Secretion Regulation of Invasion Plasmid Antigens C and D (IpaC and IpaD) of *Shigella flexneri*

by

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ABSTRACT

Shigella flexneri is an intracellular bacterial pathogen that invades the colonic mucosa of mammalian intestinal cells and causes bacillary dysentery or shigellosis. The effector proteins of *S. flexneri* are the invasion plasmid antigens (Ipa), which are secreted via the type III secretion system (TTSS) upon host-cell contact. IpaC serves as an essential effector molecule for epithelial cell invasion by forming a complex with IpaB to form a pore in the host cell, through which other effectors are secreted into the host epithelial cell. The secretion of IpaC also regulates uptake of the bacterium, by inducing actin rearrangement of the epithelial cells. Data obtained previously in the Picking lab indicated new possibilities concerning the secretion signal of IpaC. First, it was thought that the amino acids comprising the secretion signal of IpaC protein are contained at the N-terminus of the protein; secondly, it was thought that IpaC protein contained two separate secretion signals. This study examines these two hypotheses to define the secretion signal as well as in-depth analysis of the identified secretion signal to provide the first analysis of the IpaC secretion signal.

Another effector protein, IpaD, is involved in controlling secretion of all effectors delivered by the *Shigella* type-III secretion system. IpaD has been proposed to form a complex with IpaB within the type-III secretion apparatus forming a plug, thereby preventing the secretion of other effectors prior to host-cell contact. Deletion of IpaD not only results in hypersecretion of the other effector proteins but also results in a noninvasive phenotype. Therefore, it is widely thought among *Shigella*

researchers that IpaD has one role in *Shigella* invasion. It is believed that for invasion to occur, a functional IpaD must be produced to control secretion of the effector proteins, such as IpaB and IpaC. The work generated in this study examines the functional domains of IpaD, as well as studying IpaD deletion mutants and their effects on IpaB and IpaC hypersecretion. A functional comparison is also made between IpaD and its *Salmonella* homologue, *Salmonella* invasion protein D (SipD). The work presented here will provide the first functional analysis of IpaD as well as providing insight to the putative role of IpaD during *Shigella* invasion.

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TABLE OF CONTENTS

LIST OF FIGURES AND TABLES

Page

HISTORICAL REVIEW

Discovery, Description, and Epidemiology

Until 1887, when Chantemesse and Widal isolated a bacterium that caused bacillary dysentery (Kohler 2000), it was believed that only amoebae could cause dysentery. However, much credit is given to Kiyoshi Shiga for identifying *Shigella* as a bacterial dysentery-causing agent, instead of Chantemesse and Widal (Trofa 1999) (Kohler 2000). Shiga isolated a dysentery-causing bacterium in 1897 from a "skeiri" or a red diarrhea outbreak (Trofa 1999) and provided a more complete description of the organism. He found that the isolate fermented dextrose and gave a negative indole reaction and named the bacterium *Bacillus dysenteriae* (Trofa 1999) (Kohler 2000). Additionally, he used animal models to show that an isolated bacterium from a stool sample could cause dysentery when ingested (Kohler 2000) (Trofa 1999). Several researchers described similar organisms causing bacillary dysentery. Walther Kruse reported isolating the same organism from a dysentery outbreak in Westphalia and Simon Flexnor reported isolating a similar organism from an outbreak in Manila (Kohler 2000). The bacteria isolated by both Kruse and Flexnor were sent to Shiga for analysis and he determined that Kruse and Flexnor had isolated the same organism, but was slightly different from the bacterium originally identified by Shiga (Kohler 2000). More recently *B. dysenteriae* has been reclassified as *Shigella dysenteriae* by the Congress of the International Association of Microbiologists *Shigella* Commission, and has been determined to be a nonmotile,

facultative anaerobe yielding a positive catalase reaction and a negative oxidase reaction (Krieg 1984) (Kohler 2000) (Hale 1991). The genus *Shigella* now consists of four species, which are *S. dysenteriae*, *S. flexneri*, *S. boydii*, and *S. sonnei*, (groups A, B, C, and D, respectively) (Hale 1991), (Trofa 1999) (Krieg 1984).

It was shown in the 1960s that *Shigella* could infect the epithelium of guinea pigs and possessed the ability to grow intracellularly in mammalian cells (Labrec 1964). Until 1964, it was assumed that *Shigella* infection was due to the release of an endotoxin, which Shiga had noted during his investigation of the bacterium (Trofa 1999) (Hale 1991). However, it was later determined that the disease actually occurs due to the penetration of *Shigella* into the intestinal epithelium (Labrec 1964). As further research was conducted to determine the genetic basis of virulence and the mechanism of pathogenesis, one group demonstrated that a number of genes were required for *Shigella* virulence (Sansonetti 1982) (Sansonetti 1981). Sansonetti and his team of researchers showed that these genes required for *Shigella* virulence are all contained on a plasmid that can be transferred to other gram-negative bacteria (Sansonetti 1983) (Hale 1991).

Shigella infection can result from ingestion of as few as 10 to 100 organisms in healthy adults (DuPont 1989), and is often characterized as diarrhea containing mucus and blood (DuPont 1969) (Hale 1991). Members of the genus *Shigella* routinely infect humans and other primates, although a few cases of infected canines have been reported (Krieg 1984) (Hueck 1998). Even though shigellosis is not considered a major public health problem in the United States, *Shigella* species are a

major world-wide public health problem, causing an estimated one million deaths annually (Kotloff 1999) (Hueck 1998) (Venkatesan 2001). Shigellosis is usually self-limiting and requires antibiotic treatment and rehydration therapy. However, as with many other bacterial infections, antibiotic resistance is becoming a rising problem in the treatment of shigellosis (Krieg 1984) (Hueck 1998). *S. sonnei* strains have come to possess multiple resistances as reported from Japan in 1959, England in 1973, and the United States in 1975 (Krieg 1984). A separate study performed in 1981 confirmed that 50% of *Shigella* strains from subgroups A, B, and C were resistant to 3 or more antibiotics (Krieg 1984) (Hueck 1998).

Pathogenesis of *Shigella flexneri*

The pathogenesis of *Shigella* is initiated following ingestion of the organism via contaminated water or food (DuPont 1989). Once the bacteria have passed the acidic environment of the stomach and have reached the colonic mucosa, it is believed that they gain access to the basolateral surface of the intestinal epithelium by transcytosis through M cells (Wassef 1989). M cells are specialized epithelial cells that do not have mucus or glycocalyx on their apical surface and primarily function to actively sample the contents of the gut (Owen 1986). Pathogenic and nonpathogenic *Shigella* were previously shown to undergo phagocytosis by M cells, which inititated an immune response in rabbit ileal loop models (Wassef 1989). Antigenic samples acquired by the M cells are transcytosed to the basal side of the epithelial layer and exposed to the lymphoid tissues of the Peyers patches (Owen

1986) (Sansonetti 1999) (Hueck 1998). Once the bacteria are released on the basolateral side of the M cell, they are usually phagocytosed by resident macrophages (Thirumalai 1997). *Shigella* can induce apoptosis in the attacking macrophage (Zychlinsky 1992), which stimulates the release of IL-1β (Thirumalai 1997) and eventually results in the recruitment of polymorphonuclear leukocytes (PMNs) to the region (Wassef 1989) (Islam 1997). Once freed from the dying macrophage, *Shigella* can contact and invade the mucosal epithelial cells at their basolateral surface (Mounier 1992). The bacteria induce their own uptake by host epithelial cells (Mounier 1992), and actin polymerization and mysin accumulation is initiated at the site of entry (Clerc 1987). Replication of the bacterium will then occur in the cytoplasm of the host cell (Sansonetti 1986). Pathogen-directed actin-based motility then aids in cell-to-cell spread of infection (Takeuchi 1968). A large amount of the tissue destruction mostly likely occurs due to PMN-mediated inflammation (Wassef 1989) (Islam 1997).

Virulence Factors

The virulence factors of *S. flexneri* are encoded on a 230-kb virulence plasmid, which was discovered by Sansonetti and coworkers in the 1980s (Sansonetti 1981) (Sansonetti 1982) (Baudry 1988). Sansonetti demonstrated that transferring the entire *Shigella* chromosome to *E. coli* K12 did not result in an invasive phenotype for the latter (Sansonetti 1983). In contrast, an invasive *E. coli* phenotype could be achieved if only the virulence plasmid was transferred from *Shigella* to *E. coli* K12

(Sansonetti 1983) (Hale 1991). The *Shigella* virulence plasmid genes are temperature-regulated with synthesis of the plasmid-encoded proteins occurring at 37°C, but not when the organism is grown at 30°C (Hale 1985) (Hueck 1998).

Type-III Secretion System

Two operons contained on the *Shigella* virulence plasmid that are essential for the pathogenesis of shigellosis are the *mxi-spa* loci (Allaoui 1993) (Saskawa 1993) (Venkatesan 1992). These loci contain the genes necessary for the generation of a *Shigella* type-III secretion system (TTSS) (Hueck 1998), which allows insertion of virulence proteins into the host cell membrane and cytoplasm (Blocker 1999) (Tran Van Nhieu 2000). The TTSS is only one of four pathways characterized in gram-negative bacteria for the active transport of effector proteins from the cytoplasm to the surface of the bacterium or to the extracellular environment (reviewed by Hueck 1998). The secretion systems are numbered I-IV, where type-II and type-IV systems secrete proteins in conjunction with a *sec*-dependent pathway and type-I and type-III utilize a *sec*-independent pathway. In *sec*-dependent secretion pathways, a 30 amino acid hydrophobic N-terminal signal sequence allows passage of each protein to be exported across the cytoplasmic membrane and into the periplasm. Once the protein has been secreted into the periplasm, the N-terminal signal sequence is removed by a periplasmic signal peptidase. Besides the absence of N-terminal processing of the secreted proteins in *sec*-independent pathways, the secretion of effectors occurs in a continuous manner without the accumulation of periplasmic intermediates (Hueck 1998).

Many diverse gram-negative pathogens of plants and animals utilize the *sec-independent* TTSS, including *Salmonella typhimurium, Pseudomonas aeruginosa, Chlamydia* species, enteropathogenic *Escherichia coli* (EPEC), *Yersinia* species, and *Xanthomonas campestris* (Hueck 1998). The type-III secretion system apparatus is composed of approximately 20 proteins, nine of which are conserved among the previously mentioned organisms utilizing the TTSS (Bahrani 1997). The TTSS apparatus also shares sequence similarities with flagellar basal body components (Kubori 1998). Eight out of the nine proteins that are conserved between species utilizing the TTSS are homologous to the flagellar hook-basal body proteins (Young 1999) (Kubori 1998). The TTSS, like the flagellar hook-basal body, is composed of three major structures: 1) a large proximal bulb that resides in the cytoplasm of the bacterium; 2) a short cylinder that extends through both bacterial membranes and the peptidoglycan of the bacterium; and 3) an external needle (as opposed to a hook) (Kubori 1998) (Blocker 1999). The 'needle complex,' first isolated and identified by Kubori and coworkers (Kubori 1998) in *Salmonella*, is composed of the 7-8 nm wide external needle and short cylinder that is thought to extend through both bacterial membranes and the peptidoglycan. More recently, Tamano *et al.* (Tamano 2000) have provided evidence that the needle complex is assembled much like the hook of the flagellum. Furthermore, they have indicated that the needle is required for secretion of effector proteins produced by the bacterium (Tamano 2000). Further electron microscopic structural studies have identified the proximal bulb in *Shigella*

species, but the proximal bulb has not been detected in *Salmonella* species (Blocker 1999) (Tran Van Nhieu 2000).

The *ipa* Operon

Another important invasion-related operon encoded by the *Shigella* virulence plasmid is the *ipa* operon (Buysse 1987), which is included in a 31-kb gene cluster that also encodes the *mxi-spa* operons (Venkatesan 1992) (Andrews 1991) (Hueck 1998). The TTSS-exported substrates that are responsible for promoting *Shigella* uptake by epithelial cells are the invasion plasmid antigens (IpaA-D). The Ipa proteins, encoded by the *ipa* operon, are consistently detected on immunoblots carried out using serum from monkeys or humans that have been infected with *Shigella* species (Oaks 1986) (Buysse 1987). Four proteins that are consistently observed on these blots include IpaA (78kDa), IpaB62 (kDa), IpaC 43 (kDa), and IpaD (38kDa), named in the order of descending molecular mass (Buysse 1987) (Hale 1991). The *ipa* operon is expressed at 37°C and the resulting proteins are secreted via the secreton (the type-III secretion components) following host cell contact (Venkatesan 1988), (Hueck 1998). The mechanism controlling *Shigella* type-III secretion is unclear at this time; it is likely that bacterial proteins that are part of the *Shigella* TTSS apparatus interact with host cell receptors to stimulate secretion (Watarai 1996). After being secreted, it has been proposed that a complex composed of IpaB, IpaC and IpaD interacts with and binds to the eukaryotic adhesion molecule $\alpha5\beta1$ integrin (Watarai 1996), which is also a proposed receptor for the invasion protein of

Yersinia (Cossart 1997). However, some groups believe that there is little specificity towards a particular host receptor (Tran Van Nhieu 2000) (Bahrani 1997).

IpaC is believed to participate in several important steps during the course of *Shigella* invasion of epithelial cells (Blocker 1999) (Barzu 1997) (Marquart 1996). IpaC and IpaB interact to form a soluble complex following secretion through the TTSS (Menard 1996). The IpaB-IpaC complex is referred to as the pore complex because it is believed that the *Shigella* TTSS inserts the IpaB-IpaC complex into the host cell membrane to form a 25 Å pore through which other TTSS substrates subsequently pass into the host cell cytoplasm (Blocker 1999). Blocker *et al.* demonstrated that both IpaB and IpaC are necessary for pore formation and that IpaB and IpaC associate with each other only following secretion from the TTSS (Blocker 1999) (Hueck 1998). IpgC, a bacterial chaperone, is responsible for preventing the association of IpaB and IpaC prior to secretion (Menard 1994). It accomplishes this by binding individually to IpaC and IpaB while they are retained within the bacterial cytoplasm (Menard 1994). The binding of IpgC also helps to stabilize and prevent degradation of IpaC in the presence of IpaB (Page 2001). IpgC has been found to perform the same function for IpaB in the presence of IpaC (Page 2001). However, once host-cell contact has been established by the bacterium, it is proposed that IpgC disassociates from IpaC and IpaB to allow their secretion, extracellular association, and ultimately, pore formation (Blocker 1999) (Hueck 1998).

Upon insertion into the host membrane, IpaC activates Cdc42, to cause actin polymerization and the development of filopodial and lamellipodial extensions. Rac

is also believed to be involved in pathogen uptake, downstream of Cdc42 activation (Cossart 1997), (Tran Van Nhieu 2000). The IpaC-stimulated membrane extensions and ruffles probably result from host cytoskeletal changes and are thought to extend beyond the bacterium, gradually enclosing the bacterium in a phagocytosis-like vacuole [Tran Van Nhieu, 2000 #48] (Hueck 1998).

IpaB has also been implicated in several aspects of *Shigella* pathogenesis. Once the bacterium has been transcytosed through the M cell to the basolateral surface of the intestinal epithelial mucosa, it encounters a macrophage (Thirumalai 1997). The bacterium is ingested by the macrophage but avoids degradation by escaping into the cytoplasm from the vacuole (Zychlinsky 1992) (Zychlinsky 1994). At this point, secreted IpaB binds caspase-1 in the macrophage, which results in the induction of apoptosis (Hilbi 1998) (Zychlinsky 1994). IpaB was also originally implicated as the "Ipa effector" protein that triggers entry into epithelial cells (High 1992) and it is believed to exert its effect following association with the hyaluronidate receptor CD44 (Tran Van Nhieu 2000). IpaB's role as an effector of uptake has been played down, however, by the finding that IpaC possess the major effector-related functions of *S. flexneri* (Marquart 1996). IpaB also associates with IpaD, which is believed to lead to the formation of a complex within the TTSS needle complex that creates a 'plug' (Menard 1994). It is believed that the association of IpaB and IpaD functions to control secretion of all effectors that are to be secreted through the TTSS (Menard 1993) (Menard 1994). However, once the bacterium comes into contact with the host epithelial cells, IpaB and IpaD disassociate to allow the rapid secretion

of effector proteins (Hueck 1998) (Menard 1994). The absence of IpaD or IpaB results in hypersecretion of the remaining Ipa proteins and the loss of *Shigella* invasiveness (Menard 1993).

Research Goals

The research presented here focuses on the regulation of secretion of the *Shigella* invasion effector proteins by characterizing the secretion signal of IpaC and examining the role of regulation IpaD plays for secretion of other Ipa proteins. Data available on type-III secretion targets from other pathogens suggest that the N terminus of these proteins is required for their secretion (Lloyd 2002), (Lloyd 2001), (Picking 2001), but the requirements for secretion have not been well characterized and there has been no description of the type-III secretion requirements for proteins involved in *Shigella* pathogenesis. Here we present data that provide the first analysis of the IpaC secretion signal. In this study we will identify the boundaries of the IpaC export signal, determine important amino acids necessary for secretion, and initiate studies on the structural/chemical features that regulate the secretion of IpaC. As simple as the act of IpaC secretion may appear, it is clearly an essential step in the pathogenesis of *Shigella* and therefore warrants investigation.

Likewise, IpaD is known to be important in maintaining the *Shigella* invasive phenotype, but its role in invasion is largely unknown (Menard 1993). IpaD weakly associates with the Ipa complex (IpaB-IpaC pore complex) (Menard 1996) and may associate with membranes in acidic environments (pH 5.5) when at very high

concentrations (De Geyter 2000). An *ipaD* knockout not only results in a noninvasive phenotype, but will also allow in the hypersecretion of IpaB and IpaC (Menard 1994). This control of IpaB and IpaC appears to involve the formation of an IpaB/IpaD plug within the needle complex of the *Shigella* TTSS. These data have led some groups to propose that IpaD regulation of IpaB and IpaC secretion is an essential step for *Shigella* invasion to occur (Menard 1994), (Hueck 1998). These models propose that IpaD's only one role in the invasion process is to regulate effector protein secretion. However, there may be functional properties of this protein that have not yet been revealed. As previously mentioned, IpaB and IpaC carry out multiple functions during the invasion process, so it is possible that IpaD also has more than one function. Therefore, we chose to examine IpaD as a regulator of secretion and to explore the possibility that IpaD may have additional virulence-related functions. We initially set out to generate the first map of the functional organization of IpaD. In doing so, we have been able to provide evidence suggesting that IpaD has a role in the invasion process in addition to its role as a regulator of secretion. We have also shown that IpaB/IpaC hypersecretion is not, in itself, sufficient to eliminate *Shigella* invasiveness.

MATERIALS AND METHODS

Reagents and Buffers

Luria-Bertani Agar (LB)
40.0 g LB agar
in 1L H_2O

Luria-Bertani Broth (LB)
25.0 g LB broth
in 1L H_2O

Tryptic Soy Broth (TSB)
25.0 g TSB
in 1L H_2O

Tryptic Soy Agar (TSA) + 0.025% Congo Red
40 g TSA
0.25 g Congo Red
in 1L H_2O

10X Phosphate Buffered Saline (PBS)
85.0 g NaCl
10.7 g sodium phosphate, diabasic
3.9 g sodium phosphate, monobasic
in 1L H_2O, pH 7.4

Tris-Buffered Saline (TBS):
1.21 g Tris-HCl pH 7.5
8.77 g NaCl

Filler Buffer:
2% casein in TBS
0.25% Tween-20

Buffered Saline for Henle 407 Cells:
0.5 g $NaHCO_3$
1.0 g glucose
8.0 g NaCl
0.4 g KCl
filter sterilize with 0.45μl cellulose nitrate filter

MEM-10% Calf Serum
9.53 g MEM/L
2.2 g $NaHCO_3$/L
100.0 ml calf serum/L
filter sterilize with 0.45μl cellulose nitrate filter

MEM-Glucose
9.53 g MEM/L
2.2 g $NaHCO_3$/L
4.5 g glucose/L
filter sterilize with 0.45μl cellulose nitrate filter

MEM-Gentamycin
9.3 g MEM/L
2.2 g $NaHCO_3$/L
50.0 ml calf serum/L
50.0 mg gentamycin/L
filter sterilize using a 0.45 μm cellulose nitrate filter

Agarose Overlay
4.5 g agarose/L

Carbonate Coating Buffer
3.44 g Na_2CO_3
2.4 g $NaHCO_3$
0.16 g NaN_3
in 800 ml H_2O, pH 9.8

Diethanolamine Buffer
48.5 ml diethanolamine
100.0 mg NaN_3
0.5 ml $MgCl_2$ hexahydrate (100 mg/ml)
in 500 ml H_2O, pH 9.8

Casein Solution
5.2 g NaOH (mixing at very high speed)
80.0 g casein (allowing to dissolve-may take 30+ min) add slowly
4.6 g Tris HCl
8.0 g NaN_3
27.8 g NaCl
in 2 L of H_2O, dilute to 4 L and slowly pH with HCl to 7.5

3X SDS Sample Buffer
2.0 g SDS
10.0 ml glycerol
0.1 g bromophenol blue
5.0 ml 2-mercaptoethanol
add distilled water to 50 ml

SDS-PAGE Running Buffer
2.42 g Tris
14.41 g glycine
10.0 ml SDS per liter

4% SDS-PAGE Stacking Gel
2.0 ml 30% acrylamide:bisacrylamide
2.5 ml 0.5 M Tris-HCl (pH 6.8)
0.1 ml10% SDS
0.175 ml 10% ammonium persulfate
0.020 ml TEMED
adjust to 10 ml with distilled water

10% SDS-PAGE Resolving Gel
3.33 ml 39:0.8% (w/v) acrylamide:bisacrylamide
2.5 ml 0.5 M Tris-HCl (pH 8.8)
0.1 ml 10% SDS
0.150 ml 10% ammonium persulfate
0.01 ml tetramethylethylenediamine
adjust to 10 ml with distilled water

Gel Stain
0.1% (w/v) Coomassie Brilliant Blue R-250
5% (v/v) methanol
7.5% (v/v) acetic acid

Gel Destain
5% (v/v) methanol
7.5% (v/v) acetic acid

Materials and Bacterial Strains

Shigella flexneri 2a, strain 2457T, was provided by A.T. Maurelli, (Uniformed Services University of the Health Sciences, Bethesda, MD) and grown at 37°C on trypticase soy agar containing 0.025% Congo Red. *S. flexneri* mutant strains SF620 (Δ*ipaB*), SF621 (Δ*ipaC*), and SF622 (Δ*ipaD*) were provided by P.J. Sansonetti (Unite de Pathogenie Microbienne Moleculaire, Institute Pasteur). *Salmonella typhimurium* strain SL1344 (wild-type) and strain BJ68 (possessing null mutations in *sipC* and *sipD*) were provided by B.D. Jones (Department of Microbiology, University of Iowa). Bacteria were grown on Luria-Bertani (LB) agar plates or in LB broths at 37°C. All antibodies used in these studies were provided by E.V. Oaks (Department of Enteric Infections, Walter Reed Army Institute of Research, Washington, DC). Henle 407 cells (ATCC CCL6) were propagated as monolayers in Eagle's modified minimal essential medium (MEM; Fisher Scientific, St. Louis, MO) supplemented with 10% newborn calf serum (Life Technologies, Gaithersburg, MD) and grown in a 5% CO_2 environment. PCR SuperMix High Fidelity and oligonucleotides were acquired from Invitrogen (Carlsbad, CA) and NovaBlue and BL21(DE3) strains of *E.coli*, and plasmid pET15b were obtained from Novagen (Madison, WI). Buffers and reagents were prepared with deionized water. All other chemicals were of reagent grade and were purchased from Sigma (St. Louis, MO) and Fisher Scientific (St. Louis, MO).

Methods

Construction of pWPsf4 and *ipaC* mutants

pWPsfc, a pUC18 derivative containing an ampicillin resistance gene, was the original construct generated in the Picking laboratory for *ipaC* expression in *S. flexneri* SF621. However, the IpaC protein synthesized from pWPsfc posseses eight additional amino acids from β-galactosidase (MTMITNSH) at its immediate N terminus due to the polylinker cloning site residing within the *lacZ* gene of the *lac* operon (Figure 1). Even though these amino acids do not interfere with the ability of IpaC to restore invasiveness to *S. flexneri* strain SF621, pWPsf4c was designed to allow synthesis of only the actual wild-type form of *ipaC* gene (Figure 1). Furthermore, the *ipaC* coding sequence used in pWPsfc is based on the DNA sequence reported by Venkatesan and coworkers (Venkatesan 2001), which actually encodes a protein that is 19 amino acids longer than the actual native form of IpaC. Oaks *et. al.* designated the first Met codon of the Venkatesan version of *ipaC* as –19 and the second Met codon as position 1 based on a later report by Sansonetti's group describing the N-terminal protein sequence of IpaC (De Geyter 1997). The plasmids pWPsfc' and pWPsf4c' utilize this second Met codon to allow synthesis of the native form of IpaC (lacking the residues -19 to 1). The IpaC mutants used to describe the secretion signal sequence in this study were constructed using plasmid pWPsf4, which does not result in the synthesis of the eight additional amino acids from β-galactosidase (Figure 1). The strains harbouring these plasmids and the

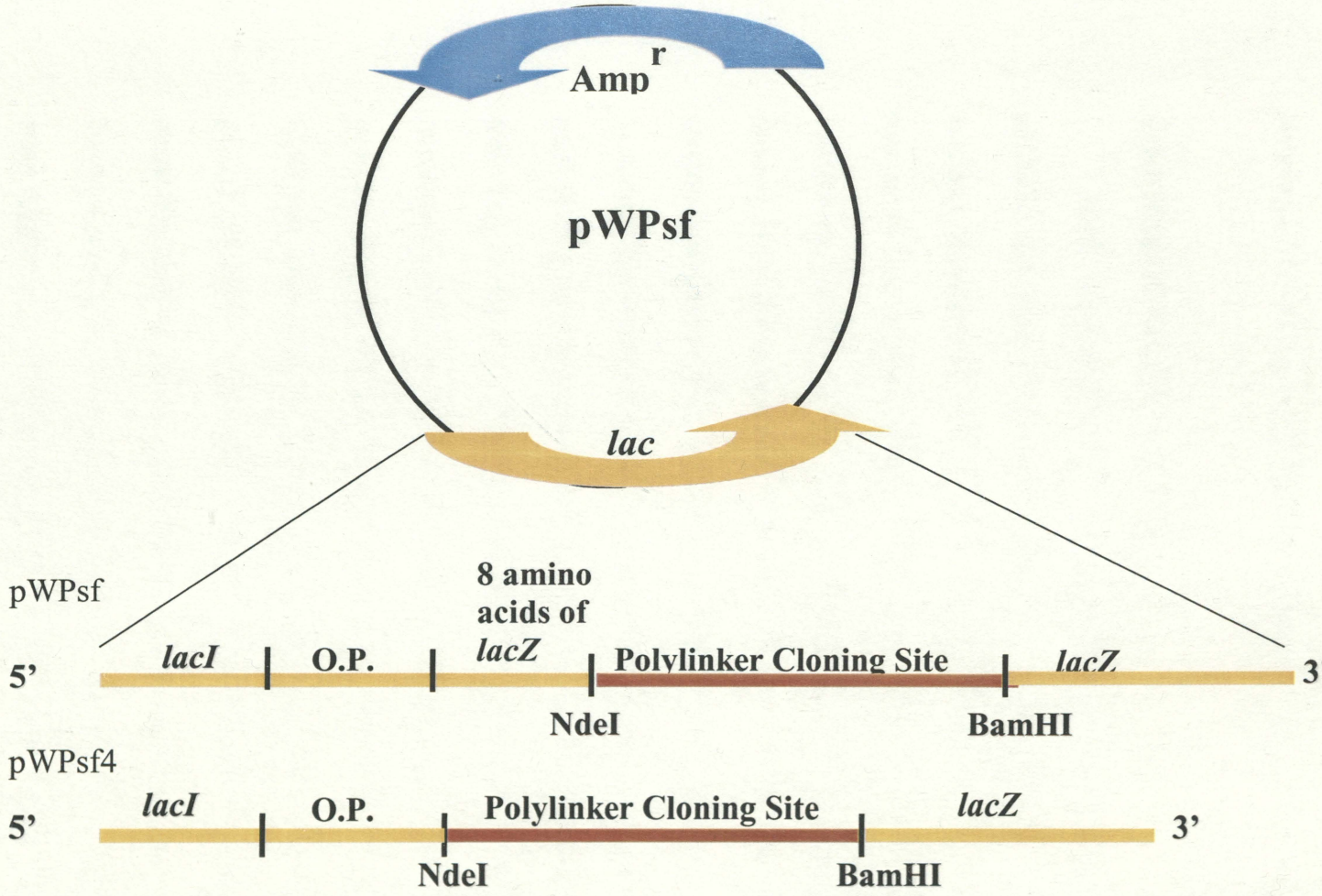


Figure 1. Schematic drawings of the *lac* operons found on the plasmids pWPsf and pWPsf4. A schematic drawing of the original plasmid used in the Picking lab (pWPsf) and the new plasmid constructed to express *ipaC* without eight residues of *lacZ* generated at the N terminus of *ipaC* (pWPsf4).

complemented genes were streaked onto LB plates and sent to the University of Nebraska, Genomic Core Lab for sequencing.

Construction of *ipaC* and *ipaD* mutants

ipaC deletion mutants were made by inverse PCR using pWPsf4c or pWPsf4c' as template, primers containing the sequence for a XhoI or NdeI restriction site, and 18 nucleotides flanking the region to be deleted. The desired linear plasmid was amplified by PCR, digested with NdeI or XhoI, intramolecularly ligated, and transformed into *E. coli* NovaBlue. The resulting plasmid was introduced into *S. flexneri* SF621 by electroporation. Ampicillin selection ensured the presence of the recombinant plasmid while kanamycin resistance and/or Congo red binding was used to ensure that the transformants still possessed the *S. flexneri* virulence plasmid. The *ipaD* deletion mutants were created by the same methods as described above, with the following exceptions: pWPsf4d was used as template and the final plasmid was introduced into *S. flexneri* SF622 by electroporation. Functional analysis studies to compare IpaD and SipD were generated in *Salmonella* and *Shigella,* respectively. SipD was produced in *S. flexneri* SF622 utilizing the plasmid pWPsf2, which is a pUC18 derivative containing a chloramphenicol resistance gene, instead of an ampicillin resistance gene. *ipaC* and *ipaD* were expressed in *S. typhimurium* BJ68, containing null mutations in *sipC* and *sipD.* To create these mutants, SipC and IpaC were expressed from pWPsf and SipD and IpaD were expressed from pWPsf2 in *S. typhimurium* BJ68. Ampicillin selection ensured the presence of the recombinant

plasmid pWPsf, while chloramphenicol selection ensured the presence of the recombinant plasmid pWPsf2. These constructs are summarized in Table 1.

ELISA Assay

Enzyme-linked immunosorbance assays (ELISAs) were routinely performed to provide quantitative data on the secretion of specific proteins by *Shigella* mutants. *S. flexneri* SF621 harboring derivatives of pWPsf4 were grown to mid-log phase and harvested by centrifugation. The supernatant fraction was saved as a source of secreted protein while the bacterial fraction was resuspended in carbonate coating buffer and sonicated to prepare a whole-cell extract sample. The cell extracts were diluted in carbonate coating buffer to a final concentration of 10μg protein/ml; 100μl of the resuspended cell extracts and their corresponding culture supernatant fractions were used to coat microtiter wells by incubating the plates stationary overnight at 4°C. After incubation, 250μl of casein solution was added and allowed to block all open protein binding sites for 30 min. The primary antibody (an anti-IpaC monoclonal antibody specific for a site near the C terminus of IpaC) was diluted 1:500 in casein solution and applied to the microtiter wells for 2 hours at ambient temperature. The wells were then washed four times with PBS, pH 7.4 containing 0.05% Tween 20. The secondary antibody (goat anti-mouse conjugated with alkaline phosphatase) was also diluted 1:500 in casein solution and applied to the microtiter wells for 1 hr at ambient temperature. The wells were washed again four times with PBS, pH 7.4 containing 0.05% Tween 20. Finally, 100μl of alkaline phosphatase

Table 1. A table describing the *Shigella* and *Salmonella* genes expressed in different plasmids used in this study.

Plasmids used	Plasmid characteristics	Plasmid introduced into Strain	Mutation in Strain	Plasmid transformed into Organism
pWPsf	Ampicillin resistance + 8 β-gal residues*	SF621	Δ *ipaC*	*S. flexneri*
pWPsf4	Ampicillin resistance	SF622	Δ *ipaD*	*S. flexneri*
pWPsf2	Chloramphenicol resistance	SF622	Δ *ipaD*	*S. flexneri*
pWPsf & pWPsf2	Ampicillin resistance + 8 β-gal residues* and Chloramphenicol resistance	BJ68	Δ *sipC* & Δ *sipD*	*S. typhimurium*

*denotes 8 β-gal residues generated at the immediate N terminus of the protein produced using this plasmid

IpaC, IpaC', IpaD, SipC and SipD were generated using plasmids that are pUC18 derivatives. An ampicillin resistance gene is located on pWPsf and pWPsf4. Chloramphenicol resistance is acquired from pWPsf2. Strains SF621 and SF622 are *Shigella flexneri* strains lacking *ipaC* or *ipaD*, respectively. BJ68 is a *Salmonella typhimurium* strain with null mutations in *sipC* and *sipD*.

substrate, Sigma 104, was applied and the plates were allowed to incubate for 30 min. at ambient temperature. The absorbance was then determined at 405 nm in an ELISA plate reader.

Henle 407 Epithelial Cell Maintenance for Invasion Assay

The Henle 407 intestinal epithelial cells used for invasion assays were grown in Modified Minimal Essential Medium Eagle (MEM) containing Earle's Salts, L-glutamine, sodium bicarbonate, and phenol red (MEM). This medium was supplemented with 1% antibiotic solution (10,000 U/ml penicillin and 10mg/ml streptomycin in 0.9% NaCl, Sigma Chemical Co., St. Louis, MO) and 10% newborn calf serum (MEM-CS-antibiotics). The cells were grown in 5% CO_2: 95% air at 37°C and were provided with MEM-CS-antibiotics every three to four days in 25 cm^2 flasks. The cell monolayers were passaged upon reaching confluence. To passage the cells, they were first washed with 5ml PBS and treated with 3ml 0.25% trypsin (Life Technologies, Grand Island, NY) for 5 min. to allow the monolayers to lift from the flasks. Then 3ml of MEM-CS-antibiotics were added to the trypsinized cells. After the cells were vigorously agitated to yield a single-cell suspension, 1ml aliquots of the suspension were added to 5ml MEM-CS-antibiotics in each of 3 new 25 cm^2 flasks. The newly passaged cells were replenished with fresh MEM-CS-antibiotics after 24 hours.

S. flexneri Invasion of Henle 407 Cells

The invasive phenotype of *S. flexneri* constructs were analyzed using a standard gentamycin protection assay. Henle 407 cells were trypsinized and transferred from the 25 cm^2 flask to a 24-well plate and were then allowed to grow to semi-confluence overnight at 37°C. The appropriate *S. flexneri* strains were then inoculated into 10ml TSB containing 100μg/ml ampicillin and 50μg/ml kanamycin. The cultures were incubated at 37°C in a shaking incubator at 200 rpm and allowed to grow until an A_{600} of 0.4 to 0.6. The Henle 407 cells were then washed once with MEM-glucose, and 250μl of MEM-glucose was added to each well. The bacteria were then diluted 1:20 in MEM-glucose and 5μl of the appropriate bacterial suspension was added to the appropriate well. The plates then were centrifuged for 5 min. at 2,000 rpm to force contact between the bacteria and the Henle 407 cells. The plates were then incubated at 37°C for 30 min. in 5% CO_2:95% air to allow invasion to progress. Following incubation, free bacteria were removed by aspiration and washing of the Henle 407 cells multiple times with MEM containing 5% calf serum and 50μg/ml gentamycin. The cells were then incubated in the gentamycin-containing medium for 2 hrs to kill adherent but noninternalized bacteria. The monolayers were then rinsed once with MEM containing glucose and then lysed by overlaying them with 250μl 0.5% agarose in water. The agarose was then overlaid with 0.5% agar containing 2x LB medium. After overnight incubation at 37°C, internalized bacteria formed subsurface colonies that were counted with the aid of a dissecting microscope.

S. typhimurium Invasion of Henle 407 cells

Salmonella mutants were assayed using the gentamycin-protection assay as described above, with the following modifications due to the motile characteristic of *Salmonella* and its less efficient invasiveness. Once the addition of *Salmonella* cultures were added to the Henle 407 monolayers, the centrifugation step was omitted and a 1 hr incubation period was allotted for invasion to occur. Gentamycin washes were performed as previously described, followed by washing the cells with 1X PBS. Ultimately, the cells were lysed using 1ml of sterile, distilled water and 0.5ml of the resulting bacterial suspension was spread onto LB plates that were incubated overnight at 37°C. After overnight incubation, the previously internalized bacteria formed subsurface colonies that were manually counted.

Contact-Mediated Hemolysis

S. flexneri were grown overnight on TSA containing 0.025% Congo red plates. A single red colony was used to inoculate TSB containing 100μg/ml ampicillin and 50μg/ml kanamycin. The inoculated bacteria were grown to mid-log phase and the cells were collected by centrifugation and resuspended in PBS at $1/40^{th}$ the original volume. Sheep red blood cells were harvested after centrifugation, washed and resuspended in PBS at a concentration of 1×10^{10} cells/ml. Red blood cells and bacteria (50μl each) were added to wells of microtiter plates. The plates were centrifuged at 2200 x g at 30°C for 15 min and then incubated at 37°C for two

hours without shaking. The pellet was resuspended by adding 100µl of cold PBS and then centrifuged for 15 min. at 2200 x g at 10°C. The supernatant fraction (100µl) was transferred to a second microtiter plate and the absorbance of the released hemoglobin measured at 545nm using a microtiter plate reader.

Testing for the secretion of IpaC and IpaB by SDS-PAGE

A single red colony from *a S. flexneri* strain grown overnight on a TSA plate containing 0.025% Congo red was used to inoculate 10 ml of TSB containing 100µg/ml ampicillin and 50µg/ml kanamycin that was placed in a shaking incubator until the bacteria reached mid-log phase (A_{600} = 0.8). The 10 ml cultures were centrifuged for 10 min at 5000 rpm and whole-cell extracts were prepared by resuspending the pellet in 200µl of water. Meanwhile, the supernatants were mixed with 2.5 ml of 100% TCA and allowed to incubate on ice for 30 min. The supernatants were then centrifuged twice for 12 min at 10,000 rpm and the protein pellets were washed with 10 ml of 5% of TCA between spins. Then the precipitated supernatant samples were washed two more times with 5% TCA and then washed with 10 ml of ice-cold acetone. The protein samples were finally resuspended in 400µl PBS and 200µl of 3X sample buffer and 30µl of DTT was added to the precipitated supernatants samples; 100µl of 3X sample buffer and 10µl of DTT were added to the whole-cell extracts. The proteins were then separated and visualized with SDS-PAGE and Coomassie-staining.

SDS-PAGE

Proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Protein samples were mixed with an equal volume of 2X-SDS sample buffer and 0.05 volumes 2-mercaptoethanol. Each protein mixture was incubated in a boiling water bath for 5 min. The proteins were loaded into wells formed in a 4% acrylamide stacking gel poured over a 10% acrylamide resolving gel. Samples were electrophoresed at 200 V until the bromophenol blue tracking dye reached the bottom of the gel and then the gel was stained overnight with 0.5% Coomassie Brilliant Blue G-250 in 25% methanol and 5% acetic acid and destained for several hours with 10% methanol and 5% acetic to observe protein bands.

CHAPTER 1:

Determination of the IpaC Secretion Signal

Introduction

IpaC is the primary effector in *Shigella* invasion of intestinal epithelial cells. In 1988 two papers were published containing the (predicted) DNA-based sequence of IpaC (Baudry 1988) (Venkatesan 1988). Sansonetti's group proposed that IpaC was 363 amino acids in length (Baudry 1988); however, Venkatesan's group reported that IpaC was 382 amino acids long based on a predicted upstream translation start site (Venkatesan 1988). Due to questions on the precise IpaC sequence, Turbyfill established what is now a universal numbering system for the amino acids of IpaC protein in 1995 (Turbyfill 1995), and it is this numbering system that is currently used in our lab. The full-length construct of IpaC used in our lab is composed of amino acids numbered, -19 to 363. A derivative of this is IpaC', which uses the downstream start site and is composed of amino acids 1 to 363. Amino acid sequencing performed in 1996 with IpaC purified from *S. flexneri* culture supernatants demonstrated that native IpaC is the shorter of the two previously proposed sequences (363 amino acids long), as predicted by Sansonetti (De Geyter 1997).

Picking *et. al.* proposed a crude model for the functional organization of IpaC (Figure 2), which was published in 2001 (Picking 2001). The functional organization was determined by constructing a series of *ipaC* deletion mutants and analyzing large regions of IpaC protein (Picking 2001), based on epitope domains of IpaC from

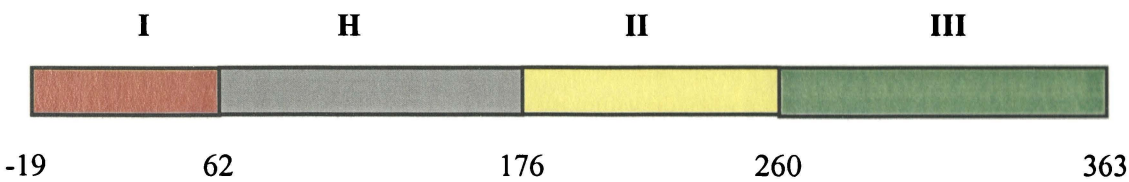


Figure 2. A schematic diagram of the functional organization for IpaC. The numbering system of Turbyfill *et al* (1995) is used here because the recombinant IpaC originally generated in this laboratory (Picking *et al.*, 1996) was based upon the sequence reported by Venkatesan *et al.* (1988) and thus possesses 19 amino acids at the N terminus that are absent from the native form of IpaC' purified by De Geyter *et al.* (1997). The presence of these additional N-terminal residues does not appear to adversely affect IpaC' activity. Region I (red) contains the TTSS secretion signal and at least part of the translocation region. Region H (gray) contains a hydrophobic region that is thought to be involved in membrane interactions. The function of region II (yellow) does not appear to have a function. Region III (green) is the effector region that directly triggers cytoskeletal changes in the host cells.

immune sera of monkeys challenged with *S. flexneri* 2a (Turbyfill 1995). Four broad regions of IpaC were determined. Region I (-19 to 62) was identified to contain the secretion signal and possibly part of the translocation region of IpaC (Picking 2001), which is consistent with the organization of other type-III secretion effector proteins found in *Yersinia* and *Salmonella* (Lloyd 2001) (Lloyd 2002). IpaC protein also contains a central hydrophobic region ('H' located within a fragment consisting of residues 63 to 170) that appears to be involved in host cell membrane interactions (Picking 2001). In contrast, region II (residues 170 to 260) is not required for IpaC invasion functions while region III (residues 260 to 363) appears to be the effector region that directly triggers the actin cytoskeletal changes that occur in host cells during *Shigella* invasion (Figure 2) (Picking 2001). Functional homologues of IpaC exist in *Salmonella* and *Yersinia* and their putative secretion signals also appear to be located near the N terminus, although there appears to be relatively little sequence homology between these different type-III secretion substrates (Lloyd 2001) (Lloyd 2002) (Kaniga 1995). The work presented here provides the first analysis of the IpaC secretion signal located in region I. Through substitution and deletional mutagenesis we have identified the minimum sequence necessary for secretion and have determined amino acids important for secretion. We show that amino acids 1 to 15 are necessary for secretion and provide initial evidence that a structural/chemical feature of the N terminus regulates IpaC secretion.

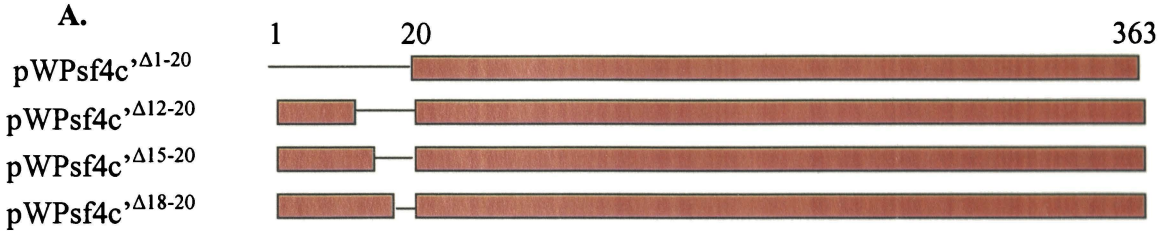
Results

Identifying the minimum sequence required for IpaC for secretion.

Deletion of the first 20 amino acids of the native form of IpaC (IpaC') abolishes its ability to be secreted into *S. flexneri* culture supernatants (Figure 3). The absence of secretion is reflected in the loss of IpaC's ability to restore invasiveness to the *ipaC* mutant *S. flexneri* SF621. In contrast, deletion of amino acids 21 to 50 does not adversely affect IpaC' secretion, invasiveness, or contact-hemolysis activity (A. Harrington, data not shown). To determine the minimum number of amino acids needed for efficient IpaC' secretion, small deletions were introduced that ended at amino acid 20 and extended toward the N terminus of the protein (Figure 3). The efficiency of IpaC' secretion was monitored using an ELISA assay in which monoclonal antibodies that specifically bind to a site near the IpaC C terminus (Turbyfill 1995) were used to detect the presence of the protein in culture supernatants. Bacterial cell extracts were also used in a similar ELISA assay as a control for *ipaC'* expression (data not shown). A negative control, *S. flexneri* SF621 with a nonpolar *ipaC* knockout, and a positive control, *S. flexneri* SF621 transformed with pWPsf4c' were included for validation of the results.

Deleting three amino acids (18 to 20, TKQ) and replacing two of the residues with an LE of the XhoI restriction site had only a minor effect on IpaC' secretion. Likewise, invasion function was nearly at wild-type levels (Figure 3). The replacement of residues 16 to 20 (ISTKQ replaced by LE of the XhoI restriction site)

A.



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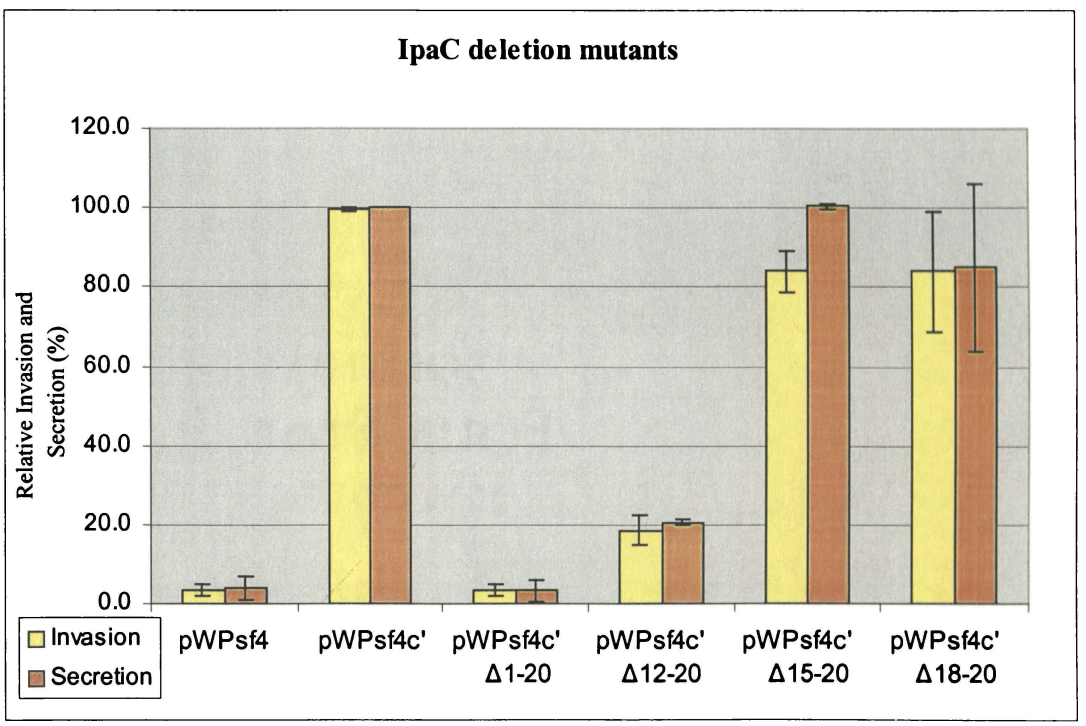

Figure 3. Identification of the minimum sequence necessary for IpaC secretion. A) A schematic diagram of the deletion mutants created to define the C-terminal boundary of the secretion signal is shown. IpaC deletion mutants (named at the left) were generated as described in Materials and Methods. B) Secretion and invasion efficiency for the deletion mutants shown in A. Negative control, pWPsf4, is the plasmid not complemented with *ipaC*. The positive control, pWPsfc', produces the native form of IpaC (363 amino acid). Data are shown as an average of three trials relative to pWPsf4c' after transformation into *S. flexneri* SF621. +/- standard deviation.

amino acids had no effect on IpaC' secretion and invasion function was retained to 85% (Figure 3). In contrast, deletion and substitution of amino acids 11 to 20 (TLYTDISTKQ replaced by LE of XhoI restriction site) resulted in an 80% reduction in IpaC' secretion from SF621. As expected, invasion was also reduced by 80% (Figure 3). These data suggest that the information needed for IpaC' to be recognized by the type-III secreton for secretion exists within the first 15 amino acids of IpaC'.

Because all the Ipa proteins are recognized for secretion by the same TTSS, it was hypothesized that the first 15 amino acids of IpaA, IpaB, IpaD, and IpgD should be able to substitute for the first 20 amino acids of IpaC' to allow proper secretion. Furthermore, they should also give rise to an IpaC derivative that is active in restoring invasiveness to *S. flexneri* SF621. Chimeras were therefore generated to replace the secretion signal of IpaC with the putative secretion signals of IpaA, IpaB, IpaD, and IpgD. These chimeras were tested for IpaC secretion by the same ELISA method already described utilizing antibodies generated against a site near the C terminus of IpaC. Invasion experiments were performed in parallel with secretion analyses. The ELISA results indicated that three of the chimeras (IpaA-IpaC, IpaD-IpaC, and IpgD-IpaC) were able to secrete IpaC at $\geq 80\%$, when compared to *S. flexneri* SF621 complemented with pWPsf4c. However, IpaB showed a reduced level of efficient IpaC secretion (Figure 4). Invasion data indicated that the putative secretion signals from IpaA, IpaD, and IpgD fused to IpaC protein allow for $\geq 60\%$ invasion (Figure 4). Interestingly, the IpaB secretion signal fused to IpaC protein was very poorly invasive ($\leq 6\%$) and reduced somewhat (40%) with respect to secretion.

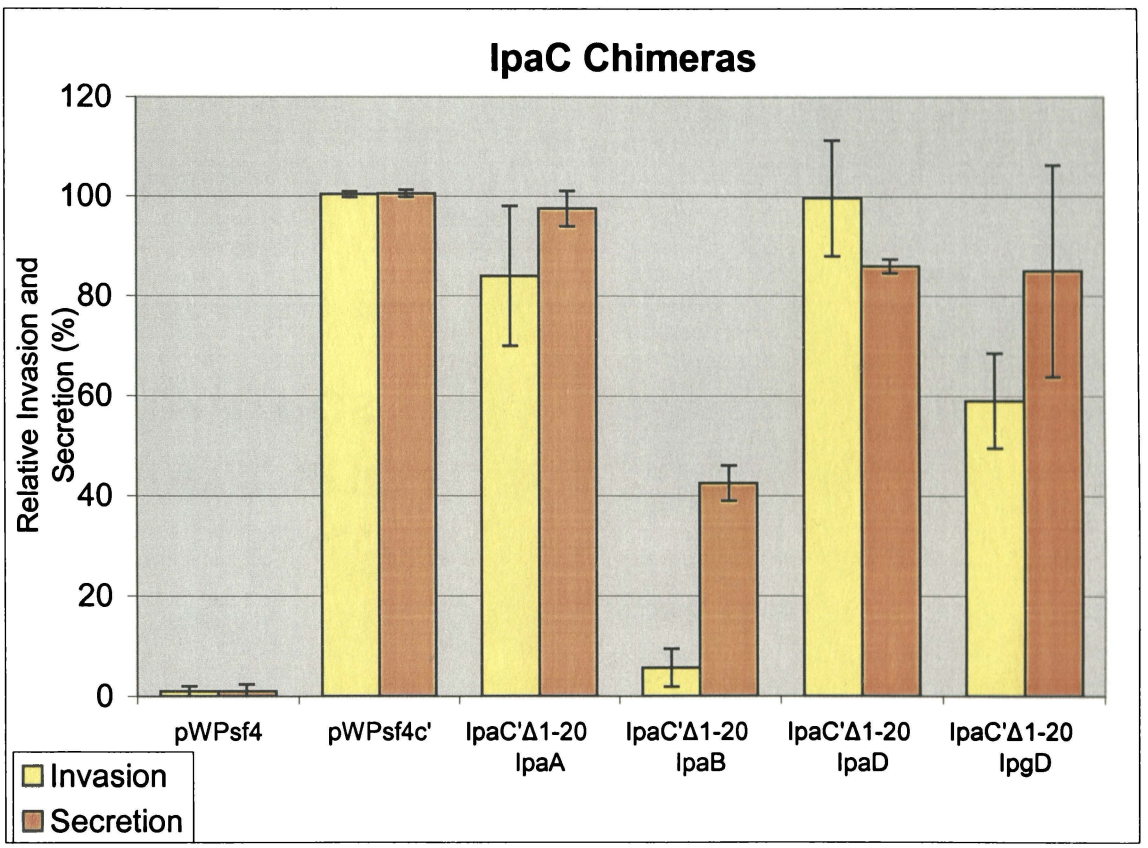

Figure 4. Generation of active IpaC following replacement of the IpaC secretion signal with those from other Ipa proteins. Invasion and secretion data were collected from IpaC' mutants after the secretion signals (presumably amino acids 1 to 15) of IpaA, IpaB, IpaD, and IpgD were fused to the N terminus IpaC'$^{\Delta 1\text{-}20}$and generated in pWPsf4. Data are shown as an average of three trials +/- standard deviation

The secretion data obtained here provides evidence that the *Shigella* type-III secreton recognizes diverse secretion signals when fused to IpaC. Although all the chimeras generated in this study were able to secrete IpaC (to at least some extent), they were not all able to demonstrate an invasive phenotype as seen with the IpaB chimera, which was found to be largely noninvasive (Figure 4).

Identification of amino acids important for IpaC secretion.

Like *S. flexneri*, *Yersinia enterocolitica* utilizes a type-III secretion system to deliver its effector proteins to target cells. One of the important TTSS substrates of *Y. enterocolitica* is YopE, which behaves as a GTPase-activating protein (GAP) for Rho in target macrophages (Von Pawel-Rammingen 2000). Wolf-Watz *et al.* constructed three mutants of YopE, in which the YopE secretion signal (also located at the protein's immediate N terminus) was replaced with a synthetic polyserine, polyisoleucine, or alternating Ser and Ile sequence (poly Ser/Ile) (Lloyd 2002). From these studies, it was discovered that replacing the original secretion signal sequence with a polyserine or polyisoleucine sequence abolished YopE secretion; however, replacing the YopE secretion signal with a synthetic alternating Ser and Ile sequence is sufficient for efficient secretion of YopE (Lloyd 2002). This suggests that a physical property or structural feature within the secretion signal is critical for targeting the protein for secretion by the *Yersinia* TTSS (Lloyd 2002). This concept was tested in *Shigella* by constructing a synthetic alternating Ile and Ser sequence to replace the first 15 amino acids of IpaC'. However, the ELISA data obtained from the

generation of this construct demonstrated that an alternating isoleucine-serine sequence is not sufficient for efficient secretion of IpaC' (Figure 5). The protein possessing the alternating sequence was only 15% secreted when compared to pWPsfc'expressed in SF621 (Figure 5). Schneewind has proposed that the secretion signal for type-III secretion substrates exists within the 5'mRNA (Anderson 1999), but other work provided by Wolf-Watz suggests that the secretion signals for most type-III secretion systems does indeed reside primarily in amino acid sequences present near the N terminus (Lloyd 2001). This was confirmed by creating a mutation that alters the *yopE* mRNA sequence, while maintaining the natural amino acid sequence (Lloyd 2001). Secretion data indicated that YopE secretion was not impaired when the 5' mRNA sequence was altered while the amino acid sequence was not (Lloyd 2001). Based on this information, our next step was to identify necessary amino acids at the immediate N terminus that are required for secretion of IpaC.

To identify necessary amino acids needed for efficient secretion, we first constructed three substitution mutants, each containing a single Ala substitution for a polar residue near the immediate N terminus of IpaC. ELISA data collected from these constructs indicated that substituting Ala for individual amino acids had no effect on secretion of IpaC (data not shown). In contrast, disrupting a group of amino acids located near the immediate N terminus of IpaC' (2E, 4Q, and 5N) was found to greatly reduce IpaC' secretion and invasiveness (Figure 6). We observed this

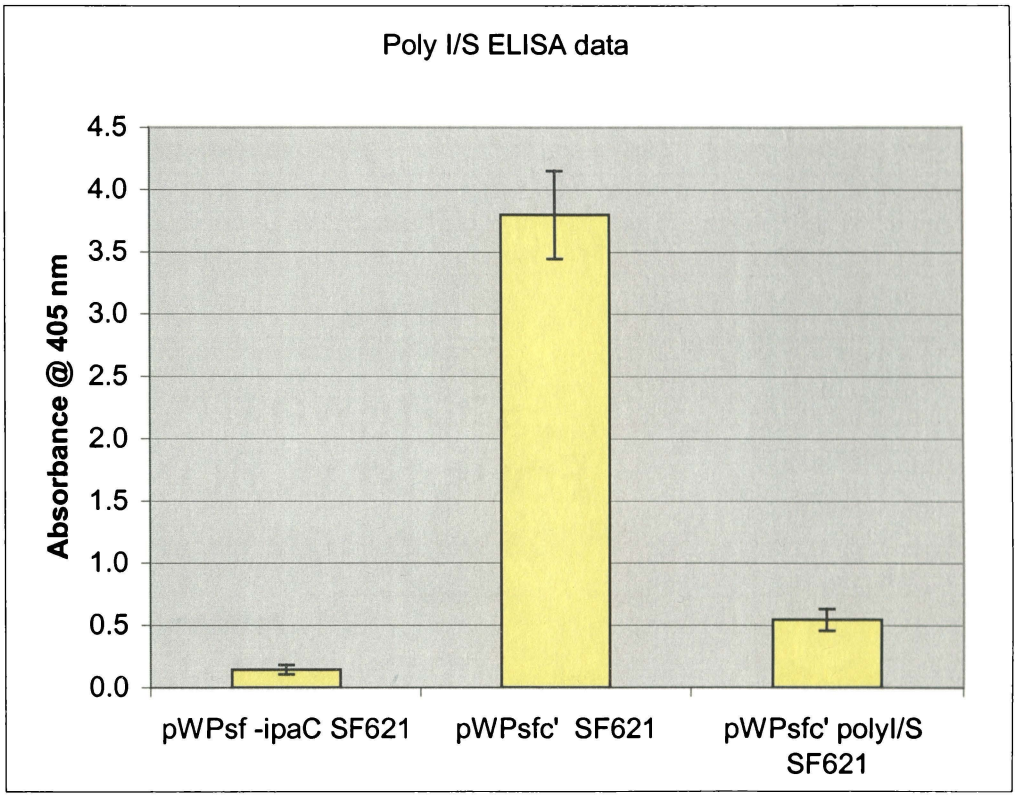

Figure 5. Secretion data generated from IpaC' containing a 15 amino acid alternating isoleucine and serine sequence. pWPsfc'polyI/S expressed in *S. flexneri* SF621 as compared to the positive control, pWPsfc' (the native form of IpaC) expressed in SF621. Data are shown as an average of three trials +/- standard deviation.

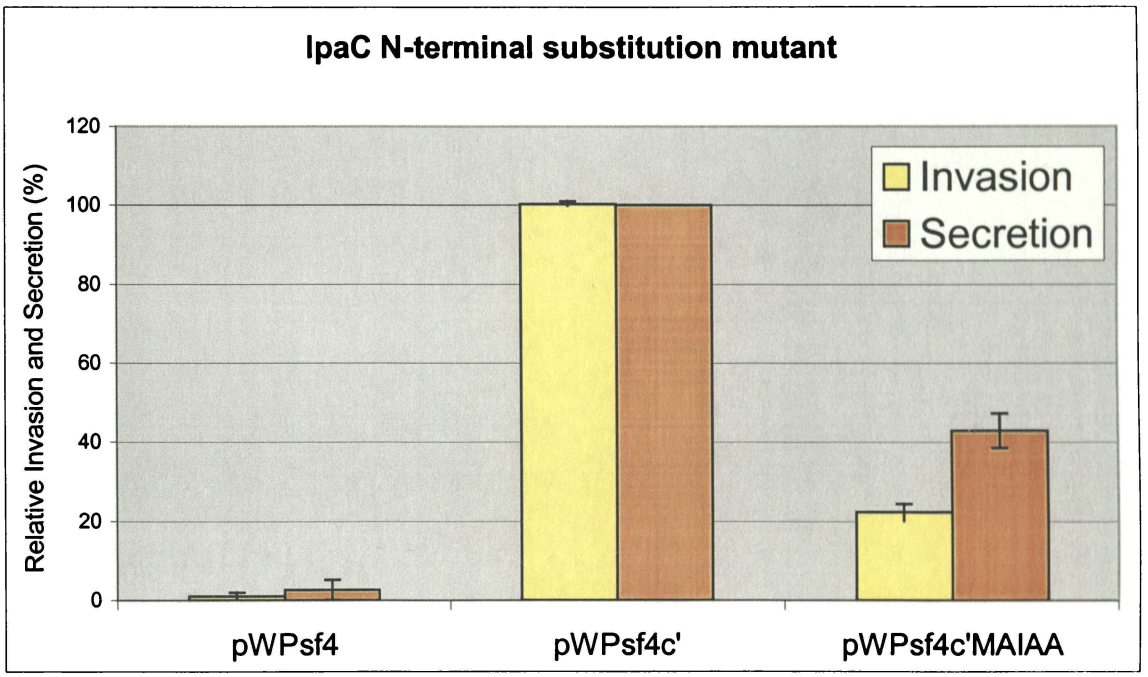

Figure 6. IpaC N-terminal substitution mutant. A graph depicting the effects on invasion and secretion of IpaC' when residues located at the immediate N terminus are altered (MEIQN → MAIAA). Data are shown as an average of three trials +/- standard deviation

phenomenon by constructing and complementing *S. flexneri* SF621 with pWPsf4c'^{MAIAA}. This mutant generates an IpaC' in which the first three polar residues are substituted with three alanine residues (MEIQN→ MAIAA). Secretion and invasion data generated from pWPsfc'^{MAIAA} indicate an 80% decrease in invasion and a 60% decrease in secretion of IpaC' (Figure 6). Collectively, these experiments indicate that disrupting a single N-terminal amino acid may have little effect on IpaC secretion. However, altering a group of N-terminal amino acids, specifically 2E, 4Q and 5N, can adversely affect the efficiency of IpaC' secretion, possibly due to disruption of the physical and/or chemical properties at the N terminus that play a role in IpaC' secretion.

Alternative IpaC secretion signals

The initial secretion data collected in our lab suggested that region I of IpaC contained two putative secretion signals, composed of amino acids –19 to 1 (seen with pWPsfc$^{\Delta\text{-}5—20}$ in SF621) or amino acids 1 to 15 (seen with pWPsfc' in SF621). However, the original plasmid constructed in the Picking lab for *ipaC* expression in SF621, pWPsfc, places eight additional amino acids from β-galactosidase at the immediate N terminus of the IpaC derivative. Although these extra residues did not appear to interfere with IpaC effector function (Figure 7), a new plasmid (pWPsf4) was constructed to express *ipaC* and *ipaC'* without the eight additional amino acids of β-galactosidase. The new plasmid was used to generate pWPsf4c (for making IpaC$^{-19\text{-}363}$) and pWPsf4c' (for making IpaC$^{1\text{-}363}$), respectively (Picking, W.L.). We

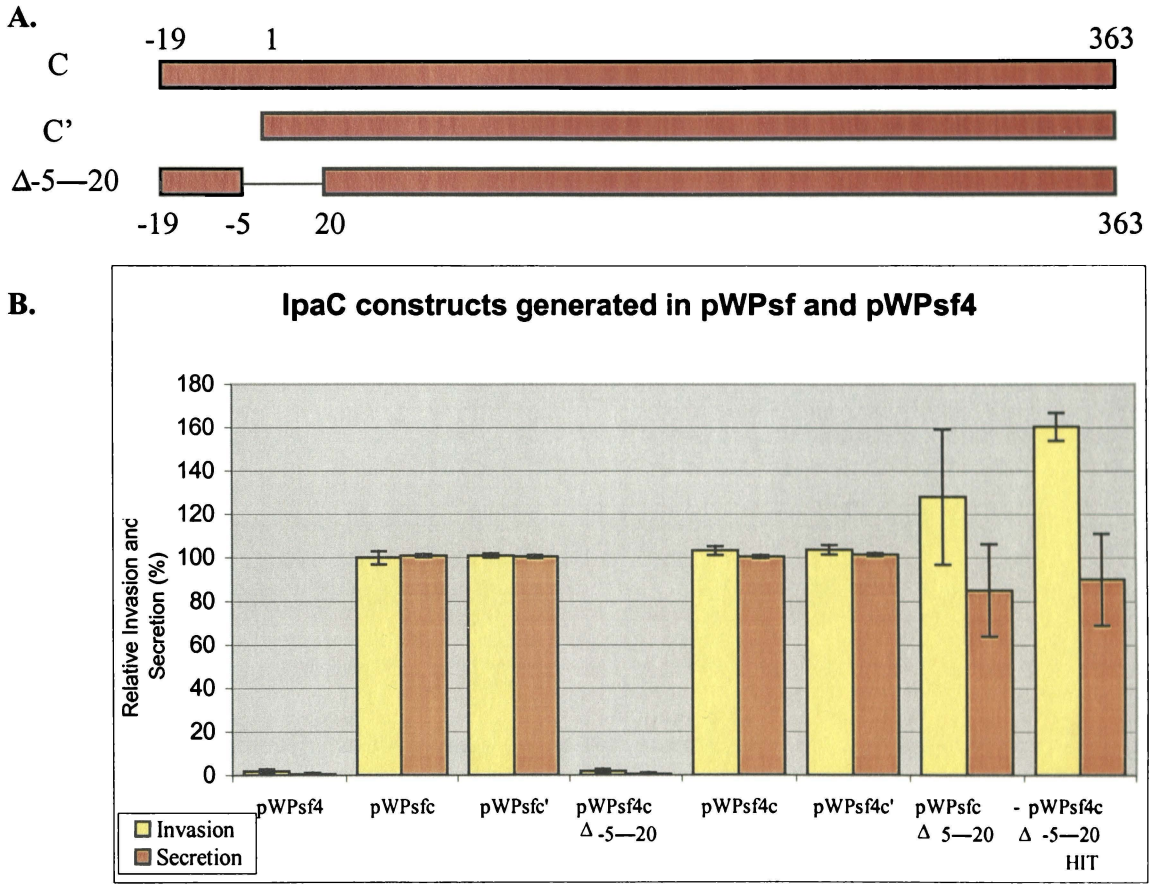

Figure 7. IpaC, IpaC', and IpaC $^{\Delta\text{-}5\text{-}20}$ generated in pWPsf or pWPsf4. A) A schematic diagram of IpaC constructs generated using either the plasmid pWPsf (contains eight β-galactosidase residues generated at the N terminus) or pWPsf4 (lacks the eight β galactosidase codons). These constructs were initially generated in *S. flexneri* complemented with pWPsf, but were later generated using pWPsf4. B) A figure comparing the secretion and invasive abilities of *S. flexneri* SF621 complemented with *ipaC*, *ipaC'* and *ipaC*$^{\Delta\text{-}5\text{-}20}$, using the original plasmid (pWPsf) and the new plasmid (pWPsf4) without the eight β-galactosidase amino acids. *ipaC*$^{\Delta\text{-}5\text{-}20HIT}$ expressed in pWPsf4 was altered to substitute three polar amino acids at the N terminus of IpaC (MLQKQF → MHQIQT). Data are shown as an average of three trials +/- standard deviation.

observed that IpaC (-19 to 363) and IpaC' (1 to 363) generated from either pWPsf or pWPsf4 in SF621 were equally able to restore invasiveness and secretion. Although pWPsfc$^{\Delta\text{-}5\text{-}20}$ gives rise to an IpaC derivative that is secreted and restores invasiveness to SF621, pWPsf4c$^{\Delta\text{-}5\text{-}20}$ (the plasmid lacking the β-galactosidase codons) does not allow for efficient secretion of IpaC and therefore does not restore invasiveness toSF621 (Figure 7). This finding suggested that there is only one IpaC secretion signal (from residues 1 to 15) that is recognized by the TTSS within the –19 to 20 region of IpaC and IpaC'; however, additional amino acids encoded immediately upstream of the IpaC N terminus do not appear to adversely affect secretion. This proposal is supported by work performed by S. Shah who constructed an IpaC mutant that encoded a 20 amino histidine-containing tag immediately upstream of the IpaC N terminus. When this construct was expressed in SF621, it was found to be efficiently secreted and completely able to restore invasiveness (S. Shah and W.L. Picking, unpublished data).

Because these findings are in agreement with the suggestion that the *Shigella* type-III secretion signal possesses a physical or chemical feature rather than a specific amino acid sequence that targets TTSS substrates for secretion, we were interested in determining whether a synthetic secretion signal could be generated that is recognized by the *Shigella* TTSS. We began with the construct pWPsf4c$^{\Delta\text{-}5\text{-}20}$, which generates an IpaC that is made but not secreted and is therefore incapable of contributing to *Shigella* invasiveness. A new mutant was generated using pWPsf4c$^{\Delta\text{-}5\text{-}20}$ as the template, with the goal of altering specific amino acids near the IpaC N terminus to

possibly generate a mutant that is secreted and functional. Based on insight gained from pWPsf4c'^{MAIAA}, we knew that substituting three polar, charged residues near the N terminus with three relatively neutral amino acids (Ala in this case) would result in the production of a protein with reduced secretion. The first six amino acids of IpaC generated from pWPsf4c$^{\Delta-5-20}$ contained three nonpolar amino acids (MLQKQF), which we substituted for three polar amino acids (MHQIQT) and named the resulting mutant pWPsf4c$^{\Delta-5—20HIT}$. ELISA data collected from experiments utilizing this construct indicated that IpaC secretion was nearly restored to 100% (Figure 7). Invasiveness was completely restored and was actually more invasive than any of the constructs generating a full-length form of IpaC (Figure 7). These data led us to conclude that synthetic secretion signals could be generated in nonsecreted (but active) mutants of IpaC and that the *Shigella* type-III secreton can recognize diverse signals based on their physio-chemical properties for efficient secretion of IpaC. These data provided the first evidence that the physical characteristics of a secretion signal are responsible for recognition by the *Shigella* TTSS.

Conclusions

Elucidating the IpaC' secretion signal that is recognized by the *Shigella* TTSS will provide novel information on the evolutional development of new TTSS substrates that is applicable not only to other Ipa proteins, but also to our understanding of the secretion signals at work in other gram-negative bacteria that use the TTSS as part of their pathogenesis. The secretion signal of IpaC' resides between amino acids 1 to 15, however, no specific consensus sequence can be assigned to this role. Instead, the accumulated data indicate that there is a physical or structural determinant that directs effector protein export. Data presented here that support this concept were acquired using the constructs pWPsf4c'^{MAIAA}, pWPsf4c$^{\Delta-5-20}$, and pWPsf4c$^{\Delta-5-20HIT}$. By substituting three polar, charged amino acids for three nonpolar, uncharged amino acids, invasion and secretion of IpaC' is greatly reduced. Alternatively, by manipulating three different amino acids with MLQKQF (from pWPsf4c$^{\Delta-5-20}$) to MHQIQT (pWPsf4c$^{\Delta-5-20HIT}$), secretion of IpaC and invasiveness is restored. These data collectively show that altering particular amino acids near the N terminus of type-III effectors can result in the creation of an artificial secretion signal that is capable of serving as an indicator to the type-III secreton for protein export.

Also supporting the hypothesis that a physical or structural determinant directs protein export are experiments involving IpaC chimeras, in which the putative secretion signals of IpaA, IpaB, IpaD, and IpgD were fused to IpaC protein. These secretion signals fused to IpaC allowed for efficient secretion of IpaC, which

indicates that other putative secretion signals, with diverse sequences, can be fused to IpaC to allow recognition by the *Shigella* type-III secreton. Although these chimeras allowed for secretion of IpaC, they did not all restore invasiveness. This could suggest an order in which the secreted effector proteins must be exported from the TTSS but the requirements for such a hierarchy are completely unknown. While it is not known whether the type-III secretion effectors are secreted in a specific order, it is widely accepted that IpaB and IpaC are first secreted to form a complex that is inserted into host epithelial cells by the *Shigella* TTSS (Menard 1996) (Blocker 1999). This IpaB-IpaC complex forms a pore through which other effectors may enter the host cell (Menard 1996). Therefore, it was expected that if an order does exist, IpaB and IpaC would be secreted first, thereby resulting in nearly 100% invasion of the IpaB-IpaC chimera. If a secretion order existed, we would more likely have expected the IpaA and IpgD chimeras to result in reduced invasion because these proteins are involved with restoring homeostasis to the host epithelial cells after invasion has occurred (Hueck 1998). However, we found the IpaA-IpaC, IpaD-IpaC and IpgD-IpaC chimeras to be invasive, and the IpaB-IpaC chimera to be only slightly invasive. Because the IpaB-IpaC chimera data showed a reduced level of secretion and inefficient levels of invasion, these data may suggest that the IpaB secretion signal is different from the secretion signals of IpaC, IpaA, IpaD, and IpgD. While replacing the native IpaC secretion signal with amino acids 1 to 15 of IpaA, IpaD, and IpgD led to efficient secretion and invasion, we can conclude that the putative secretion signals for these effectors exists between amino acids 1 to 15.

However, the replacement of IpaC secretion signal with residues 1 to 15 of IpaB resulted in lower levels of secretion and invasion, which may suggest that the entire secretion signal for IpaB does not exist within residues 1 to 15. It is possible that only a portion of the IpaB secretion signal was used to replace the native IpaC secretion signal. While it is unclear whether a secretion hierarchy exists for the TTSS-dependent export of the *Shigella* Ipa proteins, it is clear that type-III secretion by *S. flexneri* is triggered by a diverse set of N-terminal peptide sequences and that heretofore unseen sequences are able to trigger this type of secretion.

CHAPTER 2:

Functional Analysis of IpaD

Introduction

Several studies have focused on IpaB and led to the hypothesis that this protein has several roles that are essential for *Shigella* invasion of epithelial cells. Studies by Sansonetti and co-workers were key in showing that although IpaB associates with the chaperone IpgC in the bacterial cytoplasm and IpaC following type-III secretion, the auxiliary protein IpaD does not associate with IpgC prior to secretion and is not a part of the IpaB-IpaC extracellular complex (Menard 1994). On the other hand, an IpaB and IpaD complex has been proposed to have a role in controlling the secretion of the *Shigella* type-III secreted effector proteins (Menard 1994). Sansonetti's group has suggested that IpaB and IpaD associate within the type-III secretion apparatus to prevent secretion of all the Ipa proteins prior to host-cell contact (Menard 1994). Once host-cell contact has been detected by the bacterium (by an unknown mechanism), IpaB and IpaD are thought to disassociate from their position within the TTSS to allow productive secretion of themselves and the other presynthesized Ipa proteins via the *Shigella* type-III secretion apparatus. The absence of either IpaB or IpaD results in hyper-secretion of approximately 15 proteins, which include the remaining Ipa proteins (Menard 1994), (Parsot 1995). The result of uncontrolled secretion of the Ipa proteins appears to be a loss in the ability to invade cultured cells and to lyse erythrocytes (Menard 1994). Since very little information exists regarding IpaD and its role in the invasion process, we decided to

investigate this protein. The experiments presented in this chapter explore the functional organization of IpaD, the role IpaD plays in controlling secretion of other Ipa proteins, and the effect on Ipa hyper-secretion of the *Shigella* invasion phenotype when mutations are introduced in IpaD.

Experiments are also presented to compare the invasion-related functions of IpaD and its putative functional homologue from *Salmonella typhimurium*, *Salmonella* invasion protein D (SipD). Previous studies in our lab have compared the invasion-related functions of IpaC from *Shigella* and its *Salmonella* homologue, SipC. It was determined that *Salmonella* invasion could be restored by complementing Δ*sipC* mutant *Salmonella* strain SB221 with the *Shigella ipaC* gene (Osiecki 2001). In contrast, *Shigella* invasiveness could not be restored when the *Salmonella sipC* gene was expressed in the Δ*ipaC* mutant *S. flexneri* strain SF621 (Osiecki 2001). We wanted to explore the functional comparison of IpaD and SipD when generated in the Δ*sipD* mutant *Salmonella* strain SB220 and Δ*ipaD* mutant *Shigella* strain SF622, respectively, to extend these previous studies.

Results

Functional analysis of IpaD

To determine the functionally important regions of IpaD, a series of deletion mutants were constructed as described in Materials and Methods. Deletions ranging from 4 to 40 amino acids in length were created and expressed in the Δ*ipaD* mutant *S. flexneri* strain SF622 using the pWPsf4 plasmid. It has been shown that IpaD is able

to form dimers in solution (Marquart 1995), possibly via disulfide bridges formed involving the single Cys residue (at amino acid 321) near the C terminus of IpaD. Interestingly, its *Salmonella* homologue SipD, which does not encode a Cys residue, has not been found to form homodimers. We wanted to determine the importance of the single Cys at amino acid 321, by creating a substitution mutant that would encode alanine and serine instead of serine and cysteine. This double substitution mutant and the deletion mutants were expressed in pWPsf4 and transformed into *S. flexneri* SF622 (Δ*ipaD*). A schematic diagram of the deletion mutants and the double substitution mutant are summarized in Figure 8. Invasion and hemolysis functions were tested in *S. flexneri* SF622 (Δ*ipaD*) expressing each of the mutants and these experiments were followed up with ELISA and SDS-PAGE analyses aimed at determining IpaD's role in overall Ipa protein secretion.

Invasion data demonstrated that 12 of the 14 deletion mutants described in Figure 8 were unable to direct SF622 invasion of Henle 407 cells. The two mutants that were able to restore invasion contained deletions from amino acid 41 to 80 or 81 to 120, respectively (summarized in Table 2). Likewise, the substitution mutant possessing Ala and Ser in place of Ser and Cys (called $IpaD^{SC321AS}$) was able to restore *Shigella* invasion indicating that Cys321 is not essential for IpaD function. These three IpaD mutants that restored invasion SF622 were able to do so at or near 100% of the value obtained for SF622 expressing full-length IpaD. Likewise, hemolytic activity was completely restored when SF622 was complemented with $IpaD^{SC321AS}$ and partially restored (> 35%) when SF622 was complemented with

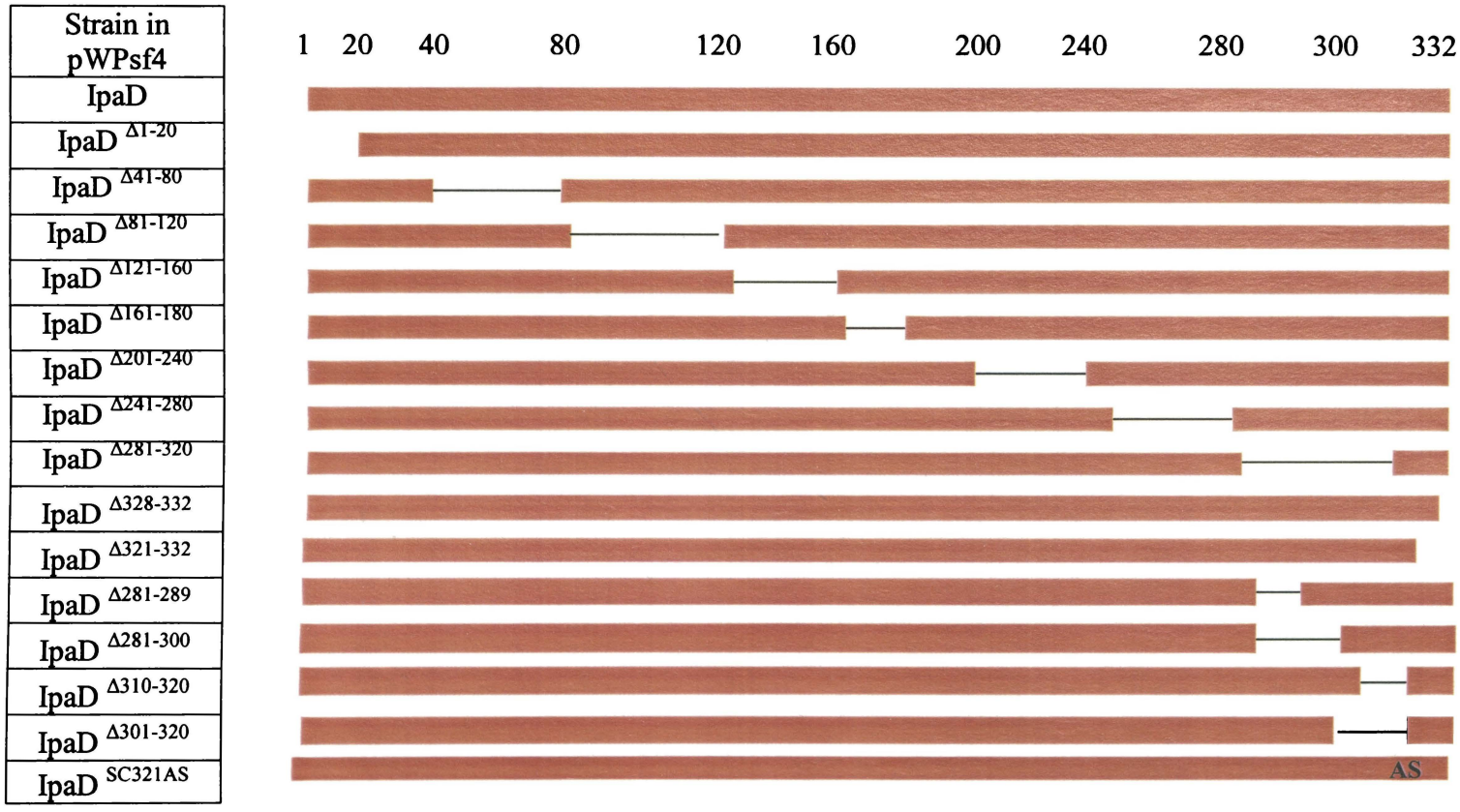

Figure 8. A schematic diagram of IpaD mutants. Fourteen deletion mutants were constructed as well as one substitution mutant. The substitution mutant IpaD^{SC321AS}, was generated to analyze a single Cys residue found near the C terminus of IpaD. All mutants were expressed in SF622 harbouring pWPsf4 plasmid.

Strain in pWPsf4	Relative Invasion (%)	IpaD Secretion	Relative Hemolysis (%)
IpaD	100.0 (+/- 16.0)	+	100 (+/- 1.0)
IpaD $^{\Delta 1\text{-}20}$	0.0 (+/- 0.0)	-	0.03 (+/- 0.03)
IpaD $^{\Delta 41\text{-}80}$	95.0 (+/- 15.0)	+	35.0 (+/- 0.5)
IpaD $^{\Delta 81\text{-}120}$	101.0 (+/- 11.0)	+	52.0 (+/- 2.0)
IpaD $^{\Delta 121\text{-}160}$	1.0 (+/- 1.0)	+	2.0 (+/- 0.2)
IpaD $^{\Delta 161\text{-}180}$	3.0 (+/- 0.2)	+	2.0 (+/- 0.2)
IpaD $^{\Delta 201\text{-}240}$	1.0 (+/- 0.4)	+	5.0 (+/- 0.2)
IpaD $^{\Delta 241\text{-}280}$	3.0 (+/- 0.2)	+	6.0 (+/- 1.0)
IpaD $^{\Delta 281\text{-}320}$	0.1 (+/- 0.2)	+	2.0 (+/- 0.2)
IpaD $^{\Delta 328\text{-}332}$	2.0 (+/- 0.6)	+	15.0 (+/- 0.2)
IpaD $^{\Delta 321\text{-}332}$	3.0 (+/- 0.0)	+	12.0 (+/- 1.0)
IpaD $^{\Delta 281\text{-}289}$	0.8 (+/- 0.1)	+	20.0 (+/- 1.0)
IpaD $^{\Delta 310\text{-}320}$	1.4 (+/- 2.0)	+	12.5 (+/- 0.1)
IpaD $^{\Delta 281\text{-}300}$	1.3 (+/- 0.0)	+	15.0 (+/- 0.0)
IpaD $^{\Delta 301\text{-}320}$	4.7 (+/- 0.1)	+	15.0 (+/- 0.1)
IpaD^{SC321AS}	147.0 (+/- 4.6)	+	100.0 (+/- 0.0)

Table 2. The percent invasion, secretion, and hemolytic activities of the IpaD mutants. Invasion, ELISA, and hemolysis data were obtained by generating these mutants in pWPsf4 *S. flexneri* strain SF622 (Δ*ipaD*). Invasion and hemolytic activities are represented as relative percents (along with their corresponding standard deviations) based on the positive control, pWPsf4d in SF622. Secretion of IpaD is indicated by a plus or minus. Data are shown as an average of three trials. +/- standard deviation

IpaD$^{\Delta 41\text{-}80}$ or IpaD$^{\Delta 81\text{-}120}$, but not with the other IpaD mutants (Table 2). ELISA assays using polycolonal antibodies specific for IpaD were then performed on all mutants to ensure that IpaD was secreted from the bacterium (Table 2). All constructs tested in this study were able to generate IpaD (data not shown) and only one construct was found to have lost the ability to secrete IpaD, which was IpaD$^{\Delta 1\text{-}20}$ (Table 2). These results indicate that the IpaD secretion signal is contained within the first 20 amino acids, consistent with data generated from studies on IpaC. Therefore, it was expected that pWPsf4d$^{\Delta 1\text{-}20}$ expressed in *S. flexneri* SF622 would not be invasive or display any hemolytic activity (Table 2).

Hypersecretion of IpaB and IpaC

ELISA assays employing IpaC monoclonal antibodies were used to monitor the IpaD mutants described in Figure 8 for hypersecretion of IpaC. It appears that only one construct generated a form of IpaD that maintained what appeared to be the proper control of IpaC secretion, which was IpaD^{SC321AS}. All other constructs gave rise to an IpaD mutant that led to oversecretion of IpaC (Figure 9). However, based on the error bars, it is possible that pWPsf4d$^{\Delta 301\text{-}320}$ is able to control secretion of IpaC, therefore not allowing hypersecretion of IpaC (Figure 9). Conclusions are difficult to draw from these results because statistical analysis has not been performed on the data obtained from these experiments. Because these results are somewhat inconclusive, we chose to perform another experiment to study hypersecretion when IpaD has been altered.

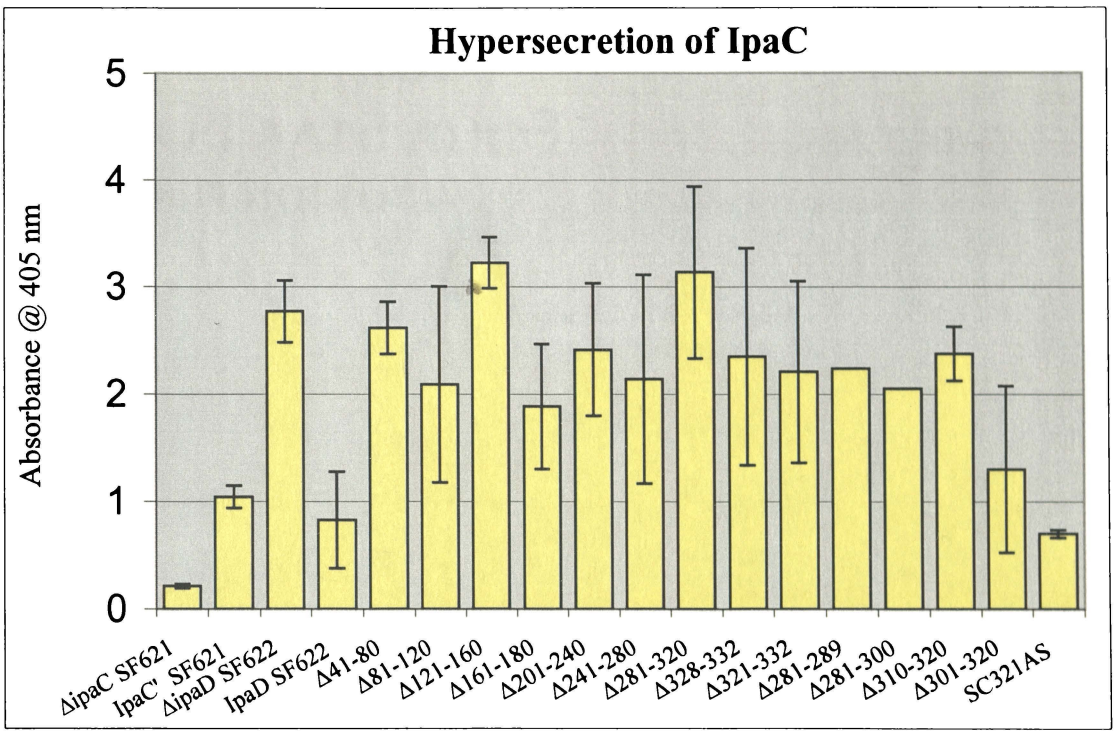

Figure 9. Hypersecretion of IpaC from ELISA data. IpaC monoclonal antibodies were used in the ELISA to detect hypersecretion of IpaC as a result of IpaD deletion and substitution mutagenesis. Antibody controls include pWPsf4 and pWPsfc' after transformation into *Shigella flexneri* SF621 (Δ*ipaC*). Secretion of IpaC is regulated when wild-type IpaD is present (pWPsf4d). In the absence of IpaD hypersecretion of IpaC occurs (pWPsf4 transformed in *S. flexneri* SF622 Δ*ipaD*). IpaD deletion and substitution mutants are compared to pWPsf4d to determine whether deleting portions of IpaD will impair the regulation of IpaC secretion. Data are shown as an average of three trials. +/- standard deviation

To further explore the role of IpaD as the controller of Ipa protein secretion, SF622 harboring the *ipaD* mutants described in Figure 8 were grown to stationary phase and the spontaneous secretion of IpaB and IpaC into the culture supernatant was monitored by SDS-PAGE. As shown in Figure 10 the absence of *ipaD* expression resulted in hyper-secretion of IpaB and IpaC. Expression of wild-type *ipaD* restored control of IpaB and IpaC secretion (Figure 10). The molecular masses of the proteins are 42 kDA (IpaC) and 62 kDa (IpaB). Typically, IpaB is observed as a single band on SDS-PAGE gels. Therefore, we believe the IpaB doublet visualized in Figure 10 to be an artifact. It would be necessary to perform a Western blot on these gels to determine which band is actually IpaB. Because the N terminus of the Ipa proteins possesses the secretion signal that is recognized by the *Shigella* TTSS, it was anticipated that pWPsf4 containing the insert $IpaD^{\Delta 1\text{-}20}$ expressed in SF622 would result in the same secretion phenotype as the *ipaD* knockout, which we observed (data not shown). However, of all the remaining IpaD mutants, only $IpaD^{SC321AS}$ retained control of the secretion of the other effector Ipa proteins. Even the deletion mutants able to restore invasion and display some level of contact-hemolysis (as shown in Table 2) were unable to control IpaB and IpaC secretion (Figure 10). Some levels of IpaA and IpgD hypersecretion can also be observed as a result of deleting portions of IpaD, but are not observed when Δ*ipaD* SF622 is expressed. Additionally, $IpaD^{\Delta 161\text{-}180}$ displayed hypersecretion of IpaA, IpaB, and IpaC, but the level of hypersecretion appears to be lower than the hypersecretion observed in other deletion mutants, which could indicate a region of possible

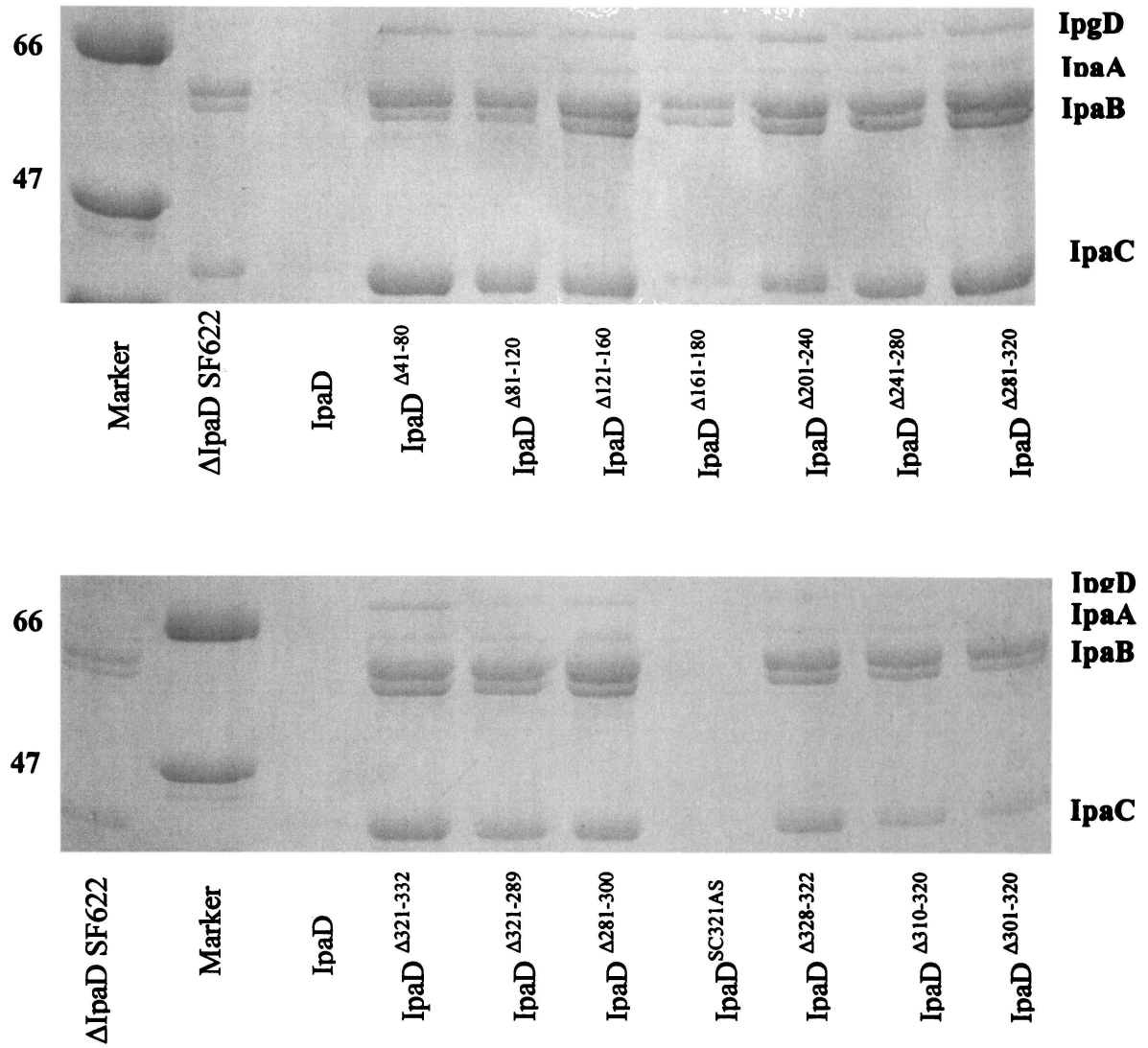

Figure 10. Hypersecretion of IpaB and IpaC in IpaD mutants. 10% SDS-PAGE gels of IpaD mutants after TCA precipitation. The SDS-PAGE marker contains BSA 66 kDa, which is the approximate size of IpaB (62 kDa) and $IpaC^{-19-363}$ plus 20 amino acid histadine-containing tag 47 kDa (native IpaC is 42 kDa). In the absence of IpaD, hypersecretion is observed (Δ*ipaD* SF622), however when IpaD is present, secretion of effectors are controlled (IpaD). All constructs were generated in *S. flexneri* SF622 harbouring the plasmid pWPsf4.

importance in controlling secretion. Interestingly, the ratio of oversecreted IpaB to IpaC does not appear to remain constant for each of the SF622 derivatives expressing different *ipaD* deletion mutants. Mutants harbouring deletions near the N terminus of IpaD and which are still able to restore invasiveness and contact-hemolysis activities to SF622 appear to secrete IpaC in greater quantities than IpaB (Figure 10). However, as deletions were introduced farther downstream, IpaB appears to be secreted in higher quantities than IpaC (Figure 10). Although we may be able to conclude that the regulation of secretion does not ensure invasiveness, we are not able to explain the difference in IpaB-IpaC ratios.

Functional Comparison of IpaD and SipD

It has been previously demonstrated that IpaC can replace SipC in *Salmonella.* SipC, however, is very inefficient at restoring invasiveness to *Shigella* SF621 (Osiecki 2001). These findings may reflect the fact that IpaC possesses specific effector activities that activate the host cytoskeleton while SipC does not. In the case of *Salmonella*, part of its effector function (actin reorganization) is carried out by another protein called SopE (Hardt 1998). To determine whether IpaD and SipD are interchangeable, each was expressed in *Shigella flexneri* SF622 or in *Salmonella typhimurium* BJ68 (Δ*sipC* Δ*sipD*). Since *S. typhimurium* BJ68 contains null mutations in both *sipC* and *sipD* double complementation was employed. Therefore, *sipC* and *ipaC* were expressed with the plasmids pWPsf, containing an ampicillin resistance gene and *ipaD* and *sipD* were expressed using pWPsf2, which

expresses a chloramphenicol resistance gene. Expression of *ipaD* was carried out in the presence of either *ipaC* or *sipC*. Likewise, the expression of *sipD* was carried out in the presence of *ipaC* or *sipC*. Expressing *sipD* in the Δ*ipaD* mutant *S. flexneri* strain SF622 did not restore invasiveness to this organism (Figure 11). Expression of *ipaD,* however, could restore invasiveness to BJ68 with complementation of either *ipa*C or *sipC* (Figure 12). We also observed a trend of higher levels of *Salmonella* invasion when the homologous effectors, SipC/SipD and IpaC/IpaD, were generated with BJ68 (Figure 12). However, the statistical differences here are in question and currently do not support the claim that higher levels of invasion will occur when homologous effector are generated in BJ68.

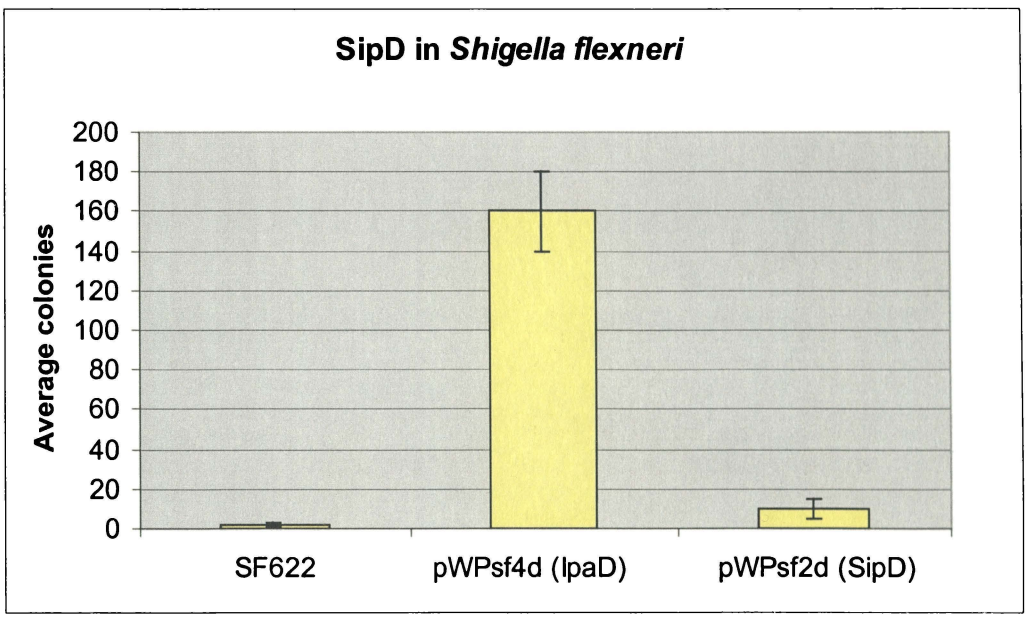

Figure 11. SipD expressed in *S.flexneri* SF622. *Shigella* invasion data representing SF622 (Δ*ipaD*) expressing either *ipaD* or *sipD*. Complementation with IpaD was carried out in pWPsf4 and SipD was produced with pWPsf2. Both plasmids were generated in *S.flexneri* SF622. Data are shown as an average of three trials +/- standard deviation

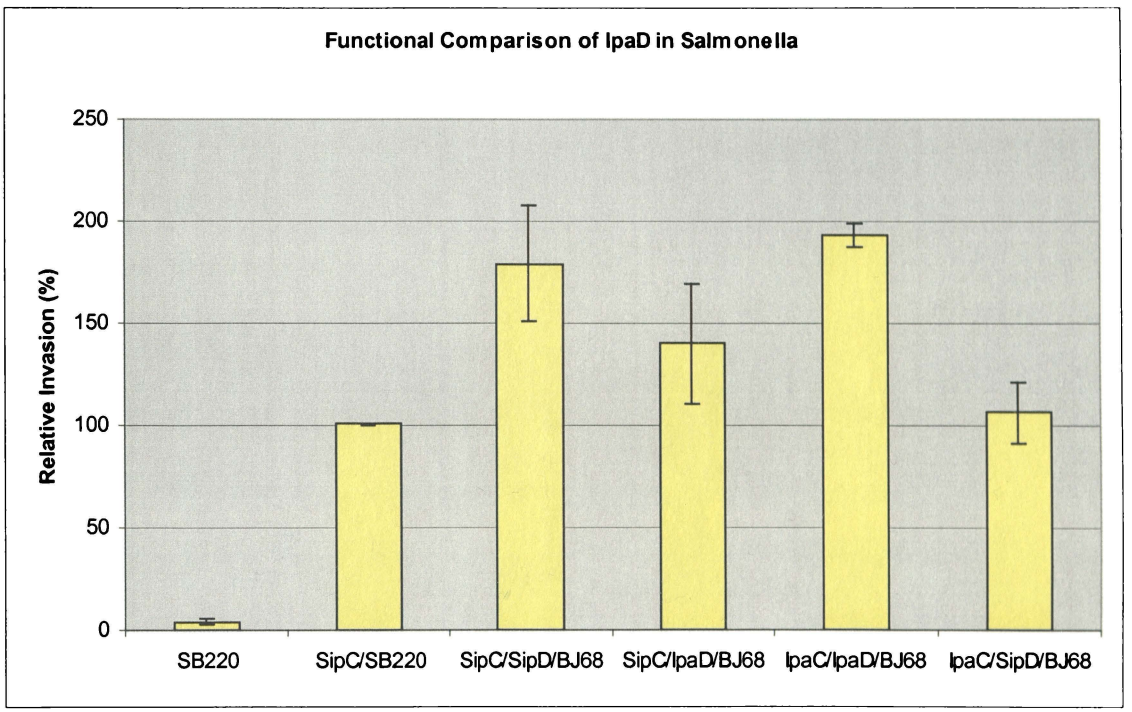

Figure 12. IpaD invasion data in *Salmonella.* Invasion data based on colonies formed. SipC generated in SB220 (Δ*sipC*) is the positive control; SB220 (Δ*sipC*) is the negative control. BJ68 was used to study the functional comparison of SipD and IpaD. BJ68 strain, containing null mutations in both *sipC* and *sipD* were complemented with *sipC/sipD*, *sipC/ipaD*, *ipaC/ipaD,* or *ipaC/sipD*. *sipC* and *ipaC* were expressed from pWPsf, containing an ampicillin resistance gene and *sipD* and *ipaD* were expressed from pWPsf2, containing a chloramphenicol resistance gene. Data are shown as an average of three trials. +/- standard deviation

Conclusions

The data presented here indicate that amino acids 1 to 20 are important for secretion of IpaD and amino acids 41 to 120 are not important for IpaD secretion, invasion and contact-hemolytic functions. In contrast, invasion function is lost when deletions are generated beyond amino acid 120 and hemolytic activity for these mutants is reduced to basal levels (6-12%). However, all the IpaD deletion mutants resulted in hyper-secretion of IpaC and IpaB (even those that restored invasiveness to SF622), indicating that control of secretion requires that the IpaD structure be completely intact. Despite allowing hypersecretion of IpaC and IpaB, mutants $IpaD^{\Delta 41\text{-}80}$ and $IpaD^{\Delta 81\text{-}120}$ generated in pWPsf4 in SF622 restored invasiveness and contact-hemolysis, disproving Sansonetti's proposal (Menard 1994) that hypersecretion was the reason *ipaD* knockouts were rendered noninvasive.

Recombinant IpaD forms homodimers *in vitro* (Marquart 1995), and separation of these IpaD dimers by SDS-PAGE requires reducing agents, which indicates that IpaD forms intramolecular disulfide bonds (Picking W.D., unpublished data). Similar dimers have been observed for IpaD in *Shigella* culture supernatants (W.L. Picking and A. Blocker, unpublished data). A single Cys residue near the C terminus of IpaD (amino acid 321) was altered to replace the Cys and Ser with Ser and Ala, under the assumption that if the disulfide bond (lending to stable dimerization) was important for IpaD function, then disrupting the disulfide bridge by mutagenesis would interfere with IpaD function. However, substituting Ser for Cys did not hinder the invasion, secretion, and hemolytic activities of IpaD, implying that

the Cys at 321 is not important for these functions and lending to the possibility that IpaD dimerization is not important for these activities.

The data obtained from the functional comparison of IpaD and SipD were consistent with SipC and IpaC comparison studies previously performed in the Picking lab. *ipaD* and *ipaC* expressed with BJ68 (*ΔsipD, ΔsipC*) was able to restore invasion in *Salmonella;* however, *sipD* did not restore invasion when expressed from pWPsf2 in *S. flexneri* strain SF622.

To date, the only function proposed for IpaD is to associate with IpaB within the needle complex of the TTSS to prevent secretion of the other TTSS effectors prior to host-cell contact (Menard 1994). It has also been demonstrated that IpaD forms homodimers in culture supernatants (Marquart 1995), but the functional importance of these dimers are not clear. Likewise, there is not information available with regard to the functional organization of IpaD. From the invasion, hemolysis, and secretion data presented here, a preliminary functional organization can be proposed for IpaD (Figure 13). Secretion data show that amino acids 1 to 20 are important for the export of the protein, which is not surprising based on our findings for IpaC and findings by others regarding the TTSS effector proteins of *Yersinia* (Lloyd 2001),(Lloyd 2002). Interestingly, amino acids 41 to 120 of IpaD can be eliminated without altering the invasive ability. Although 161 to 180 displayed a lower level of Ipa hypersecretion, all the deletions introduced into IpaD gave rise to a hypersecretion phenotype. More importantly, even those deletions that do not eliminate IpaD's invasion-related function hypersecreted IpaB and IpaC. Together, these data show that IpaD is

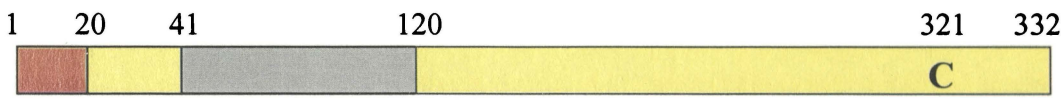

Figure 13. A schematic diagram of the functional organization of IpaD. The IpaD secretion signal is located between amino acids 1 to 20 (red); regions important for invasion and regulating the secretion of other effectors are shown in yellow 21 to 40 and 121 to 332. Amino acids 41 to 120 are not needed for invasion (gray). A single cysteine is located at amino acid 321 that is not required for maintaining the regulation of other secreted effector proteins.

involved in controlling IpaB and IpaC secretion. Loss of secretion control, however, is not sufficient to render *S. flexneri* noninvasive. It is not yet clear what invasion-related functions, other than regulation of secretion, IpaD possesses.

DISCUSSION

We have demonstrated here that only one secretion signal exists within native IpaC and it is contained within the first 15 amino acids of the protein. Interestingly, during the process of identifying the native secretion signal, we have shown that it is possible to synthesize artificial secretion signals that are recognized by the *Shigella* type-III secretion system. Two synthetic signals were created in our lab. The first of these is composed of eight β-galactosidase residues (MTMITNSH) attached to the N terminus of the –19 to –1 region of the IpaC first generated in our lab. The second is encoded on the plasmid pWPsf4c$^{\Delta\text{-}5\text{—}20\text{HIT}}$ This secretion signal is composed of amino acids –19 to –6, except that residues –18 (Leu), -16 (Lys), and –14 (Phe) have been replaced by His, Ile, and Thr, respectively. Without these substitutions, the IpaC derivative is not secreted and therefore cannot contribute to invasion of epithelial cells. Both of the "artificial secretion signals" allow for IpaC secretion and invasion at levels equal to that observed when the native secretion signal is present. A summary of the active (and inactive) TTSS-specific secretion signals generated as part of this investigation is given in Table 3. Collectively, the data presented here imply that sequences at the N terminus of the native form of IpaC can be altered to promote or eliminate that protein's secretion. The first synthetic secretion signal (eight β-galactosidase residues attached to the N terminus of the –19 to –1 region of IpaC) was created unintentionally and was not actually identified until generation of the deletion mutant IpaC$^{\Delta\text{-}5\text{—}20}$ (from the plasmid pWPsfc$^{\Delta\text{-}5\text{—}20}$).

Table 3. Sequences used to construct a synthetic IpaC secretion signal and relative levels of secretion and invasion.

Construct in *S. flexneri* SF621	N-terminal IpaC sequence	Secretion (%)	Relative Invasion
pWPsfc	MTMITNSH*MLQKQFCNKLLLDTNKENV***MEIQNTKPTQTLYTDISTKQ**	99	77
pWPsfc'	MTMITNSH**MEQNTKPTQTLYTDISTKQ**	100	100
pWPsf4c	*MLQKQFCNKLLLDTNKENV***MEIQNTKPTQTLYTDISTKQ**	97	90
pWPsf4c'	**MEIQNTKPTQTLYTDISTKQ**	100	105
pWPsfc$^{\Delta-5-20}$	MTMITNSH*MLQKQFCNKLLLDT*	100	85
pWPsf4c$^{\Delta-5-20}$	*MLQKQFCNKLLLDT*	0	0
pWPsf4c$^{\Delta-5-20HIT}$	*MHQIQTCNKLLLDT*	100	100

The sequences shown are all the amino acids preceding what would be residue 21 of native IpaC. The remainder of the protein (21 to 363) is not changed. Bold faced amino acid sequences are native IpaC sequences; italicized amino acids are residues –19 to –1 of IpaC$^{-19-363}$, which were originally described as representing the first 19 amino acids of native IpaC according to the DNA sequence data reported by Venkatesan and colleagues (Venkatesan 1988). Underlined amino acids are the eight β-galactosidase residues added to the N terminus when IpaC is generated from the plasmid pWPsf.

Synthetic secretion signals have previously been created in the *Yersinia* with the type-III secretion effector, YopE (Lloyd 2002). The YopE synthetic secretion signal was created by substituting the native secretion signal with an alternating isoleucine and serine sequence (Lloyd 2002). When this was idea was tested in our system, the alternating sequence of isoleucine and serine could not restore IpaC secretion. Although it is agreed upon that the secretion signals of the type-III secretion effectors resides at the N terminus of the proteins (Lloyd 2001) (Lloyd 2002) (Harrington 2003), the secretion signals may possess unique qualities. While the alternating sequence of isoleucine and serine restore secretion in *Yersinia*, this sequence did not restore secretion in IpaC. Furthermore, replacing the IpaC secretion signals with other putative secretion signals from *Shigella* were shown to restore invasion (except with IpaB) and secretion, although there appears to be no sequence homology between these secretion signals. It would be interesting to replace the IpaC secretion signal with the YopE secretion signal to further explore sequences that are recognized by they type-III secreton.

Once we discovered that it is possible to generate artificial secretion signals in *Shigella*, we chose to better define the precise region making up the native IpaC secretion signal, by investigating what type of site-specific mutations could be used to destroy this signal's activity, and to see if a "nonfunctional secretion signal" (i.e. that of IpaC synthesized from pWPsf4c$^{\Delta\text{-}5\text{—}20}$) could be converted into an active signal. Based on the native secretion signals from IpaA IpaB, IpaD and IpgD, all of which restore some degree of secretion function to IpaC$^{\Delta\text{-}5\text{—}20}$, it appeared that there was

some periodicity with respect to hydrophilic and hydrophobic amino acids that allowed recognition for export by the *Shigella* TTSS. This and the data generated by Wolf-Watz, *et. al.* from the *Yersinia* type-III secretion substrate YopE (Lloyd 2002), prompted us to convert the IpaC generated from pWPsf4c$^{\Delta\text{-}5\text{—}20}$ into IpaC$^{\Delta\text{-}5\text{—}20\text{HIT}}$ which has some degree of alternating polar/nonpolar residues, which resulted in active secretion. In all, the data suggest that the *Shigella* TTSS recognizes a general physical or structural feature at or near the N terminus of proteins targeted for secretion. However, each protein may possess a unique characteristic that is specific for recognition by the secretion.

Our lab has continued to make progress toward developing a clear understanding of the functional and structural features of IpaC from *S. flexneri.* Since the first published information on the functional organization of IpaC in 2001, the Picking lab has continued to provide more and more detail on the functional organization of IpaC. Large functional domains have been more carefully examined to identify smaller and sometimes more subtle functional regions within the IpaC protein (Figure 14). Based on the data presented here, we can now confidently conclude that the secretion signal lies within the first 15 amino acids; however, it may be possible to artificially add additional components upstream without affecting secretion (W. L. Picking and S. Shah, unpublished data). Deleting amino acids beyond amino acid 15 does not negatively affect secretion of IpaC nor does it negatively affect invasion (until amino acid 50). It has previously been shown that IpaC binds to IpaB as well as the chaperone, IpgC (Menard 1994). Recently, findings

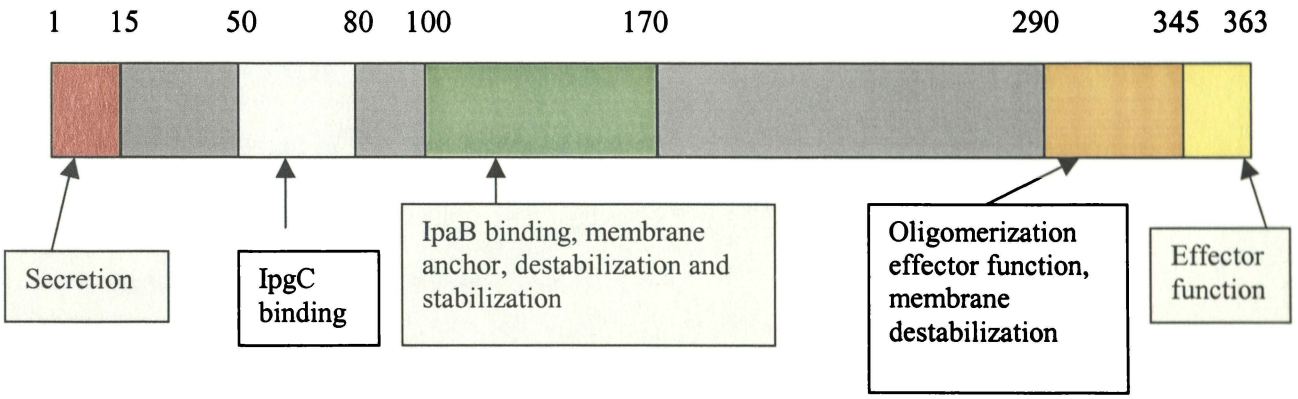

Figure 14 Schematic diagram of the current functional organization of IpaC. This diagram is based on data generated by the Picking lab. Amino acids required for IpaC secretion are shown in red (1 to 15). The IpgC chaperone binding site is located between 50 to 80 amino acids, shown in white. The IpaB binding site, membrane anchor, membrane destabilization, and stabilization of protein structure are shown green (amino acids 100 to 170). Oligomerization via predicted coiled-coil, effector function, and membrane destabilization 290-345 (orange); effector function 345-363 (yellow).

from our lab have identified binding sites for both chaperone and IpaB binding, which are near the IpaC N terminus, but downstream from the secretion signal described here (Harrington 2003). Other studies in our lab suggest a coiled-coil exists near the C terminus of the protein and is part of an important effector region along with the 19 amino acid C-terminal tail (Kueltzo 2003). Membrane destabilization and protein stabilization regions have also been identified near the center of IpaC (Kueltzo 2003). The generation of these data has allowed us to propose an updated model for the functional organization of IpaC (Figure 14).

On another front, also related to Ipa protein secretion, we began investigating the stucture-function relationships of IpaD. The only function thus far associated with IpaD has been the regulation of secretion in type-III effectors by *Shigella* (Menard 1994). Sansonetti and co-workers previously proposed that IpaD must properly regulate secretion for *Shigella* to efficiently invade epithelial cells (Menard 1994). Based on data generated in this study, we partially agree with Sansonetti's hypothesis. We agree that IpaD is able to prevent over-secretion of other Ipa effectors prior to contact with a host cell. However, we propose that IpaD must have another role related to the invasion process, as well. Deletion of amino acids 41 to 120 of IpaD does not affect its ability to contribute to *Shigella* invasion of Henle 407 cells. However, deletion of these residues knocks out IpaD's ability to properly regulate the secretion of IpaB and IpaC. Based on this observation, we also propose that IpaD does not have to maintain proper secretion control to be able to contribute to cellular invasion, which contradicts Sansonetti's group.

A shared mechanism in which many gram-negative pathogens deliver their effector proteins to host cells is via a type-III secretion system. TTSS effectors from one gram-negative pathogen often have functional homology with those produced by at least one other bacterium. Therefore increasing our understanding of a small set of type-III secretion effector proteins, such as IpaC or IpaD, may not only provide useful information to other groups working with *Shigella,* but will also aid other researchers in elucidating functions for their homologous proteins, from *Salmonella*, for example.

Salmonella and *Shigella* invasion occur in a similar fashion, both utilizing a type-III secretion system for the delivery of their effectors as well as inducing a ruffling of the membrane (Kubori 1998) (Osiecki 2001) (Young 1999) (Hueck 1998). Based on data generated over years of work by other scientists studying *Shigella* pathogenesis and the work presented here, a model for *Shigella* invasion can be proposed. Prior to host cell contact, IpgC individually binds to IpaB or IpaC, preventing the association of IpaB to IpaC. IpaB and IpaD are bound, possibly within the needle, preventing the secretion of other effectors. Upon host cell contact, IpaB and IpaD partially dissociate from each other allowing the secretion of other effectors and other IpaB proteins. IpgC disassociates from IpaB and IpaC and allows their secretion signals to be exposed to the type-III secretion apparatus. Once the secreton recognizes their secretion signals, they are allowed to be exported. IpaD guides IpaB to the host cell membrane. Once IpaB has been firmly established with the host membrane, IpaD completely disassociates, which allows IpaB to complex with IpC. This complex forms a pore within the host cell membrane to allow the direct secretion

of *Shigella* effector proteins to the host cytoplasm. IpaC can then interact with Cdc42 to initiate a cascade of events, eventually resulting in a cytoskeletal rearrangement and the uptake of the bacterium. IpaA and IpgD then function to restore homeostasis to the cell membrane once the bacterium has been engulfed.

SOURCES CITED

Allaoui, A., Sansonetti, P.J., and Parsot, C. (1993). "MxiD, an outer membrane protein necessary for the secretion of the *Shigella flexneri* Ipa invasins." Molecular Microbiology **7**(1): 59-68.

Anderson, D. M., Schneewind, O. (1999). "*Yersinia enterocolitica* type III secretion: an mRNA signal that couples translation and secretion of YopQ." Molecular Microbiology **31**(4): 1139-48.

Andrews, G. P., Hromockyj, A.E., Coker, C., and Maurelli A.T. (1991). "Two novel virulence loci, *mxiA* and *mxiB*, in *Shigella flexneri* 2a facilitate excretion of invasion plasmid antigens." Infection and Immunity **59**: 1997-2005.

Bahrani, F. K., Sansonetti, P. J., Parsot, C. (1997). "Secretion of Ipa proteins by *Shigella flexneri*: inducer molecules and kinetics of activation." Infection and Immunity **65**(10): 4005-10.

Barzu, S., Benjelloun-Touimi, Z., Phalipon, A., Sansonetti, P., Parsot, C. (1997). "Functional analysis of the *Shigella flexneri* IpaC invasin by insertional mutagenesis." Infection and Immunity **65**(5): 1599-605.

Baudry, B., Kaczorek, M., Sansonetti, P. J. (1988). "Nucleotide sequence of the invasion plasmid antigen B and C genes (*ipaB* and *ipaC*) of *Shigella flexneri*." Microbial Pathogenesis **4**(5): 345-57.

Blocker, A., Gounon, P., Larquet, E., Niebuhr, K., Cabiaux, V., Parsot, C., Sansonetti, P. (1999). "The Tripartite Type III Secreton of *Shigella flexneri*

Inserts IpaB and IpaC into Host Membranes." Journal of Cell biology **147**(3): 683-693.

Buysse, J. M., Stover, C.K., Oaks, E.V., Venkatesan, M., Kopecko, D.J. (1987). "Molecular Cloning of Invasion Plasmid Antigen (*ipa*) Genes from *Shigella flexneri*: Analysis of *ipa* Gene Products and Genetic Mapping." Journal of Bacteriology: 2561-2569.

Clerc, P., Sansonetti, P.J. (1987). "Entry of Shigella flexneri into HeLa cells: evidence for directed phagocytosis involving actin polymerization and myosin accumulation." Infection and Immunity **55**(11): 2681-8.

Cossart, P. (1997). "Subversion of the Mammalian Cell Cytoskeleton by Invasive Bacteria." Journal of Clinical Investigation **99**(10): 2307-2311.

De Geyter, C., Vogt, B., Benjelloun, Z., Sansonetti, P.J., Ruysschaert, J.M., Parsot, C., and Cabiaux, V. (1997). "Purification of IpaC, a protein involved in entry of *Shigella flexneri* into epithelial cells and characterization of its interaction with lipid membranes." Federation of European Biochemical Society **400**: 149-54.

De Geyter, C., Wattiez, R., Sansonetti, P., Falmagne, P., Ruysschaert, J. M., Parsot, C., Cabiaux, V. (2000). "Characterization of the interaction of IpaB and IpaD, proteins required for entry of *Shigella flexneri* into epithelial cells, with a lipid membrane." European Journal of Biochemistry **267**(18): 5769-76.

DuPont, H. L., Hornick, R.B., Dawkins, A.T., Snyder, M.J., and Formal, S.B. (1969). "The response of man to virulent Shigella flexneri 2a." Journal of Infectious Disease **119**(3): 296-9.

DuPont, H. L., Levine, M.M., Hornick, R.B., Formal, S.B. (1989). "Inoculum size in shigellosis and implications for expected mode of transmission." Journal of Infectious Disease **159**(6): 1126-8.

Hale, T. L. (1991). "Genetic basis of virulence in *Shigella* species." Microbiological Reviews **55**(2): 206-24.

Hale, T. L., Oaks, E.V., Formal, S.B. (1985). "Identification and antigenic characterization of virulence-associated, plasmid-coded proteins of *Shigella* spp. and enteroinvasive *Escherichia coli*." Infection and Immunity **50**(3): 620-9.

Hardt, W. D., Chen, L.M., Schuebel, K.E., Bustelo, X.R., and Galan, J.E. (1998). "*S. typhimurium* encodes an activator of Rho GTPases that induces membrane ruffling and nuclear responses in host cells." Cellular Microbiology **93**: 815-826.

Harrington, A. T., Hearn, P.D., Picking, W.L., Barker, J.R., Wessel, A., and Picking, W.D. (2003). "Structural Characterization of the N-terminus of IpaC from *Shigella flexneri*." Infection and Immunity **71**(3): 1255-1264.

High, N., Mounier, J., Prevost, M.C., Sansonetti, P.J. (1992). "IpaB of *Shigella flexneri* causes entry into epithelial cells and escape from the phagocytic vacuole." European Molecular Biology Organization Journal **11**(5): 1991-9.

Hilbi, H., Moss, J.E., Hersh, D., Chen, Y., Arondel, J., Banerjee, S., Flavell, R.A., Yuan, J., Sansonetti, P.J., Zychlinsky, A. (1998). "*Shigella*-induced apoptosis is dependent on caspase-1 which binds to IpaB." Journal of Biological Chemisty **273**(49): 32895-900.

Hueck, C. J. (1998). "Type III protein secretion systems in bacterial pathogens of animals and plants." Microbiology and Molecular Biology Reveiw **62**(2): 379-433.

Islam, D., Veress, B., Bardhan, P.K., Lindberg, A.A., Christensson, B. (1997). "*In situ* characterization of inflammatory responses in the rectal mucosae of patients with shigellosis." Infection and Immunity **65**(2): 739-49.

Kaniga, K., Tucker, S., Trollinger, D., Galan, J.E. (1995). "Homologs of the *Shigella* IpaB and IpaC invasins are required for *Salmonella typhimurium* entry into cultured epithelial cells." Journal of Bacteriology **177**: 3965-3971.

Kohler, W. (2000). "Zentralblatt fur Bakteriologie--100 years ago: the isolation of Shigella flexneri." Int Journal of Medical Microbiology **290**(1): 5-6.

Kotloff, K. L., Winickoff, J.P., Ivanoff, B., Clemens, J.d., Swerdlow, D.L., Sansonetti, P.J., Adak, G.K., and Levine, M.M. (1999). "Global burden of *Shigella* infections: implications for vaccine development and implementation of control strategies." Bulletin of the World Health Organization **77**: 651-666.

Krieg, N. R. a. H., J.G. (1984). Bergey's Manual of Systematic Bacteriology. Baltimore, MD, Williams and Wilkins.

Kubori, T., Matsushima, Y., Nakamura, D., Uralil, J., LaraTejero, M., Sukhan, A., et al (1998). "Supramolecular structure of the *Salmonella typhimurium* type III protein secretion system." Science **280**: 602-605.

Kueltzo, L. A., Osiecki, J., Barker, J., Picking, W.L., Ersoy, B., Picking, W.D., Middaugh, C.R. (2003). "Structure-Function Analysis of Invasion Plasmid Antigen C (IpaC) from *Shigella flexneri*." Journal of Biological Chemistry **278**(5): 2792-8.

Labrec, E. H., Schneider, H., Magnani, T.J., and Formal, S.B. (1964). "Epithelial Cell Penetration as an Essential Step in the Pathogenesis of Bacillary Dysentery." Journal of Bacteriology **88**(5): 150-1518.

Lloyd, S. A., Norman, M., Rosqvist, R., and Wolf-Watz, H. (2001). "*Yersinia* YopE is targeted for type III secretion by N-terminal, not mRNA, signals." Molecular Microbiology **39**(2): 520-31.

Lloyd, S. A., Sjostrom, M., Andersson, S., and Wolf-Watz, H. (2002). "Molecular characterization of type III secretion signals via analysis of synthetic N-terminal amino acid sequences." Molecular Microbiology **43**: 51-59.

Marquart, M. E., Picking, W.L., and Picking, W.D. (1995). "Structural analysis of invasion plasmid antigen D (IpaD) from *Shigella flexneri*." Biochemical and Biophysical Research Communications **214**(3): 963-970.

Marquart, M. E., Picking, W.L., Picking, W.D. (1996). "Soluble invasion plasmid antigen C (IpaC) from *Shigella flexneri* elicits epithelial cell responses related to pathogen invasion." Infection and Immunity **64**(10): 4182-7.

Menard, R., Prevost, M.C., Gounon, P., Sansonetti, P., and Dehio, C. (1996). "The secreted Ipa complex of *Shigella flexneri* promotes entry into mammalian cells." Proceedings from the National Academy of Science U.S.A. **93**(3): 1254-8.

Menard, R., Sansonetti, P., Parsot, C., and Vasselon, T. (1994). "Extracellular association and cytoplasmic partitioning of the IpaB and IpaC invasins of *S. flexneri*." Cell **79**(3): 515-25.

Menard, R., Sansonetti, P.J., and Parsot, C. (1993). "Nonpolar mutagenesis of the *ipa* genes defines IpaB, IpaC, and IpaD as effectors of *Shigella flexneri* entry into epithelial cells." Journal of Bacteriology **175**(18): 5899-906.

Menard, R., Sansonetti, P.J., and Parsot, C. (1994). "The secretion of the *Shigella flexneri* Ipa invasins is activated by epithelial cells and controlled by IpaB and IpaD." European Molecular Biology Organization Journal **13**: 5293-5302.

Mounier, J., Vasselon, T., Hellio, R., Lesourd, M., and Sansonetti, P.J. (1992). "*Shigella flexneri* enters human colonic Caco-2 epithelial cells through the basolateral pole." Infection and Immunity **60**(1): 237-48.

Oaks, E. V., Hale, T.L., Formal, S.B. (1986). "Serum immune response to Shigella protein antigens in rhesus monkeys and humans infected with Shigella spp." Infection and Immunity **53**(1): 57-63.

Osiecki, J. C., Barker, J., Picking, W.L., Serfis, A.B., Berring, E., Shah, S., Harrington, A., and Picking, W.D. (2001). "IpaC from *Shigella* and SipC from

Salmonella possess similar biochemical properties but are functionally distinct." Molecular Microbiology **42**(2): 469-81.

Owen, R. L., Peirce, N.F., Apple, R.T., Cary, Jr. W.C. (1986). "M cell transport of *Vibrio cholerae* from the intsetinal lumen into Peyer's patches: a mechansm for antigen sampling and for microbial transepithelial migration." Journal of Infectious Disease **153**: 1108-1118.

Page, A. L., Fromont-Racine, M., Sansonetti, P., Legrain, P., and Parsot, C. (2001). "Characterization of the interaction partners of secreted proteins and chaperones of *Shigella flexneri*." Molecular Microbiology **42**(4): 1133-45.

Parsot, C., Menard, R., Gounon, P., and Sansonetti, P.J. (1995). "Enhanced secretion through the *Shigella flexneri* Mxi-Spa translocon leads to assembly of extracellular proteins into macromolecular structures." Molecular Microbiology **16**(2): 291-300.

Picking, W. L., Coye, L., Osiecki, J.C., Serfis, A.B., Schaper, E., and Picking, W.D. (2001). "Identification of functional regions within invasion plasmid antigen C (IpaC) of *Shigella flexneri*." Molecular Microbiology **39**(1): 100-11.

Sansonetti, P., Ryter, A., Clerc, P., Maurelli, A.T., and Mounier, J. (1986). "Multiplication of *Shigella flexneri* within HeLa Cells: Lysis of the Phagocytic Vacuole and Plasmid-mediated Contact Hemolysis." Infection and Immunity **51**(2): 461-469.

Sansonetti, P. J. (1999). "*Shigella* plays Dangerous Games." American Society of Microbiology News **65**(9): 611-617.

Sansonetti, P. J., Hale, T.L., Dammin, G.J., Kapfer, c., Collins, H.H. Jr., and Formal, S.B. (1983). "Alterations in the pathogenicity of *Escherichi coli* K-12 after transfer of plasmid and chromosomal genes from *Shigella flexneri.*" Infection and Immunity **39**: 1392-1402.

Sansonetti, P. J., Kopecko, D.J., and Formal, S.B. (1981). "*Shigella sonnei* plasmids: evidence that a large plasmid is necessary for virulence." Infection and Immunity **34**(1): 75-83.

Sansonetti, P. J., Kopecko, D.J., and Formal, S.B. (1982). "Involvement of a plasmid in the invasive ability of Shigella flexneri." Infection and Immunity **35**(3): 852-60.

Saskawa, C., Komatsu, K., Suzuki, T., and Yoshikawa M. (1993). "Eight genes in region 5 that form an operon are essential for invasion of epithelial cells by *Shigella flexneri* 2a." Journal of Bacteriology **175**(8): 2334-2346.

Takeuchi, A., Formal, S.B., Sprinz, H. (1968). "Experimental acute colitis in the Rhesus monkey following preoral infection with *Shigella flexneri:* An electron microscope study." American Journal of Pathology **52**(3): 503-29.

Tamano, K., Aizawa, S., Katayama, E., Nonaka, T., Imajoh-Ohmi, S., Kuwae, A., Nagai, S., and Sasakawa, C. (2000). "Supramolecular structure of the *Shigella* type III secretion machinery: the needle part is changeable in length and essential for delivery of effectors." European Molecular Biology Organization Journal **19**(15): 3876-87.

Thirumalai, K., Kim, K.S., and Zychlinsky, A. (1997). "IpaB, a *Shigella flexneri* invasin, colocalizes with interleukin-1 beta- converting enzyme in the cytoplasm of macrophages." Infection and Immunity **65**(2): 787-93.

Tran Van Nhieu, G., Bourdet-Sicard, R., Dumenil, G., Blocker, A., and Sansonetti, P.J. (2000). "Bacterial signals and cell responses during *Shigella* entry into epithelial cells." Cellular Microbiology **2**(3): 187-193.

Trofa, A. F., Ueno-Olsen, H., Oiwa, R., and Yoshikawa, M. (1999). "Dr. Kiyoshi Shiga: discoverer of the dysentery bacillus." Clinical Infectious Diseases **29**(5): 1303-6.

Turbyfill, K. R., Joseph, S.W., and Oaks, E.V. (1995). "Recognition of three epitopic regions on invasion plasmid antigen C by immune sera of rhesus monkeys infected with *Shigella flexneri* 2a." Infection and Immunity **63**: 3927-35.

Venkatesan, M., Buysse, J., and Kopecko, D. (1988). "Characterization of invasion plasmid antigen genes (*ipaBCD*) from *Shigella flexneri*." Proceedings from the National Academy of Science U.S.A. **85**: 9317-9321.

Venkatesan, M. M., Buysse, J.M., and Oaks, E.V. (1992). "Surface presentation of *Shigella flexneri* invasion plasmid antigens requires the products of the spa locus." Journal of Bacteriology **174**(6): 1990-2001.

Venkatesan, M. M., Goldberg, M.B., Rose, D.J., Grotbeck, E.J., Burland, V., and Blattner, F.R. (2001). "Complete DNA sequence and analysis of the large virulence plasmid of *Shigella flexneri*." Infection and Immunity **69**(5): 3271-85.

Von Pawel-Rammingen, U., Telepnev, M.V., Schmidt, G., Aktories, K., Wolf-Watz, H., and Rosqvist, R. (2000). "GAP activity of the *Yersinia* YopE cytotoxin specifically targets the Rho pathway: a mechanism for disruption of actin microfilament structure." Molecular Microbiology **36**(3): 737-48.

Wassef, J. S., Keren, D.F., Mailloux, J.L. (1989). "Role of M cells in initial antigen uptake and in ulcer formation in the rabbit intestinal loop model of shigellosis." Infection and Immunity **57**(3): 858-63.

Watarai, M., Funato, S., Sasakawa, C. (1996). "Interaction of Ipa proteins of *Shigella flexneri* with alpha5beta1 integrin promotes entry of the bacteria into mammalian cells." Journal of Experimental Medicine **183**(3): 991-9.

Young, G. M., Schmiel, D.H., Miller, V. L. (1999). "A new pathway for the secretion of virulence factors by bacteria: the flagellar export apparatus functions as a protein-secretion system." Proceedings from the National Academy of Science U.S.A **96**(11): 6456-61.

Zychlinsky, A., Kenny, B., Menard, R., Prevost, M.C., Holland, I.B., and Sansonetti, P.J. (1994). "IpaB mediates macrophage apoptosis induced by *Shigella flexneri*." Molecular Microbiology **11**(4): 619-27.

Zychlinsky, A., Prevost, M.C., and Sansonetti, P.J. (1992). "*Shigella flexneri* induces apoptosis in infected macrophages." Nature **358**: 167-169.