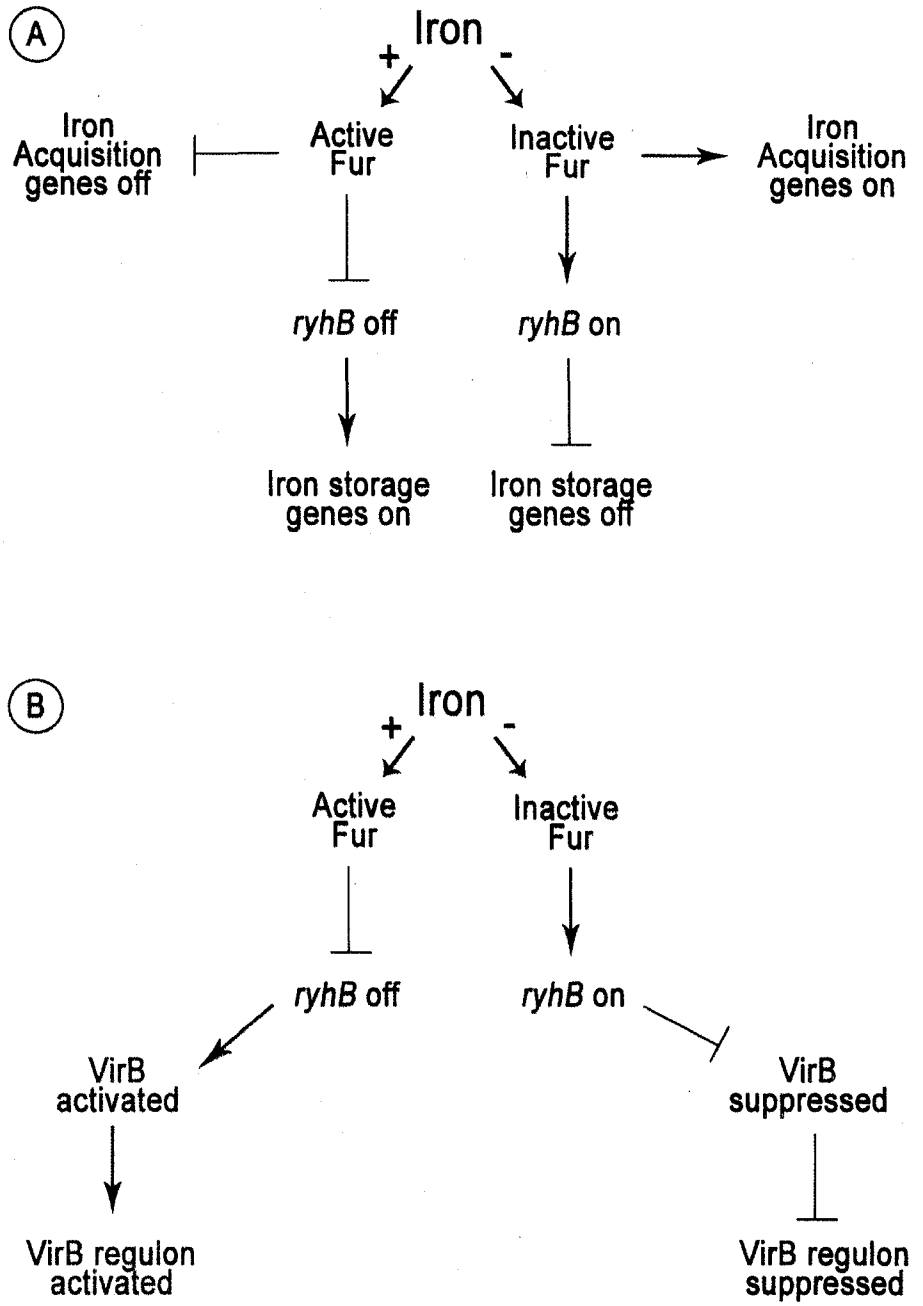

Figure 3.4: Iron regulation in *Shigella*

A. Model of Fur and RyhB interaction to regulate iron utilization

B. Model of Fur, RyhB, and VirB interaction to regulate virulence

Repression of *virB* leads to the reduced expression of certain genes within the VirB regulon, suggesting that all or most genes in the VirB regulon will be down regulated. However, this remains to be tested.

3.2 Materials and Methods

3.2.1 Bacterial strains, growth media, growth

Bacteria were grown as single colonies by streaking glycerol stocks onto agar plates containing antibiotics, where appropriate and incubating at 37°C overnight. Liquid cultures were prepared by inoculating sterile media containing antibiotics, where appropriate, with a single colony from an agar plate and then incubating at 37°C with aeration. Bacterial strains used in this study are listed in Table 3.3. *S. flexneri* strains were routinely grown in TSB. *E. coli* and *S. typhimurium* strains were routinely grown in LB medium. Antibiotic concentrations used were as follows: ampicillin, 100 $\mu g\ mL^{-1}$; chloramphenicol, 25 $\mu g\ mL^{-1}$; kanamycin 45 $\mu g\ mL^{-1}$ for TG61; kanamycin 50 $\mu g\ mL^{-1}$ for EHK01 and TMS02. All experiments were performed in triplicate.

3.2.2 Plasmid constructions

Plasmids used in this study are listed in Table 3.4.

3.2.2.1 Construction of pEHK19, pgtE in pBAD33

To construct pEHK19, *pgtE* was amplified from CS022 with oligonucleotides 9 and 10 (Table 3.5). The PCR product was digested with *XmaI* and *HindIII* and ligated to linearized *XmaI*, *HindIII* pBAD33. To confirm the correct sequence of *pgtE* in pBAD33, pEHK19 was sequenced by Nevada Genomics Center.

Table 3.3 - Bacterial Strains

Strain	Relevant Genotype	Source
E. coli		
DH10B	K-12 cloning strain	Invitrogen
DH5a	K-12 cloning strain	Invitrogen
MBG263	MC1061 Δ*ompT*::Kan^R	Goldberg & Theriot 1995
MC1061	F$^-$ *araD139 Δ(ara-leu)7696 galE15 galK16 Δ(lac) X74 rpsL (Str^R) hsdR2 (r_k- m_k+)mcrA mcrB1*	Meissner *et al.*, 1987
S. typhimurium		
CS022	*pho-24* (PhoP constitutive)	Miller *et al.*, 1990
TG61	CS022 Δ*pgtE*::Kan^R	Guina *et al.*, 2000
S. flexneri		
2457T	Wild-type serotype 2a	Labrec *et al.*, 1964
2457T-Δ*wzy*	2457T-Δ*wzy*, Congo Red negative	Carter *et al.*, 2007
M90T	Wild-type serotype 5	Sansonetti *et al.*, 1982
BS109	2457T *galU*::Tn*10*	Maurelli *et al.*, 1985
MBG341	2457T Δ*icsP*:: Amp^R	Shere *et al.*, 1997
AWY3	2457T Δ*virB*::Tn*5*	Wing *et al.*, 2004
MBG283	2457T Δ*icsA*::Spec^R	Steinhauer *et al.*, 1999
TMS02	2457T Δ*wzy*::Kan^R	This work
EHK01	MBG341 Δ*wzy*::Kan^R	This work

Table 3.4 - Plasmids

Plasmid	**Relevant Genotype**	**Reference/Source**
IcsP Roles Study		
pAJH02	pBAD33-*icsP*	This work
pBAD33	Cloning vector	Stratagene
pEHK19	pBAD33-*pgtE*	This work
pMBG270	pBR322-*icsA*	Magdalena & Goldberg, 2002
icsP Regulation Study		
pHJW20	*icsp::lacZ*	Unpublished data
pryhB	pQE-2-*ryhB*	Murphy *et al.*, 2007
pQE-2	Expression Vector	Murphy *et al.*, 2007

Table 3.5: Oligonucleotides - 5' to 3'

Oligo 9	CCTCATTCGCCCGGGTGACAAGATGAAA ACTTCATCACC	Amplify *pgtE* off CS022, Forward
Oligo 10	CGAATGAGGAAGCTTCCGGTTATGACCG ATGACATCCCG	Amplify *pgtE* off CS022, Reverse
Oligo 21	CCTCATTCGCCCGGGCTTATAAAGTAAG AAGATCATGG	Amplify *icsP* off pHJW6, Forward
Oligo 22	CGAATGAGGAAGCTTGCCATGAAAACG TAATCAACTCGG	Amplify *icsP* off pHJW6, Reverse
Oligo 58	GGAAGTTAAGGCGGAAAAAGGCTG	Amplify *wzy,* Forward
Oligo 59	CCCTATTTTTAACATCCTTTATTTTGCTC CAG	Amplify *wzy,* Reverse
Oligo 11	GCCATCACAGGAAGCAGCCTC	Amplify *icsA*, Forward
Oligo 12	GCCATCACAGGAAGCAGCCTC	Amplify *icsA*, Reverse
Oligo 13	GGATATAGAAGAGCGGTTTG	Amplify *virK*, Forward
Oligo 14	ACTTTATAATTTCAAGGGTACGGGTCCG	Amplify *virK*, Reverse
Oligo 15	GCACTTTGTGTACCTGCGATC	Amplify *icsP*, Forward
Oligo 16	GCACTATTTTTAATGGTGCCAG	Amplify *icsP*, Reverse
Oligo 17	CGAATCGCTGCAGGATATTATGATGCTG GAGTTTTGCGAAGC	Amplify *virF*, Forward
Oligo 18	CGAATCGAATTCCCATCTGGCAATAGCG ATGGGC	Amplify *virF*, Reverse
Oligo 19	CGAATCGAATTCTGAATTGGGCAGTTTA CATCAGTG	Amplify *virB*, Forward
Oligo 20	CGAATCGCTGCAGATTCTCTTTCTCTGAT TGAAATGCTGG	Amplify *virB*, Reverse

3.2.2.2 Construction of pAJH02, icsP in pBAD33

To construct pAJH02, *icsP* was amplified from pHJW6 with oligonucleotides 21 and 22 (Table 3.5). The PCR product was digested with *HindIII* and *XmaI* and ligated to linearized *XmaI, HindIII* pBAD33. To confirm the correct sequence of *icsP* in pBAD33, pAJH02 was sequenced by the Nevada Genomics Center.

3.2.3 Strain Constructions

3.2.3.1 Construction of TMS02 and EHK01

3.2.3.1.1 Preparation of transducing lysates

To prepare transducing lysates, 2457T-Δ*wzy* (Congo Red negative), the donor strain was grown overnight in tryptic soy broth containing kanamycin, 45 μg ml^{-1}. The following day, the cultures were back diluted (1:100) to an OD_{600} 0.5-1.0. 0.25 M $CaCl_2$ was added to the cultures and incubated for five minutes at 25°C. P1 phage stock, 6 $x10^9$ PFU ml^{-1} was added to the donor strains and incubated for two minutes at 25°C. Cells lysed after vigorous shaking at 37° C for three hours. Chloroform was added and incubated for 5 minutes at room temperature. Cultures were then centrifuged for two minutes at 12,800 x *g* at 25°C. The supernatant was transferred to a new tube and chloroform was added and ultimately stored at 4°C.

3.2.3.1.2 P1 transduction

To create TMS02 and EHK01, 2457T and MBG341 (recipient strains) were grown overnight in L broth medium without $CaCl_2$. The following day the cultures were back diluted (1:100) into L broth medium supplemented with 2.5 mM $CaCl_2$ to a density of 2 x 10^8 cells ml^{-1} at 37°C. Phage lysates were added to the recipient strains in L broth containing 2.5 mM CaCl2 to achieve MOIs of 0, 0.5, 2.5, and 10 and incubated at 25°C

for 30 minutes to allow for absorption. One ml of L broth medium, supplemented with 50 mM sodium citrate was added to each mixture and incubated for one hour at 37°C. Cells were then centrifuged at 12,800 x *g* for two minutes and the pellet was resuspended in 100 µl of 1 M sodium citrate. Cells were ultimately spread on minimal medium with appropriate antibiotics.

To determine whether resultant colonies were transductants carrying the interrupted *wzy* gene, oligonucleotides 58 and 59 were used to amplify the *wzy* locus by PCR (Table 3.5). To determine whether the *wzy* mutants still contained significant virulence genes, 5 loci were verified by PCR amplification, *virB, virF, virK, icsP, icsA* using oligonucleotides pairs 11, 12; 13, 14; 15, 16; 17, 18; and 19, 20 (Table 3.5).

3.2.4 Determining cleavage of IcsA by IcsP in S. flexneri, western blot analysis

To show that IcsP cleaved IcsA in *S. flexneri,* 2457T (wild-type), MBG341 (2457T-Δ*icsP*), and MBG283 (2457T-Δ*icsA*) carrying pMBG270 (*icsA*) were grown overnight in TSB medium. The following day, cultures were back-diluted (1:50) in TSB medium to an OD_{600} 0.4-1.0.

Whole cell proteins were prepared from this culture by harvesting equivalent number of cells and hence protein. Samples were then centrifuged at 12,800 x *g* at 4°C for two minutes. The pellets were resuspended in 0.2 M Tris (pH 8.0) at 4°C in half of the original harvested volume. The samples were then centrifuged at 12,800 x *g* at 4°C for two minutes. The supernatants were discarded and the pellets were resuspended in 200 µl 10 mM Tris (pH 7.4) plus 50 µl 4X SDS-PAGE loading buffer containing β-mercaptoethanol.

To harvest the supernatant proteins, the supernatants were passed through a 0.22 μM filter and precipitated with 10% (v/v) trichloroacetic acid. The samples were centrifuged at 12,800 x *g* for 10 minutes at 4°C, washed with cold 95% (v/v) ethanol, and re-centrifuged for 5 minutes at 12,800 x *g*. The pellets were resuspended in 200 μl of 10 mM Tris (pH 7.4) and 5 mM $MgSO_4$ and 50 μl 4X SDS-PAGE loading buffer containing β-mercaptoethanol.

The whole cell and supernatant protein preparations were loaded onto a 10% (v/v) SDS-polyacrylamide gel and separated by electrophoresis at 30 mA. Proteins were then transferred to polyvinylidene fluoride (PVDF) membrane for two hours at 150 mA. The PVDF membrane was blocked with 1X PBS containing 5% (w/v) non-fat powdered milk overnight at 4°C. The following day, the PVDF membrane was probed with a rabbit IcsA polyclonal antibody (1:10,000) for two hours at 37°C, washed with 1X PBS/Tween-20 for 15 minutes three times, and incubated with the secondary antibody, anti-rabbit IgG, horseradish peroxidase linked $F(ab')^2$ fragment from donkey (1:10,000) for one hour at 25°C. The membrane was washed with PBS/Tween-20 for 15 minutes three times. IcsA was detected by ECL kit (Amersham) according to the manufacturer's instructions and visualized on a Typhoon scanner using ImageQuant Software.

3.2.5 Determining cleavage of IcsA by OmpT in E. coli, western blot analysis

To determine whether OmpT cleaved IcsA in *E. coli*, pMBG270 (full-length *icsA)* was introduced into MC1061 (wild-type *E. coli*) and MBG263 (MC1061-Δ*ompT*) by electroporation. Cultures were grown overnight in LB medium, containing ampicillin, 100 μg mL^{-1}. The following day, cultures were back-diluted (1:50) in the LB medium for

3.5 hours. Whole cell and supernatant proteins were prepared as previously described in section 3.2.4 and subjected to western blot analysis.

3.2.6 Determining cleavage of IcsA by PgtE in Salmonella, western blot analysis

To determine whether PgtE cleaved IcsA in *Salmonella*, pMBG270 (full-length *icsA*) was introduced by electroporation into CS022 (wild-type *Salmonella*) and TG61 (CS022-Δ*pgtE*). Cultures were grown overnight in LB medium. The following day, cultures were back diluted (1:50) in LB medium for 3.5 hours. Whole cell and supernatant proteins were prepared as previously described in section 3.2.4 and subjected to western blot analysis.

3.2.7 Determining cleavage of IcsA by PgtE in S. flexneri, western blot analysis

To determine whether PgtE cleaved IcsA in *S. flexneri*, pEHK19 (pBAD33-*pgtE*) was introduced into MBG341 (2457T-Δ*icsP*). Cultures were grown overnight in TSB, supplemented with glucose, 0.02% (w/v) and chloramphenicol, 25 μg mL^{-1}. The following day, cultures were back-diluted (1:50) in the TSB medium, lacking glucose, to an OD_{600} 0.4-1.0 and subsequently induced with 0.2% (w/v) L-arabinose for one hour at 37°C. Whole cell and supernatant proteins were prepared as previously described in section 3.2.4 and subjected to western blot analysis.

3.2.8 Determining whether IcsP and PgtE promote resistance to cationic antimicrobial peptide (CAMP), LL-37 in Shigella and Salmonella, respectively

Minimum inhibitory concentrations (MICs) of CAMPs, required to kill *Salmonella* and *Shigella* cells either encoding or lacking omptins, were measured using bacterial sensitivity assays with the following strains: *Salmonella* spp. (CS022, wild-type; TG61, CS022-Δ*pgtE*) and *Shigella* spp. (2457T,wild-type; MBG341, Δ*icsP*; TMS02,

Δ*wzy*; EHK01, Δ*wzy*Δ*icsP*). *Shigella* cultures were grown overnight in minimal medium containing: Na_2HPO_4, KH_2PO_4 NaCl, NH_4Cl, casamino acids, nicotinic acid, tryptophan, thiamine, 2 mM $CaCl_2$, and 10 mM $MgSO_4$ and appropriate antibiotics. *Salmonella* cultures were grown overnight in N minimal medium containing: 5 mM KCl, 7.5 mM $(NH_4)_2SO_4$, 0.5 mM K_2SO_4, 1 mM KH_2PO_4, thiamine, casamino acids, 10% (v/v) glycerol, and 10 mM $MgCl_2$ and the appropriate antibiotics. The following day, cultures were diluted to 2 x 10^5 bacteria per ml in N minimal medium or minimal medium. Test peptide, LL-37 was assayed at final concentrations of 10 μg per ml to 320 μg ml^{-1} in 96-well microtiter plates. The MICs were determined as the lowest concentration of the peptide that did not allow visible bacterial growth after 18 hours.

3.2.9 Bacterial strains, growth media, growth

Bacteria were grown as single colonies by streaking glycerol stocks onto agar plates containing antibiotics, where appropriate and incubating at 37°C overnight. Liquid cultures were prepared by inoculating sterile media containing antibiotics, where appropriate, with a single colony from an agar plate and then incubating at 37°C with aeration. Bacterial strains used in this study are listed in Table 3.3. *S. flexneri* strains were routinely grown in TSB. Antibiotic concentrations used were as follows: ampicillin, 100 μg mL^{-1} and chloramphenicol, 25 μg mL^{-1}. All experiments were performed in triplicate.

3.2.10 Determining IcsP expression levels in RyhB-expressing Shigella cells, western blot analysis

To determine whether RyhB decreased IcsP expression levels, pryhB or pQE2 was introduced into 2457T (wild-type) and AWY3 (2457T-Δ*virB*). Cultures were grown

overnight in TSB medium. The following day, cultures were back diluted (1:50) to an OD_{600} 0.4-1.0, and induced with 200 μM IPTG for one hour at 37°C.

Whole cell proteins were prepared from this culture by harvesting equivalent number of cells and hence protein. Samples were then centrifuged at 12,800 x *g* at 4°C for two minutes. The pellets were resuspended in 0.2 M Tris (pH 8.0) at 4°C in half of the original harvested volume. The samples were then centrifuged at 12,800 x *g* at 4°C for two minutes. The supernatants were discarded and the pellets were resuspended in 200 μl 10 mM Tris (pH 7.4) plus 50 μl 4X SDS-PAGE loading buffer containing β-mercaptoethanol.

The whole cell protein preparations were loaded onto a 12.5% (v/v) SDS-polyacrylamide gel and separated by electrophoresis at 30 mA. Proteins were then transferred to polyvinylidene fluoride (PVDF) membrane for two hours at 150 mA. The PVDF membrane was blocked with 1X PBS/5% (w/v) milk overnight at 4°C. The following day, the PVDF membrane was probed with a rabbit IcsP polyclonal antibody (1:25) for two hours at 37°C, washed with PBS/Tween-20 for 15 minutes three times, and incubated with the secondary antibody, anti-rabbit IgG, horseradish peroxidase linked $F(ab')^2$ fragment from donkey (1:10,000) for one hour at 25°C. The membrane was washed with PBS/Tween-20 for 15 minutes three times. IcsP was detected by ECL kit (Amersham) according to the manufacturer's instructions and visualized on a Typhoon scanner using ImageQuant Software.

3.2.11 Determining icsP promoter activity levels in RyhB-expressing Shigella cells, β-galactosidase assay using a PicsP::lacZ fusion

Transcription from the *icsP* promoter was determined by measuring β-galactosidase activity. 2457T pryhB pHJW20 (*icsP::lacZ*), 2457T pQE2 pHJW20, AWY3 pryhB pHJW20, and AWY3 pQE2 pHJW20 were grown overnight in tryptic soy broth containing the appropriate antibiotics. The following day, 5 ml of tryptic soy broth was inoculated with 150 µl of overnight culture and grown to an OD_{650} 0.3-0.6. Cells were lysed with the addition of a drop of toluene and a drop of 1% (v/v) deoxycholate. The sample was vortexed and aerated for 20 minutes at 37°C to eliminate the toluene. The reaction was started by the addition of 100 µl of lysates to a mixture of 1.9 ml Z buffer and 0.5 ml of ONPG solution. When a yellow color had developed at 25°C, the reaction was stopped by adding 1 ml of 1M Na_2CO_3 solution. The tube was vortexed and the OD_{420} was measured. Each set of reactions was repeated three times and the mean β-galactosidase activities were calculated. The β-galactosidase activity of the cell lysate was calculated using the following equation:

$$\textbf{Activity} = \frac{OD_{420} \times 1000 \times 2.5 \times 3.5}{OD_{650} \times 4.5 \times t \times v} \quad \textbf{nmol / min / mg bacterial mass}$$

2.5 = conversion factor to convert OD_{650} into mg bacterial mass based on the assumption that an OD_{650} of 1 corresponds to 0.4 mg bacterial mass ml^{-1}.
3.5 = total assay volume after addition of 1M Na_2CO_3
1000 / 4.5 = conversion factor to convert OD_{420} of a 1 nmol ml^{-1} solution of ONPG in a 1 cm light path is 0.0045
t = time in minutes
v = volume of lysate added in ml

3.2.12 Media

Liquid and solid media were prepared by dissolving the specified quantities of reagents in distilled water.

3.2.12.1 *Tryptic Soy Broth Medium* (TSB) (per liter): As described in section 2.2.8.1.

3.2.12.2 *Luria-Bertani* (per liter): As described in section 2.2.8.2.

3.2.12.3 *Z buffer* (per liter): 0.75g KCl, 0.25g $MgSO_4 \cdot 7\ H_2O$, 8.5g Na_2HPO_4, 4.9g $NaH_2PO_4 \cdot 2H_2O$, 2.7 ml β-mercaptoethanol

3.2.12.4 *A Buffer* (per liter): 11.6 g K_2HPO_4, 4.54g KH_2PO_4

3.2.12.5 *ONPG*: 13 mM in A buffer

3.2.12.6 *Minimal Medium*: 40 mM Na_2HPO_4, 17 mM KH_2PO_4, 8.5 mM NaCl, 18.6 mM NH_4Cl, 0.4% (w/v) glucose, 0.4% (w/v) casamino acids, 0.01 mg ml^{-1} nicotinic acid, 0.01 mg ml^{-1} tryptophan, 0.01 mg ml^{-1} thiamine, 0.1 mM $CaCl_2$, 0.5 mM $MgSO_4$

3.3 Results and Discussion

3.3.1 OmpT and PgtE cleave IcsA differently than IcsP

To characterize the proteolytic activity of omptin proteases found in *Shigella* (IcsP), *E. coli* (OmpT), and *Salmonella* (PgtE), experiments were conducted to determine whether these proteases recognized and cleaved one substrate, IcsA in a similar manner. IcsA, a known substrate of IcsP was introduced into *Shigella*, *E. coli*, and *Salmonella,* on a medium copy plasmid, pMBG270. IcsA and its cleavage products found in whole cell and supernatant protein preparations from *Shigella*, *E. coli* and *Salmonella* were compared and analyzed by western blot analysis.

3.3.2 IcsP cleaves IcsA

Previous work has shown that IcsP cleaves IcsA (Egile *et al.*, 1997). To confirm this observation, whole cell and supernatant proteins of two *Shigella* strains: 2457T (wild-type) and MBG341 (2457T-Δ*icsP*), were compared (Figure 3.5). In whole cell protein extracts of both wild-type *Shigella* and the *Shigella icsP* mutant, full-length IcsA

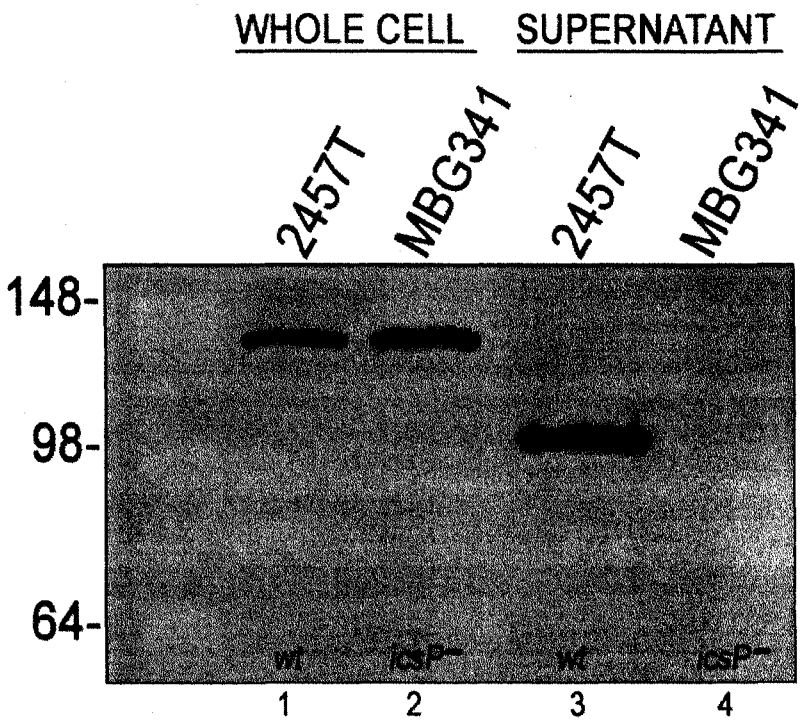

Figure 3.5: IcsP cleaves IcsA in *Shigella* cells and results in a ~95 kDa IcsA fragment.

2457T (wild-type) and MBG341 (2457T-Δ*icsP*) were grown overnight and back-diluted (1:50) to an OD_{600} 0.4-1.0. Equivalent amounts of proteins were loaded by normalizing the harvest volume to cell density. Whole cell and supernatant proteins were separated by SDS-PAGE, and transferred to a PVDF membrane. IcsA was detected by immunoblotting with an anti-IcsA antibody. A lower level of IcsA was present in wild-type *Shigella* revealing that IcsP cleaves IcsA. A ~95 kDa IcsA fragment was present in the supernatant from wild-type *Shigella* cultures and absent in the supernatant from the *icsP* mutant cultures. Collectively these data show that IcsP cleaves IcsA and removes a ~95 kDa fragment off the bacterial surface.

protein (~125 kDa) was observed, however, less IcsA was observed in the wild-type strain (Figure 3.5, lane 1) than in the *Shigella icsP* mutant (Figure 3.5, lane 2). In the supernatant protein extracts, a ~95 kDa IcsA fragment was present in samples collectedfrom cultures of wild-type *Shigella* (2457T) (Figure 5, lane 3), but nothing was detectable in samples collected from the *Shigella icsP* mutant (MBG341) (Figure 5, lane 4). Collectively, the decrease in the amount of IcsA in whole cell protein preparations of wild-type *Shigella* and the presence of a ~95 kDa fragment only in the supernatant protein preparations of wild-type *Shigella* indicates that IcsP cleaves IcsA. These data are consistent with previous observations.

3.3.3 Multi-copy IcsA is cleaved by IcsP in a similar manner to native IcsA

Having confirmed that IcsP cleaves IcsA, our goal was to assess whether other omptins cleave IcsA. To do this we first chose to examine how IcsA is cleaved when it is expressed in a variety of omptin containing bacteria. Since IcsA is unique to *Shigella*, we chose to express IcsA from a medium copy plasmid in each of the strains to be tested.

As a starting point for this work, we first chose to examine whether IcsA, expressed from a medium-copy plasmid, was cleaved by IcsP in a similar manner to that observed in our previous experiments. These experiments would allow us to assess whether expression of IcsA from a multi-copy plasmid overwhelmed the *Shigella* protease or not. To do this, pMBG270 (pBR322-*icsA*) was introduced into MBG283 (2457T-Δ*icsA*). Cells were grown in similar conditions, whole cell and supernatant protein preparations were normalized to cell density, separated by SDS-PAGE, analyzed by western blot and IcsA levels were compared to those observed in wild-type *Shigella* (2457T) (Figure 3.6). In whole cell protein extracts, a ~125 kDa IcsA band was present

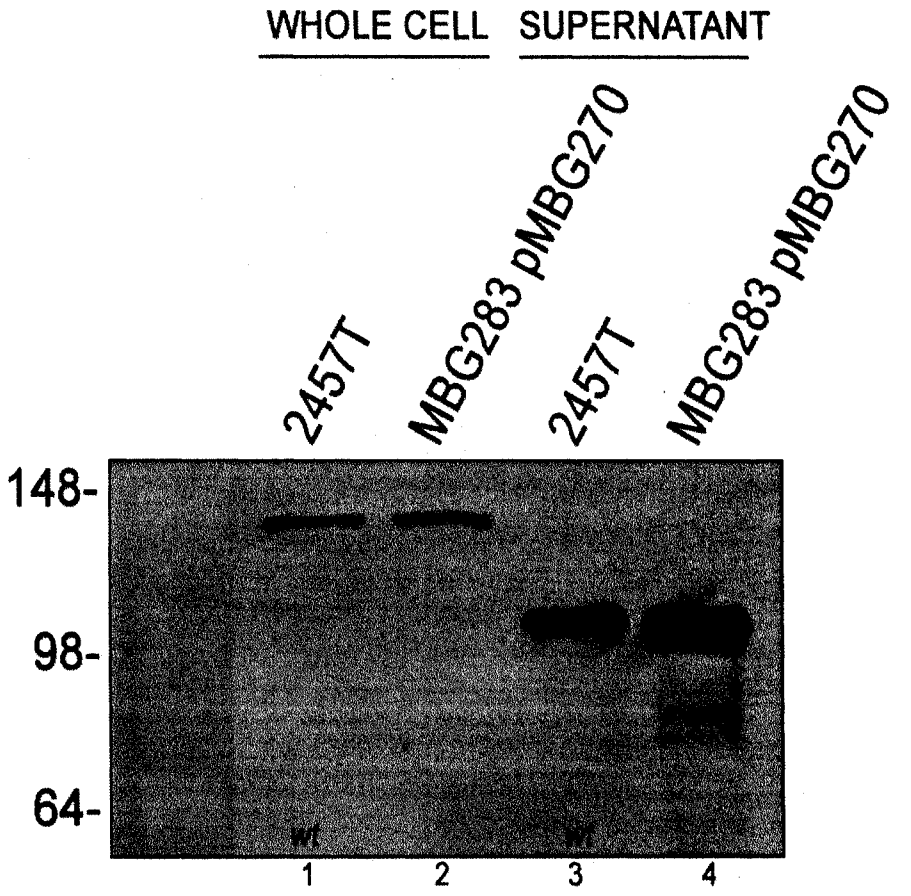

Figure 3.6: IcsP cleaves IcsA expressed from pMBG270 in *Shigella* cells and results in the release of ~95 kDa IcsA fragment, similar to wild-type.
pMBG270 (*icsA)* was introduced into a *Shigella* strain: MBG283 (2457T-Δ*icsA*). Equivalent amounts of proteins were loaded by normalizing the harvest volume to cell density. Whole cell and supernatant proteins were separated by SDS-PAGE, and transferred to a PVDF membrane. IcsA was detected by immunoblotting with an anti-IcsA antibody. IcsA, expressed from pMBG270, is cleaved by IcsP in a similar manner to IcsP cleaving natively expressed IcsA.

in both wild-type *Shigella* (2457T) and MBG283 pMBG270 (Figure 3.6, lanes 1 and 2). Additionally, cleavage of native IcsA and non-native IcsA, expressed from pMBG270, resulted in a ~95 kDa fragment appearing in the supernatant protein extracts of both strains (Figure 3.6, lanes 3 and 4). These data suggest that although IcsA was expressed from a multi-copy plasmid in these experiments, the *Shigella* protease was not overwhelmed and retained its ability to cleave its substrate. Overall, these experiments confirmed that the plasmid system could be used to examine cleavage of IcsA by the other omptin proteases.

3.3.4 OmpT cleaves IcsA differently than IcsP

To determine whether the *E. coli* omptin, OmpT recognizes and cleaves IcsA in a similar manner to the other omptins, whole cell and supernatant protein extracts from two *E. coli* strains: MC1061 (wild-type) and MBG263 (MC1061-Δ*ompT*) each carrying pMBG270 (*icsA*) were examined. Whole cell and supernatant protein extracts were normalized to cell density, separated by SDS-PAGE, and analyzed by western blot (Figure 3.7). Strikingly, IcsA was absent in whole cell protein extracts of wild-type *E. coli* (Figure 3.7, lane 1) and present in whole cell protein extracts of an *E. coli ompT* mutant (Figure 3.7, lane 2). In the supernatant protein extracts of wild-type *E. coli*, an ~85 kDa was present (Figure 3.7, lane 3), but IcsA was not detected in the supernatant protein extracts of the *E. coli ompT* mutant (Figure 3.7, lane 4). Collectively, these data suggest OmpT cleaves IcsA, resulting in an ~85 kDa IcsA fragment which is released into the growth medium (Figure 3.7, lane 3 and 4). Furthermore, when compared to the results observed in *Shigella*, these data indicate that OmpT cleaves IcsA in a dissimilar manner to IcsP. It is important to note that these *E. coli* strains contain a second omptin

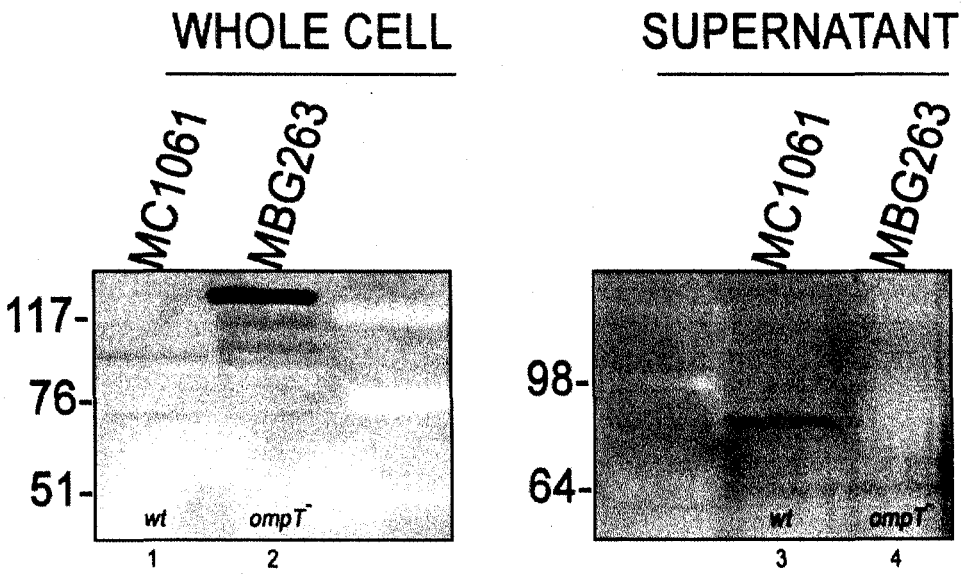

Figure 3.7: OmpT cleaves IcsA in *E. coli* cells and results in an ~85kDa IcsA fragment.

pMBG270 (*icsA)* was introduced into these two *E. coli* strains: MC1061 (wild-type) and MBG263 (MC1061-Δ*ompT*). Equivalent amounts of proteins were loaded by normalizing the harvest volume to cell density. Whole cell and supernatant proteins were separated by SDS-PAGE, and transferred to a PVDF membrane. IcsA was detected by immunoblotting with an anti-IcsA antibody. IcsA is present in whole cell protein preparation in an *E. coli* strain lacking OmpT. However, IcsA cannot be detected in wild-type *E. coli*. In an *E. coli* strain containing OmpT, a ~85 kDa IcsA band is detected in the supernatant protein preparations.

protease, OmpP, however, OmpP is not likely expressed in these conditions (glucose-replete) because it is regulated by the cyclic AMP system (Kaufmann *et al.*, 1994). Consequently, OmpP should not interfere or compound the proteolytic activity of OmpT in these experiments.

3.3.5 PgtE cleaves IcsA differently than IcsP and OmpT

Next, to examine whether the *Salmonella* omptin, PgtE recognizes and cleaves IcsA in a similar manner to the other omptins, whole cell and supernatant protein extracts from two *Salmonella* strains: CS022 (constitutive *phoP*) and TG61 (CS022-Δ*pgtE*) each carrying pMBG270 (*icsA*) were examined. Previous work has shown that PgtE is regulated by PhoP/PhoQ activation system (Guina *et al.*, 2000). Consequently, these *Salmonella* mutants constitutively express PgtE. Whole cell and supernatant protein extracts were normalized to cell density, separated by SDS-PAGE and analyzed by western blot (Figure 3.8). IcsA was not detected in whole cell protein extracts of wild-type *Salmonella* (Figure 3.8, lane 1), but a ~125 kDa IcsA and was present in the whole cell protein extracts of a *Salmonella pgtE* mutant (Figure 3.8, lane 2). These data resemble our observations made in the *E. coli* strains. In supernatant protein extracts of wild-type *Salmonella*, two fragments (~85 kDa and ~95kDa) were present (Figure 3.8, lane 3), but IcsA was not detected in supernatant protein extracts of the *Salmonella pgtE* mutant (Figure 3.8, lane 4). Together, these data suggest that PgtE cleaves IcsA, resulting in two fragments being released into the growth medium (Figure 3.8, lanes 3 and 4). Moreover, PgtE cleaves IcsA differently that IcsP and OmpT.

These experiments show that the omptins tested recognize and cleave IcsA differently. Yet, it remained unclear whether the altered pattern of IcsA cleavage was a

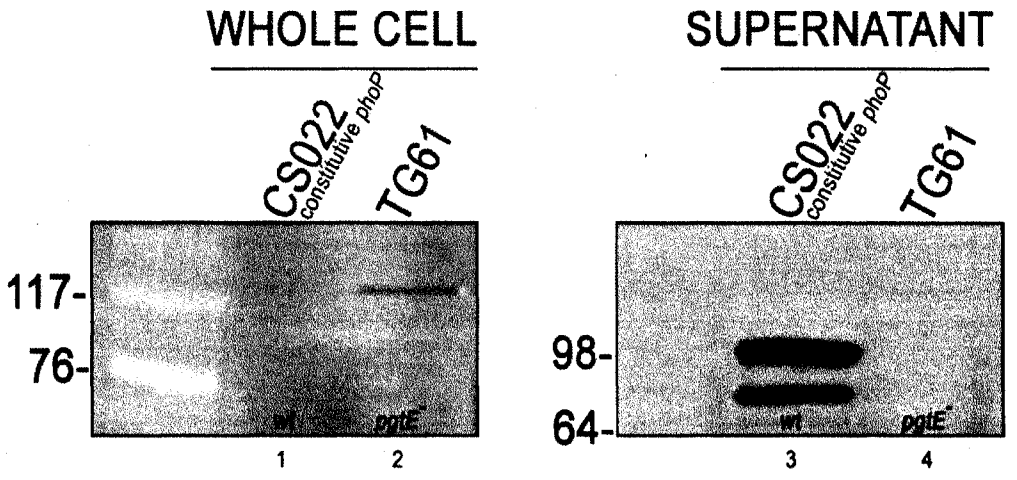

Figure 3.8: PgtE cleaves IcsA in *Salmonella* cells and results in two IcsA fragments. pMBG270 (*icsA)* was introduced into these two *Salmonella* strains: CS022 (wild-type) and TG61 (CS022-Δ*pgtE*). Equivalent amounts of proteins were loaded by normalizing the harvest volume to cell density. Whole cell and supernatant proteins were separated by SDS-PAGE, and transferred to a PVDF membrane. IcsA was detected by immunoblotting with an anti-IcsA antibody. Two bands, a ~95 kDa and ~85 kDa, are present in the supernatant from CS022. IcsA was not detected in the supernatant from TG61 cultures.

result of amino acid differences in the surface loops of the omptins and/or LPS binding region of the proteins or whether the different cellular backgrounds, and more specifically the LPS composition of these different strains, influence the specificity of activity of these proteases (described further in section 3.1.3). To determine whether the LPS composition affected the cleavage of IcsA, an omptin switching experiment was conducted. The *Salmonella* omptin *pgtE* was introduced in a *Shigella icsP*- background and IcsA and IcsA cleavage products were analyzed. We hypothesized that if the *Salmonella* cellular background affected the activity of PgtE to recognize and cleave IcsA, then *Shigella* cells expressing PgtE, would cleave IcsA in a dissimilar manner. However, if the IcsA banding pattern were the same in both conditions tested, the most likely explanation for the different cleavage pattern of IcsA would be inherent differences in the amino acid residues of the extracellular loops and/or the LPS binding region of the PgtE protein, leading to alteration of its substrate recognition and/or site specificity and hence its cleavage of the substrate IcsA.

3.3.6 Multi-copy IcsP cleaves IcsA similar to native IcsP

To determine whether the pattern of IcsA cleavage was similar in strains carrying medium-copy *icsP* to that seen in strains carrying the native copy of this gene, pAJH02 (pBAD33-*icsP*) was introduced in a *Shigella* background carrying an interrupted *icsP* gene, MBG341 (2457T-Δ*icsP*). Gene expression from pAJH02 was induced with 0.2% (w/v) L-arabinose for one hour, and whole cell and supernatant proteins were analyzed. In whole cell protein extracts of both wild-type *Shigella* and the *Shigella icsP* mutant (MBG341), each carrying pAJH02, full-length IcsA (~125 kDa) was observed, however, less IcsA was observed in the wild-type strain (Figure 3.9, lanes 3 and 4). In the

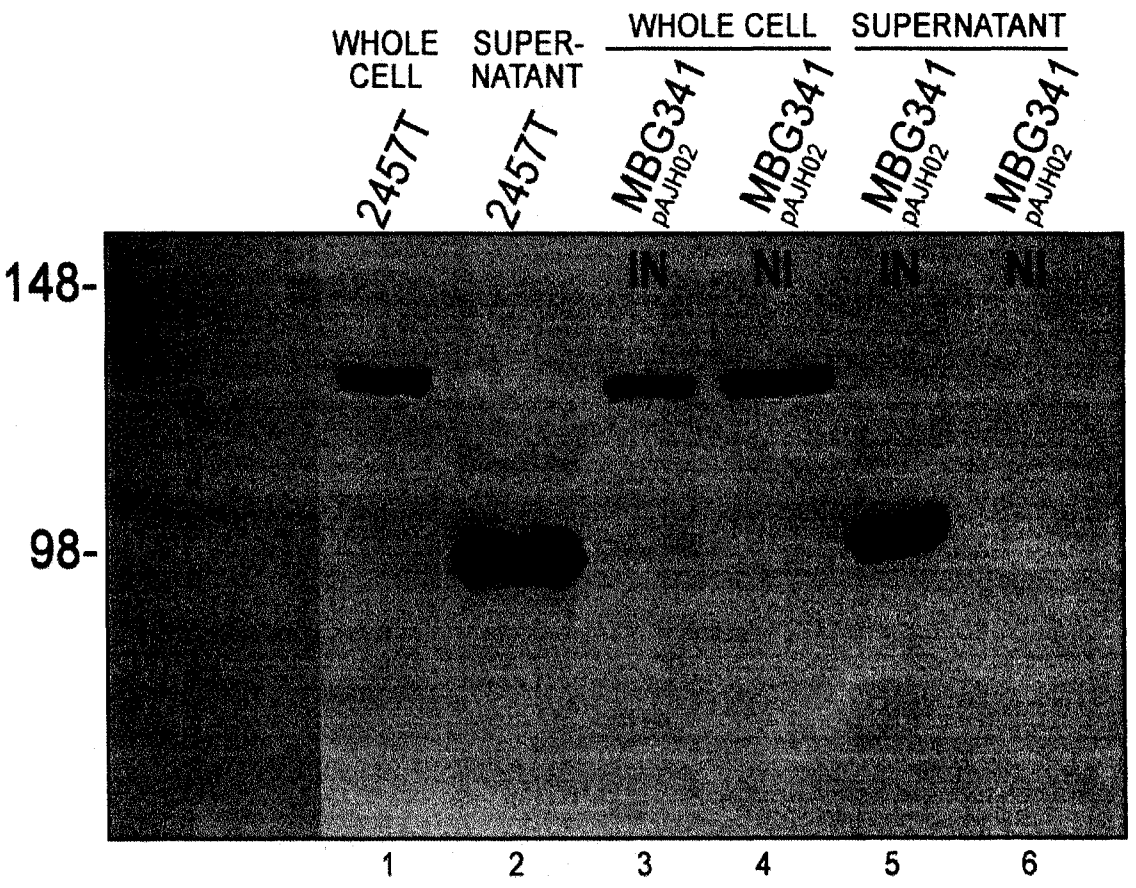

Figure 3.9: IcsP, expressed from pAJH02, cleaves IcsA in MBG341, similar to wild-type *Shigella*.

pAJH02 (pBAD33-*icsP)* was introduced into *Shigella*: MBG341 (2457T-Δ*icsP*) and induced with 0.2% (w/v) L-arabinose for one hour at 37° C. Samples were normalized to total and supernatant protein, separated by SDS-PAGE, and transferred to a PVDF membrane. IcsA was detected by immunoblotting with an anti-IcsA antibody.

supernatant protein extracts of MBG341, carrying pAJH02 (induced), a ~95 kDa fragment was present (Figure 3.9, lane 5), but nothing was detectable in samples collected from the non-induced cells (figure 3.9, lane 6). This showed IcsA is cleaved by IcsP and is released into the supernatant. This was similar to what was observed in wild-type *Shigella* expressing *icsP* (Figure 3.9, lanes 1 and 2). Overall, the inducible-plasmid system reflected similar banding patterns seen in wild-type *Shigella* cells expressing native IcsP.

3.3.7 The cellular environment does not affect the proteolytic specificity of PgtE

The next question we sought to address was: Does the LPS environment surrounding the omptin influence its activity or site specificity? To address this, pEHK19 (pBAD33-*pgtE*) was introduced in an *icsP Shigella* mutant, MBG341 (2457T-Δ*icsP*) and *pgtE* was expressed using conditions described above for *icsP*. In whole cell protein extracts, IcsA was not detected in cells expressing *pgtE* (Figure 3.10, lanes 1 and 2), whereas IcsA was present as a ~125 kDa band in cells not expressing *pgtE* (non-induced pEHK19), cells containing the empty expression vector, either induced or non-induced (Figure 3.10, lanes 3, 4, and 5). In supernatant protein extracts of cells expressing multi-copy *pgtE*, two fragments of IcsA (~85 kDa and ~95 kDa) were detected in cells expressing *pgtE* (Figure 3.10, lanes 6 and 7), but IcsA was not detected in supernatant proteins of cells not expressing multi-copy *pgtE* (Figure 3.10, lanes 8, 9, and 10). When IcsA fragments from *Shigella* cells expressing PgtE (Figure 3.10) were compared to those detected in *Salmonella* cells expressing PgtE (Figure 3.8), IcsA was found to be cleaved in a similar manner. Collectively, these data suggest that the cellular background does not affect the proteolytic specificity of PgtE. It is also possible that there is no difference

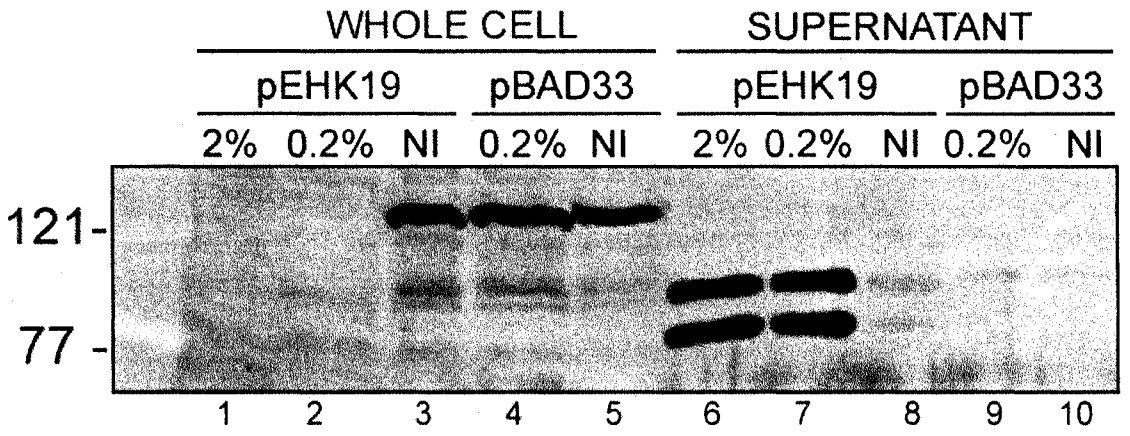

Figure 3.10: PgtE cleaves IcsA in *Shigella* cells and results in two IcsA fragments. pEHK19 (pBAD33-*pgtE*) and pBAD33 (empty vector) were induced and non-induced for one hour at 37°C with 2% (w/v) or 0.2% (w/v) L-arabinose in a *Shigella* background, MBG341 (2457T-Δ*icsP*). Samples were normalized to total protein and supernatant protein, separated by SDS-PAGE, and transferred to a PVDF membrane. IcsA was detected by immunoblotting with an anti-IcsA antibody. IN, induced; NI, non-induced. Two bands, a ~95 kDa and ~85 kDa, are present in the supernatant from MBG341. No IcsA was detected in the supernatant of *Shigella* containing the empty vector.

in the LPS of *Salmonella* and *Shigella* with the growth conditions used. Moreover, these results suggest that the observation that the omptins cleave IcsA differently is likely caused by the amino acid differences present in the surface loops of the active site and/or the LPS binding motif.

3.3.8 Omptin family conclusions

Although the catalytic amino acids are completely conserved among IcsP, OmpT, and PgtE, it is clear that these proteases cleave IcsA differently, as judged by western blot analysis. These differences seen in IcsA-cleavage can be attributed to the differences in amino acid residues within the surface loops of the active site and in the LPS binding motif. When comparing amino acid residues in the surface loops of IcsP, OmpT, and PgtE, a difference is found at residue 223 (comparison of surface loops in 3D homology models in Figures 3.1 and 3.11). In OmpT, serine, an uncharged polar amino acid, is found at residue 223, and the corresponding amino acids in IcsP and PgtE are aspartic acid, a negatively charged polar amino acid and glutamic acid, a negatively charged polar amino acid, respectively. The inherent amino acid differences at this residue in the surface loops may contribute to differences observed in the proteolytic specificity among these omptins.

The activity of omptin proteases has been found to be dependent on the presence of LPS (Kramer *et al.*, 2000). Kramer *et al.* (2002) examined the affect of LPS on the enzymatic activity of OmpT. Results showed that the refolding of OmpT into its active form required the addition of LPS. Consequently, any amino acid modification within the LPS binding motif may alter the specificity and activity of IcsP, OmpT, and PgtE. When comparing the LPS binding region of IcsP to OmpT, one amino acid difference is

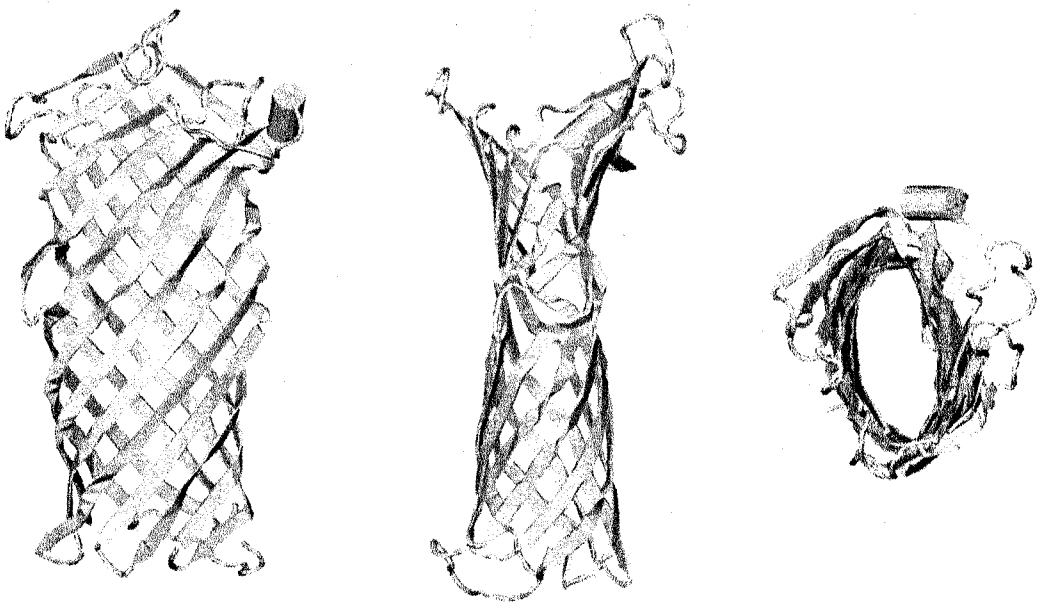

Figure 3.11: IcsP homology model to OmpT.
Predicted 3D structure of IcsP. The IcsP homology model was created using MEME/MAST software by Christian Ross.

found at.residue 175. In OmpT, arginine, a positively charged amino acid is present in the LPS binding region and the corresponding amino acid in IcsP is leucine, a nonpolar amino acid. This amino acid change may affect the proteins interaction with the LPS and hence its structure, thereby altering the proteolytic specificity of IcsP with respect to OmpT.

Additionally, the *Salmonella* omptin, PgtE, requires LPS to be proteolytically active (Kukkonen *et al.*, 2004). Previous work has shown that substitution of Arg_{138} and Arg_{171} of the LPS protein binding motif abolished proteolytic activity, suggesting that PgtE requires LPS for enzymatic activity (Kukkonen *et al.*, 2004). When comparing the LPS binding region of PgtE to IcsP, there are two amino acid differences. In IcsP, an amino acid, leucine, a nonpolar amino acid is present and the corresponding amino acid found in PgtE is arginine, a positively charged polar amino acid. The other amino acid difference is lysine, a positively charged polar amino acid in IcsP to glutamic acid, a negatively charged polar amino acid in PgtE. Consequently, these amino acid differences may alter the conformation and placement of IcsP and PgtE within the LPS, thereby affecting their proteolytic specificity for IcsA.

3.3.9 PgtE promotes resistance to cationic antimicrobial peptide, LL-37 in Salmonella and Shigella

It has been shown that some of the omptins specifically cleave between paired basic residues and after a basic residue that is followed by a nonpolar amino acid (Sugimura *et al.*, 1988). Consequently, cationic antimicrobial peptides are potential targets for PgtE and IcsP. Moreover, previous work has shown that PgtE promotes resistance to antimicrobial peptides, C18G and LL-37 in *Salmonella* (Guina *et al.*, 2000).

To determine whether IcsP promotes resistance to cationic antimicrobial peptide LL-37, minimum inhibitory concentration (MIC) assays were conducted. To determine whether our MIC assays worked properly, resistance of wild-type *Salmonella* (CS022), compared to a *Salmonella pgtE* mutant (TG61) was tested initially to determine whether PgtE promotes resistance to LL-37 in *Salmonella*, as shown previously (Guina *et al.*, 2000). Our results show that in the *pgtE* mutant strain (TG61) there was increased sensitivity to LL-37, 10 $\mu g\ ml^{-1}$ when compared to wild-type *Salmonella* in which PgtE appeared to promote resistance to LL-37, 80 $\mu g\ ml^{-1}$ (Table 3.6).

3.3.10 IcsP does not promote resistance to cationic antimicrobial peptide, LL-37 in Shigella

We next examined whether IcsP, the outer membrane protease of *Shigella,* could promote resistance to LL-37. MICs of wild-type *Shigella* (2457T) and a *Shigella icsP* mutant (MBG341) were compared. The *Shigella icsP* mutant did not show increased sensitivity to LL-37, 30 $\mu g\ ml^{-1}$ when compared to wild-type *Shigella*, 30 $\mu g\ \mu l^{-1}$, suggesting that IcsP does not protect *Shigella* from LL-37 (Table 3.6).

Previous studies have shown that the O-side chain of smooth lipopolysaccharide may sterically inhibit the activity of omptins. An example of steric inhibition by the O-antigen, affecting the proteolytic activity, has been shown in Pla and PgtE (Kukkonen *et al.*, 2004; Pouillot *et al.*, 2005). Additionally, O-antigen modulation in reduction and mutations in genes encoding for the biosynthesis of the O-antigen pathway have been found in *Salmonella* and *Yersinia*, respectively. It is possible that the long O-antigen side chains of LPS may hinder the proteolytic activity of IcsP. Moreover, the lengths of the O-antigen side chains of *Shigella* in an *in vivo* and *in vitro* system are not well-

Table 3.6. MICs of cationic antimicrobial peptide, LL-37

Strain	Genotype	MIC (μg/ml) LL-37
Salmonella		
CS022	wild-type *Salmonella*	80
TG61	CS022-Δ*pgtE*	10
TG61 pBAD33	CS022-Δ*pgtE*, parental expression vector *Induced*	80
TG61 pBAD33	CS022-ΔpgtE, parental expression vector *Non-induced*	80
TG61 pAJH02	CS022-Δ*pgtE*, pBAD33-*icsP* *Induced*	80
TG61 pAJH02	CS022-ΔpgtE, pBAD33-icsP *Non-induced*	80
Shigella		
2457T	wild-type	30
MBG341	2457T-Δ*icsP*	30
TMS02	2457T-Δ*wzy*	10
EHK01	2457T-Δ*wzy*-Δ*icsP*	10
EHK01 pAJH02	2457T-Δ*wzy*-Δ*icsP*, pBAD33-*icsP* *Induced*	80
EHK01 pAJH02	2457T-Δ*wzy*-Δ*icsP*, pBAD33-*icsP* *Non-Induced*	80
MBG341 pBAD33	2457T-Δ*icsP*, parental expression vector *Induced*	160
MBG341 pBAD33	2457T-Δ*icsP*, parental expression vector *Non-Induced*	160
MBG341 pEHK19	2457T-Δ*icsP*, pBAD33-*pgtE* *Induced*	320
MBG341 pEHK19	2457T-Δ*icsP*, pBAD33-*pgtE* *Non-Induced*	160

characterized. Consequently, we created *Shigella* O-antigen polymerase (*wzy*) mutants, which produce only one O-antigen side chain, to determine whether IcsP could promote resistance to LL-37 in a *Shigella* background.

When comparing MICs of a *Shigella wzy* mutant (TMS02) to a *Shigella wzy, icsP* mutant (EHK01) in the antimicrobial resistance assay, EHK01 did not show increased sensitivity to LL-37, 10 μg ml^{-1} (Table 3.6) when compared to TMS02, 10 μg μl^{-1}. To further examine the role of IcsP in resistance to LL-37, *icsP* was introduced in EHK01 on an L-arabinose inducible plasmid (pAJH02). MICs of cells expressing *icsP* and cells not expressing *icsP* in EHK01 were compared. Again, the MICs of *icsP*-expressing and *icsP*-nonexpressing *Shigella* were the same. Take together, these data suggest that IcsP does not promote resistance to α-cationic antimicrobial peptide, LL-37.

We next examined whether the cellular background was affecting the proteolytic activity of IcsP by conducting omptin-switching experiments. PgtE was introduced in a *Shigella* background and IcsP was introduced in a *Salmonella* background to determine whether each promoted resistance to LL-37 in a non-native background. *pgtE* (pEHK19) was expressed in a *Shigella icsP* mutant (MBG341) and compared to a non-expressing *pgtE Shigella icsP* mutant. PgtE protected *Shigella* (MBG341) from the bactericidal effects of LL-37 2-fold higher (320 μg ml^{-1}) than a *Shigella icsP* mutant not expressing PgtE (160 μg ml^{-1}) (Table 3.6). However, IcsP did not protect *Salmonella* from the bactericidal effects of LL-37. *icsP* (pAJH02) was expressed in a *Salmonella pgtE* mutant (TG61) and compared to a non-expressing *icsP Salmonella* mutant. IcsP did not promote resistance to LL-37 in a *Salmonella* background, 80 μg μl^{-1}. These data suggest that the resistance to LL-37 is affected by the inherent differences between omptins rather than

the LPS environment influencing the activity of the protein. This is most likely a result of amino acids differences present in the surface loops, or it is possible that differences in LPS binding motif may influence the activity of the omptins.

3.3.11 RyhB does not affect IcsP expression levels or icsP promoter activity

Previous work has shown that RyhB represses the expression of several genes within the *S. dysenteriae* VirB regulon (Figure 3.6 B) (Murphy *et al.*, 2007). Additionally, VirB, a transcriptional activator, has been shown to regulate the expression of *icsP* (Wing *et al.*, 2004). Consequently, we wanted to determine whether RyhB regulated the expression of IcsP. *ryhB* was expressed in *Shigella* strains lacking *virB* and IcsP protein levels and transcriptional activity from the *icsP* promoter were measured. IcsP levels were compared in *Shigella* wild-type (2457T) and *virB* mutant strains (AWY3) each expressing *ryhB* from an IPTG inducible plasmid (pryhB), or carrying the vector control (pQE2) (Figure 3.12, top). Additionally, IcsP levels were compared in *Shigella* wild-type (2457T) and *virB* mutant strains (AWY3), each containing *icsP* (pHJW6) and expressing *ryhB* from an IPTG inducible plasmid (pryhB), or carrying the vector control (pQE2). *icsP* was introduced on a low-copy plasmid in *Shigella* cells expressing *ryhB* because differences in IcsP protein levels may not be detected with native IcsP protein levels. Overall, there was no detectable difference in IcsP protein levels in *Shigella* expressing *ryhB* from uninduced *Shigella* strains and *ryhB*-expressing *Shigella* carrying the vector control (Figure 3.12, bottom). IcsP was not detected in the *Shigella icsP* mutants (AWY3) as predicted because of the absence of *virB*, a known transcriptional activator of *icsP*. Taken together, these data suggest that RyhB does not decrease the protein levels of IcsP.

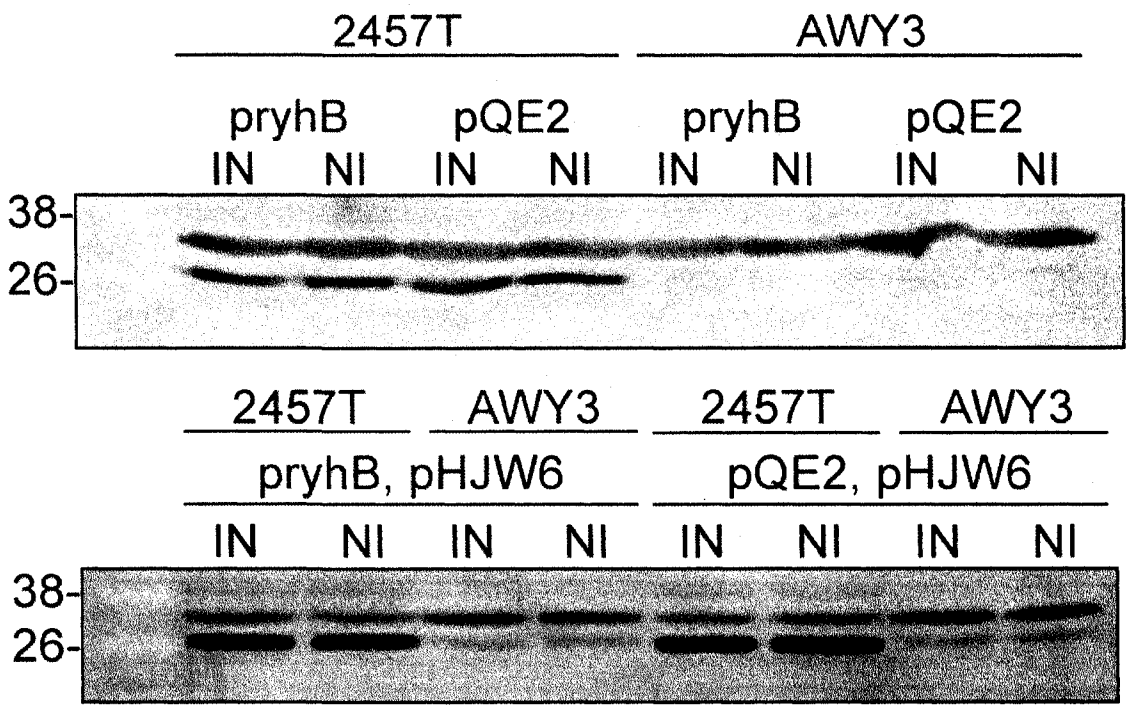

Figure 3.12: RyhB does not decrease IcsP expression levels.
pryhB, pQE2 , pryhB and pHJW6, or pryhB and pQE2 were introduced into *Shigella*: 2457T (wild-type) and AWY3 (2457T-Δ*virB*). Samples were normalized to total protein, separated by SDS-PAGE, and transferred to a PVDF membrane. IcsP was detected by immunoblotting with an anti-IcsP antibody. IN, induced with 200 μM IPTG for 1 hour at 37°C; NI, not induced.

To examine the effect of RyhB on *icsP* transcription, we measured β-galactosidase production from an *icsP-lacZ* transcriptional reporter (pHJW20) in wild-type *Shigella* (2457T) and a *Shigella virB* mutant (AWY3). Transcription from the *icsP* promoter was measured in wild-type and *virB* mutant *Shigella* strains, each carrying pryhB or the vector control, pQE2. When *ryhB* was induced in each strain, transcription from the *icsP* promoter was not significantly different from the non-induced *ryhB* strains (Table 3.7). These data suggest that RyhB does not decrease *icsP* transcription.

Although RyhB has been shown to suppress many genes encoded on the VirB regulon, the molecular mechanism of RyhB-dependent repression of *virB* is not entirely clear. Murphy *et al.* (2007) did not find any significant complementarity between RyhB and *virB* mRNA, but suggested that that limited complementarity between these RNA molecules may be sufficient to mediate repression. Because of the limited complementarity, it is possible that VirB is expressed at low levels and that VirB has a higher affinity for *icsP* than the affinity of RyhB for *virB*. If the affinity of VirB for *icsP* is higher, then a difference in IcsP protein levels may not be detected.

3.4 Conclusions

This chapter addresses experiments that focus on three objectives. The first objective was to further enhance our knowledge of members of the omptin family. To characterize members of the omptin family, we examined whether three omptins, IcsP, OmpT, and PgtE recognized and cleaved a substrate, IcsA, in a similar manner. These experiments identified whether the cellular background or the amino acid differences present in the surface loops and/or LPS binding motif affected the proteolytic activity of

Table 3.7: Activation of *icsP-lacZ* transcriptional fusions by RyhB

Strain	Genotype[a]	β-Galactosidase expression (Miller units)		Fold activation (mean ± SD)
		Uninduced	Induced	
2457T pryhB	wild-type *ryhB*	5,467	5,846*	NA
2457T pQE2	wild type vector control	4,999	6,210*	NA
AWY3 pryhB	2457T-Δ*virB ryhB*	402	409*	NA
AWY3 pQE2	2457T-Δ*virB* vector control	395	514*	NA

[a] All strains carried pHJW20 (*icsP-lacZ)*
* There was no significant difference between uninduced and induced

the omptins. The second objective was to determine whether the *Shigella* outer membrane protease, IcsP could promote resistance to cationic antimicrobial peptides. Finally, the third objective was to determine whether IcsP was regulated in a RyhB-dependent manner.

Our results revealed that IcsP of *Shigella*, OmpT of *E. coli*, and PgtE of *Salmonella* all cleave a substrate, IcsA, differently, as judged by western blot analysis. These results raised the question of whether the differences seen in IcsA cleavage were a result of the differences in the amino acids present in the surface loops of the active site or the cellular background in which these omptins reside. The omptin switching experiment addressed this question and revealed that the cleavage specificity of PgtE for IcsA remained the same in both a *Salmonella* and *Shigella* background. These results suggest that the cellular background is not influencing the differences observed in the cleavage of IcsA, but the amino acid differences present in the extracellular loops of the proteins were responsible for the differences in the proteolytic cleavage of IcsA. It is also possible that the LPS of *Salmonella* and *Shigella* are not significantly different and that LPS may have an effect but cannot be detected. Although there are only one to two differences in the surface loops of the active site (Table 3.2, Figure 3.1) and LPS binding motif among the omptins found in *E. coli*, *Salmonella*, and *Shigella*, this work has shown that these slight differences can significantly affect proteolytic specificities of a specific substrate (Figures 3.5 - 3.8). Additionally, the amino acid differences within the LPS binding motif may alter the conformation and placement of the protein in the outer membrane, which can also affect the proteolytic specificity of these proteases. From our results, we can conclude that the different cleavage specificities of each protease is likely

from the amino acid differences instead of the cellular background (Figures 3.8 and 3.10). It would be interesting to determine whether, in fact, the differences in IcsA cleavage are a result of the amino acid differences by substituting key amino acids in the surface loops and the LPS binding motif to make these omptins more similar to each other.

As previously reported, the only known function of IcsP is cleavage and maintenance of IcsA at the bacterial pole. Because of the high sequence identity and the 100% conserved catalytic site with the other proteases of the omptin family, it was suspected that IcsP might serve an additional role in *Shigella* pathogenesis. Consequently, the second objective was to determine whether IcsP could promote resistance to cationic antimicrobial peptides (CAMPs). One member of the omptin family, PgtE has been shown to promote resistance to α-cationic antimicrobial peptides by cleaving CAMPs into smaller fragments (Guina *et al.*, 2000). Cleavage of CAMPs into smaller fragments prevents the destabilization of the bacterial membrane. MICs of 2457T (wild-type *Shigella*) were compared to MBG341 (2457T-Δ*icsP*). In these strains examined, no differences in MICs were observed. Our results showed that IcsP did not promote resistance to LL-37 in *Shigella*. These results raised the question of whether the cellular background was affecting the proteolytic activity of IcsP. Studies have shown that LPS length can sterically inhibit the proteolytic activity of outer membrane proteases (Kukkonen *et al.*, 2004). Consequently, we addressed this issue in two ways: using *Shigella* mutants that created only one O-antigen side chain (*wzy*) and conducting omptin switching experiments. MICs of EHK01 (2457T-Δ*icsP* Δ*wzy*) expressing *icsP* (pAJH02) were compared to EHK01 not expressing *icsP*. There was no difference in MICS observed. We also addressed this question by switching IcsP and PgtE from a *Shigella*

into a *Salmonella* background. IcsP did not promote resistance in a *Salmonella* background. However, PgtE did promote resistance to LL-37 in a *Shigella* background. Our studies suggest that IcsP does not recognize LL-37 as a substrate and that this may be caused by the differences in the amino acids of the surface loops of the active site and/or the LPS binding region from PgtE. Only one antimicrobial peptide was used in this study. Previous work has shown that OmpT promotes resistance to protamine in *E. coli* (Stumpe *et al.*, 1998). It would be interesting to examine whether IcsP can promote resistance to other antimicrobial peptides such as protamine.

RyhB has been shown to repress several genes within the *S. dysenteriae* VirB regulon. In *S. flexneri,* VirB derepresses *icsP*. Consequently, we hypothesized that increased levels of RyhB would result in decreased protein levels of VirB and ultimately decreased protein levels and transcriptional activity of *icsP*. However, when comparing IcsP protein levels in RyhB expressing cells to uninduced cells, IcsP protein levels were the same. Furthermore, when determining the effect of RyhB on *icsP* transcription, there was no difference in *icsP* promoter activity in RyhB expressing cells from uninduced cells. Although RyhB has been shown to suppress many genes encoded on the VirB regulon, the exact mechanism of RyhB-dependent repression has not been elucidated. It would be interesting to examine whether *icsP* in an *S. dysenteriae* background is affected by RyhB-repression. It is also possible that the affinity of VirB for *icsP* is higher than the affinity of RyhB for *virB.* This could reflect the results seen in the western blot analysis and β-galactosidase assays.

Taken together these data shed light on members of the omptin family and allow us to better understand the specificity of a protease and the interaction of an outer

membrane protein with its environment. Moreover, this work has focused specifically on a *Shigella* virulence plasmid-encoded protease, IcsP. It is important to highlight that the only ascribed function of IcsP is to cleave IcsA However, due to high similarity among other omptins, we hypothesize that there are additional roles and regulation of this key virulence determinant in *Shigella.* Whether there are additional roles, are yet to be elucidated. These studies expand our knowledge of the roles of IcsP in pathogenesis and protein regulation. Ultimately, gaining knowledge of this significant key virulence factor better enables us to understand why this pathogen has acquired this protease and what function it has in the pathogenesis of *Shigella.*

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CHAPTER 4

CONCLUSIONS

Shigella spp. pose a major health problem worldwide warranted by the damaging dysentery that the bacteria may cause in humans. Research in the past 30 years has contributed to our understanding of shigellosis. This study further contributes to our knowledge of the molecular biology of these important pathogens. A thorough understanding of the molecular mechanisms underlying *Shigella* biology and pathogenicity is essential for the design of protective and prevention strategies, particularly ideal vaccine development. This section highlights the four main objectives addressed in this thesis and concludes with the overall significance and relevance of our findings.

4.1 Conserved mechanism of protein targeting in γ-proteobacteria

Accurate localization of proteins to specific spatial sites within bacterial cells is essential for many biological functions in bacteria. However, the mechanisms by which these proteins localize to their specific addresses are not completely understood. To acquire a better understanding of this process, we sought to determine whether the mechanism of polar targeting found in *Shigella* is conserved in three closely related families in the γ-proteobacteria. We have concluded that the elusive mechanism of protein targeting used by this protein is conserved in the γ-proteobacterial species

investigated in this study (discussed in chapter 2). This study provides insight on this process and can essentially influence the development in prevention strategies against bacterial infections and disease.

4.2 Characterization of the omptin family

Our second aim in this study was to further characterize omptin proteins found in three pathogenic organisms: *Shigella* (IcsP), *E. coli* (OmpT), and *Salmonella* (PgtE). It is not entirely clear how the LPS environment affects the activity of outer membrane proteases. Experiments described in chapter 3 of this thesis address whether the LPS environment and/or the inherent amino acid differences in the surface loops and LPS binding motif affect the cleavage specificity of three omptin proteases for a specific substrate. Understanding the activity and interaction between the omptin protease and their LPS environment allows us to dissect the interplay of these proteases with their environment. Although our results showed that the LPS environment might not affect the proteolytic activity of these proteases, further work is needed to clarify the complex relationship outer membrane proteases have with their cellular environment. The comparative approach described here using IcsP, OmpT, and PgtE has provided more clues about the proteolytic mechanism of these proteases. Ultimately these data could be used as a starting point of obtaining omptin-inhibiting drugs, and thus preventing severe infections and disease.

4.3 The roles and regulation of IcsP in *Shigella flexneri*

Our third aim in this study was to determine whether IcsP, an outer membrane protease, plays an additional role in the pathogenesis of *S. flexneri* by promoting resistance to cationic antimicrobial peptides. To date, the only known function for IcsP is cleavage of IcsA. However, the significance of IcsP in *Shigella* pathogenesis is not completely understood. Since it has been shown that the *Salmonella* homolog to IcsP, PgtE promotes resistance to cationic antimicrobial peptides, we hypothesized that IcsP may play a similar role in the pathogenesis of *Shigella*. Our results show that IcsP, at least under the conditions tested, does not promote resistance to a cationic antimicrobial peptide, LL-37 (discussed in chapter 3). However, its homolog, PgtE of *Salmonella*, does. Currently, it remains unclear whether IcsP promotes resistance to other antimicrobial peptides such as protamine. Moreover, there are additional roles such as cleavage of C3 of complement system and adhesion to human epithelial cells, for IcsP that have yet to be investigated.

The final aim of this study was to determine whether RyhB regulates the expression of *icsP*. RyhB is a regulatory RNA that suppresses a key virulence transcriptional activator, VirB. It has been suggested that the role of RyhB may be to coordinate and fine-tune the expression of *S. dysenteriae* virulence genes with the local concentration of iron (Murphy *et al.*, 2007). Since VirB is required for maximal expression of *icsP*, we reasoned that RyhB may regulate *icsP*. To test this we examined *icsP* expression in the presence of multi-copy RyhB. Our results suggested that RyhB does not down-regulate the expression of *icsP* in *S. flexneri* (discussed in chapter 3).

However, further studies are needed to better understand whether iron and/or RyhB are involved in fine-tuning the expression of virulence genes in *S. flexneri*.

Ultimately, this work has attempted to improve our understanding of how IcsP functions. Moreover, IcsP can be a suitable target for antimicrobial drugs and vaccine development. Ultimately, the applied relevance of this study has potential use for the development of prevention and intervention strategies against shigellosis.

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