EXPORT OF THE VIRULENCE FACTORS FROM SHIGELLA FLEXNERI AND CHARACTERIZATION OF THE MXI LOCI

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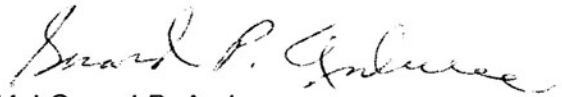

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ABSTRACT

Title of Dissertation:

Export of Virulence Factors from *Shigella flexneri* and Characterization of the *mxi* Loci.

Gerard P. Andrews: Candidate, Doctor of Philosophy, 1992

Dissertation directed by: Anthony T. Maurelli, Ph.D., Associate Professor, Department of Microbiology.

Virulence of *Shigella flexneri* requires a 220 kilobase (kb) plasmid which encodes temperature-regulated genes necessary for early steps in *Shigella* pathogenesis. To identify temperature-regulated virulence genes on the plasmid, *lacZ* protein fusions were randomly generated in *S. flexneri* and screened for fusions to temperature-regulated promoters. Analysis of one non-invasive mutant revealed that it made wild-type intracellular levels of invasion plasmid antigens (Ipa) but was deficient in their export. Furthermore, an analysis of cellular fractions showed that the normally excreted *ipa* gene products were absent in the outer membrane of the mutant. Thus, export of these antigens to the extracellular environment is essential for *Shigella* virulence. The locus defined by this mutant was designated *mxiA*.

The *mxiA* product was characterized as a 76 kilodalton (kDa) polypeptide homologous to the inner membrane regulatory protein of the low calcium response locus, *lcrD,* of *Yersinia pestis,* which is implicated in the export of *Yersinia* antigens. Thus, MxiA, a homolog of LcrD, may function either by directly affecting the excretion of virulence factors or by regulating expression of accessory genes of a multi-component protein export system.

An additional locus, *mxiC*, encoding a 40 kDa putative cytoplasmic protein, was identified upstream of *mxiA*. Homology searches revealed no similarities between *mxiC* and any known prokaryotic gene. A mutation in this locus conferred the Mxi$^-$ phenotype and was found to affect virulence of *S. flexneri* at the level of invasion, which correlated with reduced excretion of IpaC.

Protease protection experiments indicated the presence of high intracellular reservoirs of Ipa proteins in wild-type *S. flexneri* as well as *mxi* mutants. Excretion of these pre-existing protein pools in wild-type *Shigella* was stimulated by both Congo red and an unidentified component in HeLa cells, but was not stimulated in the *mxi* mutants. The failure of *mxi* mutants to be modulated by either extrinsic factor suggested that *mxi* gene products may also be responsible for controlling the response of wild-type *Shigella* to environmental cues, possibly from the host cell. Furthermore, exported Ipa virulence proteins may be selectivley released at different stages in infection which implies functions for these antigens beyond the initial step of invasion.

Export of Virulence Factors from *Shigella flexneri* and Characterization of the *mxi* Loci

by

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INTRODUCTION

Steps in the pathogenesis of *Shigella* and their *in vitro* correlates

All four species of *Shigella* have been well characterized as invasive intestinal pathogens of man and primates and are the causative agents of bacillary dysentery. While *S. flexneri* is the predominant etiologic agent of dysentery in developing countries, the prototype species, *S. dysenteriae,* has been associated with epidemic levels of shigellosis throughout the world (Bennish, et al., 1990). The two other species, *S. boydii* and *S. sonnei*, have also been associated with outbreaks of the disease in endemic areas.

Infection with *Shigella* is by the oral route and can occur with as little as 10 to 100 bacteria. This low infectious dose may be due to increased resistance of the organism to low gastric pH (DuPont et al., 1989). The target tissue for *Shigella* is the colonic epithelium, and early studies demonstrated that contact of these cells with the bacteria first results in a cytopathic alteration of the microvilli, followed by entry of the bacteria into the cells by an induced phagocytic event (Takeuchi, et al., 1963). This event appears to be initiated by bacterial-specified signals which cause a condensation of filamentous actin beneath the plasma membrane of the host cell (Clerc and Sansonetti, 1987). Contact and invasion, therefore, represent the earliest steps in the pathogenesis of *Shigella.* The observation that the bacteria are readily capable of penetrating HeLa cell monolayers in culture has allowed investigators to simulate these early events *in vitro* (Labrec, et al., 1964). In fact, by centrifuging bacterial suspensions onto host cell monolayers to enhance contact, greater than 90% of the HeLa cells are capable of becoming infected by wild-type strains (Hale and Formal, 1981). The use of ordinary light microscopy to readily visualize bacteria which have invaded individual host cells has established the HeLa cell invasion assay as a standard method of assessing *Shigella* virulence.

Following internalization, *Shigella* are quickly released from the phagocytic vacuole into the host cell cytoplasm (Sansonetti, et al., 1986). This event has been correlated with the ability of the bacteria to lyse sheep erythrocytes as determined by an *in vitro* contact hemolysis assay (Sansonetti, et al., 1986). Thus, the ability of the organism to make a hemolysin is a requirement for intracellular proliferation which is independent of the constraints of the phagosome.

Intracellular multiplication and spread of the organism to adjacent cells are the next events which take place in the pathogenesis of *Shigella* infection. These steps are simulated *in vitro* by an assay developed by Oaks and coworkers (1985). The plaque assay measures the ability of virulent *Shigella* strains to form areas of clearing (plaques) in HeLa cell monolayers which result from the destruction of invaded cells by intracellular bacteria which have spread to adjacent host cells.

As a consequence of the general tissue destruction caused by the multiplication and localized spread of *Shigella* in the colonic mucosa, the formation of inflammatory lesions generally occurs (Labrec, et al., 1964). A means to measure the ability of *Shigella* to elicit an inflammatory ressponse *in vitro* was first described by Sereny (1955). Virulent strains of *Shigella* induce a characteristic keratoconjunctivitis over a five day incubation period when inoculated onto guinea pig eyes. The response in the infected corneal epithelium mimics that seen in the intestine, and is characterized by leukocyte infiltration, swelling, and pus.

The progression of shigellosis into its later stages results in fluid loss accompanied by blood and mucous in the stool, which are the hallmarks of the disease. The full range of pathology associated with the disease, presently, can only be studied in a monkey model for *Shigella* infection. The *in vitro* assays just described have been routinely used to closely examine each of the steps in *Shigella* pathogensis.

The temperature-regulated virulence phenotype of *Shigella*.

Virulence and virulence associated loci of *Shigella* are temperature-regulated (Maurelli, et al., 1984; Maurelli and Curtiss, 1984). Virulent strains of *Shigella* spp. when grown at 30°C are non-invasive on tissue culture cells and are Sereny test negative. This temperature dependent virulence phenotype is reversible, since the same strains shifted back to 37°C for 2 hrs regain their ability to invade Henle cells. Additionally, the restoration of virulence requires the synthesis of proteins as demonstrated by the inhibition of recovery of the invasive phenotype upon temperature shift in the presence of chloramphenicol (Maurelli, et al., 1984). The temperature-regulated phenotype was mapped to a single locus on the *S. flexneri* chromosome near *galU* (Maurelli and Sansonetti, 1988). A mutation in this locus, designated *virR*, was shown to temperature de-regulate all virulence-associated phenotypes in the organism, such that the bacteria are invasive, contact hemolytic, and able to form plaques at both 30°C and 37°C. Further, through an analysis of *lacZ* transcriptional fusions on the 220 kb plasmid, it was concluded that *virR* effects expression of plasmid-encoded virulence genes. The pleiotropic effects of this locus on virulence strongly suggest that it is a regulatory gene which may modulate expression of virulence factors when the bacteria experience temperatures associated with the host extracellular environment. It has also been demonstrated that an *Escherichia coli virR* homolog is capable of restoring the temperature-regulated phenotype in an *S. flexneri virR* deletion mutant (Hromockyj and Maurelli, 1989). Subsequent studies have suggested that the *virR* locus is allelic to *osmZ* and *hns* which encode a histone-like protein which appears to increase DNA supercoiling (Dorman, et al., 1990; Hulton, et al., 1990). Recently, a sequence analysis of the *virR* locus in *S. flexneri* has confirmed homology with *hns* (Hromockyj, et al., 1992). The *virR* gene product may, therefore, act to regulate expression of virulence genes, in response to temperature, by modulating the degree of DNA supercoiling of regions of the virulence plasmid.

Congo red binding (Crb) and *Shigella* virulence.

The ability of some bacterial pathogens to bind the planar dye Congo red *in vitro* has been associated with virulence or the expression of virulence determinants (Payne and Finkelstein, 1977). *S. flexneri* was shown to become avirulent after spontaneous loss of the ability to bind the dye, and this phenotype was correlated with the loss of the 220 kb invasion plasmid (Maurelli, et al., 1984b). Thus, the gene or genes which confer the Congo red binding (Crb) phenotype are plasmid-encoded. Additionally, the dye-binding phenotype was found to be temperature-regulated (Maurelli, et al., 1984a). Daskaleros and Payne (1985) were able to clone dye-binding determinants on a 9 kb restriction fragment and identified the presence of an IS*1* element closely linked to the locus which they suggested may account for the high frequency of deletions and loss of the Crb phenotype observed with virulent *S. flexneri* (Maurelli, et al., 1984b). Subsequently, a 1 kb DNA fragment (which actually encoded the trans-activator*virF*) was cloned from the *Shigella* invasion plasmid and was found to be sufficient for expression of the dye-binding phenotype in *E. coli* (Sakai, et al., 1986). Interestingly, other unlinked restriction fragments from the plasmid are also able to partially confer dye-binding to *E. coli*. Additionally, the 1 kb clone was not able to complement a Crb^- *S. flexneri* mutant with large deletions in the invasion plasmid. This suggested that additional determinants were required to confer the Congo red-binding phenotype in *Shigella*. Similarly, Daskeleros and Payne (1986) had found that two or more separate cloned restriction fragments from the invasion plasmid were able to confer Congo red binding to *E. coli*, lending support to the complexity of the dye-binding phenotype in *Shigella.* Thus, it appears, by these studies, that the Crb^+ phenotype not only may require expression from multiple loci which encode the "structural" elements involved in binding Congo red, but also expression of regulatory elements which coordinately control the Congo red binding structural genes. In this regard, the loss of virulence, concomitant with the loss

of dye binding, may be related to the loss of expression of a regulatory gene or genes which also regulates virulence loci unrelated to Congo red binding. This in fact, may be the case with *virF*, which encodes a trans-activating product which has pleiotropic effects on virulence gene expression as well as effecting the Crb phenotype (Sakai, et al., 1986). Moreover, some Crb^+ isolates of *S. flexneri* have been isolated which were non-invasive on HeLa monolayers strongly suggesting that determinants not related to virulence may effect the dye-binding phenotype and are merely linked to virulence genes (Maurelli, et al., 1984b). Also, the mechanism by which Congo red binds to the bacteria has yet to be clarified. In fact, the studies just described imply the involvement of more than one mechanism for dye binding.

In an interesting series of experiments, Daskalaros and Payne (1987) demonstrated that both Congo red and hemin, an iron-binding porphyrin, are able to compete for the same binding site on the *S. flexneri* outer membrane. A 101 kDa heme-binding surface protein was identified in *S. flexneri* which also correlates strongly with Congo red binding (Stugard, et al., 1989). Although these experiments suggested that the gene product for Congo red binding had been identified, a non-denaturing electrophoresis gel system was used to separate heme-bound protein. Therefore, one can not rule out the possibility that the 101 kDa species represented a complex of proteins which are involved in heme and/or Congo red binding (Stugard, et al., 1989). Thus, it remains unclear whether a protein complex must be assembled to confer the Congo red binding phenotype or if this phenotype can be affected by a single gene product, or multiple separate components in the cell envelope.

Protein export in gram negative bacteria and its relevance to pathogenesis.

From an understanding of the disease process of *Shigella*, it becomes evident that certain bacterial products associated with virulence are required to reach target cells of host tissues through export pathways specified by the bacteria. Bacterial protein export

thus represents an important aspect of infection at the molecular level, and as will be discussed below, is a common requisite for all Gram negative pathogens.

Gram negative microorganisms excrete[1] a variety of proteins which provide them with assets for survival in their immediate growth environment. Excreted proteins serve several functions in gram negative bacteria. Some serve to facilitate the passage of smaller hydrophilic molecules (*e.g.*, *ompCF* porins, Nikaido and Vaara, 1985), thus providing the mechanism for movement of these compounds across the outer membrane. Others act as structural components of extracellular organelles which provide the bacteria with some physical attribute such as motility (flagella) or adherence (pili). Additionally, many examples of proteins which are excreted by Gram negative bacterial pathogens have been reported (Table 1). While the exact role of some of these excreted proteins in virulence still remains to be elucidated (*e.g.*, *Vibrio* proteases; *Klebsiella* pullulinase), others function as true virulence determinants which give the pathogen a survival advantage in its animal host (*e.g.*, *E. coli* α-hemolysin; *S. flexneri* invasion plasmid antigens B and C). Clearly, a requisite exists for most bacterial virulence factors which have been directly correlated with disease production to interact with the host. Thus, these proteins must be exposed to the bacterial extracellular environment.

A common mechanism for bacterial protein translocation across the cytoplasmic membrane of Gram negative bacteria has been well characterized and appears to be necessary for general protein export in virtually all living cells, since analogous systems have been identified in Gram positive bacteria as well as eukaryotes (Schatz and Beckwith, 1990; Saier, et al., 1989). This type of export is characterized by the proteolytic processing of a signal (leader) sequence at the N-terminus of the secreted protein followed by membrane translocation. Several genes, *secABDEFY*, *lep*, and *lspA*, have been identified which carry out this process (Schatz and Beckwith, 1990). The *sec*

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1. Translocation of proteins across the outer membrane which exposes them to the extracellular environment.

Table 1. Excreted Proteins of Gram Negative Bacterial Pathogens and their Export Machinery

Organism	Product	Export Mechanism		Reference
		Inner membrane	Outer membrane	
Serratia marcescens	Hemolysin	*sec*	*shlB*	Schiebel, et al. (1989)
	Serine Protease	*sec*	-COOH	Miyazaki, et al. (1989)
Vibrio cholerae	Cholera Toxin	*sec*	?	Hirst, et al. (1984)
	Hemolysin	?	?	
Escherichia coli	P Pili	*sec*	*papCDHJ*	Lindberg, et al. (1989)
	Colicin	*cel*, -COOH	*cel, pldA*	Pugsley and Schwartz (1984)
	Hemolysin	*hylBD*, -COOH	*hylBD, tolC*, -COOH	Wandersman and Delepelaire (1990)
Pasteurella haemolytica	Leukotoxin	*lktBD*	*lktBD*	Highlander, et al. (1990)
Pseudomonas aeruginosa	Exotoxin A	*sec*	*xcp*, Bayer's Junct.?, NH-	Bally, et al. (1992)
	Alkaline Protease	?	?	
Neisseria gonorrhoeae	IgA Protease	*sec*	-COOH	Pohlner, et al. (1987)
Shigella flexneri	IpaB/C	*mxi*	*mxi*	Hromockyj and Maurelli (1989)
	VirG	?	?	
Shigella dysenteriae	Shiga Toxin	*sec*	?	
Klebsiella pneumoniae	Pullulanase	*sec*	*pulDGHIJK, pulS*	Pugsley, et al. (1990)
Yersinia pestis	Yop's	*lcrD*	*lcrD*	Plano, et al. (1990)
	V antigen	?	?	

NH-, -COOH: N- or C- terminus of the excreted product.

gene products include an ATPase (SecA) which interacts directly with the signal sequence of the exported protein (Akita, et al., 1990). Both *lep* and *lspA* specify signal peptidases (I and II, respectively) which are localized in the inner membrane. Signal peptidase II (LspA) recognizes a consensus sequence present only in the N-terminus of exported lipoproteins (Wu and Tokunaga, 1986). The gene products of *secDEFY* encode inner membrane proteins which play a direct role in protein export, although their specific functions have not been clearly defined (Schatz and Beckwith, 1990). Translocation of most essential membrane and periplasmic proteins is directed by the *sec* system. Consequently, mutations in any of these genes, with the exception of *secB,* are lethal to the bacteria (Schatz and Beckwith, 1990). Interestingly, the product of *secB* appears to act only on a subset of exported proteins and consequently is not an essential bacterial gene (Kumamoto, 1991). SecB has been identified as a cytoplasmic protein which prevents premature folding of proteins destined for export, thus "holding" them in an export competent conformation (Weiss, et al., 1988). Many Gram negative pathogens utilize the *sec* system to move excreted virulence factors across their inner membrane (Table 1). Movement of proteins destined to reach the extracellular environment, however, is not only hindered by the cytoplasmic membrane, but is further restricted by the barrier presented by the outer membrane. While the excretion of some outer membrane proteins such as OmpF has been linked to lipopolysaccharide biogenesis (Pages, et al., 1990), specialized protein export systems have evolved in many Gram negative pathogens which facilitate translocation of excreted virulence factors across the second lipid bilayer. Genes in several Gram negative species have, in fact, been identified for a number of accessory proteins which facilitate outer membrane translocation (Table 1). Unlike the *sec* loci, however, mutations in these genes are not lethal. In some cases, excreted virulence factors are moved across the cytoplasmic membrane in the absence of any processing event (*e.g.*, α-hemolysin, IpaA-D, and Yops, Table 1). Hence, protein translocation in these systems is independent of the *sec* loci.

Export of these gene products usually does not involve the periplasm in terms of the establishment of a significant periplasmic pool of proteins destined to be excreted. In contrast, periplasmic accessory proteins (chaperones) have been identified in some *sec*-dependent inner membrane mechanisms which are highly specific for the proteins being moved across the outer membrane from the periplasm (*e.g.*, PapD, Table 1). Some export models propose the involvement of inner/outer membrane fusion sites (Bayer's junctions; Bayer, 1968), such as in the *sec*-dependent system of exotoxin A excretion from *Pseudomonas aeruginosa* (Table 1). Although these fusion sites have been implicated in several Gram negative export systems (Hirst and Welch, 1988), some individuals believe that they are artifactual and actually represent sites of membrane-spanning proteins or complexes thereof (Kellenberger, 1990). "Auto-translocation" of IgA protease from *Neisseria gonorrhoeae* across the outer membrane has also been reported, which circumvents the need for a specific outer membrane transport complex for excretion of this virulence factor (Table 1).

Since cleavable (N-terminal) signal sequences are necessary for the movement of the *sec*-dependent excreted virulence factors across the cytoplasmic membrane, the lack of these N-terminal hydrophobic amino acid sequences in the *sec*-independent systems implies that these excreted proteins may provide some other signal for targeting by the export machinery. Both amino- and carboxy-terminal amino acid domains of excreted proteins have been found to confer specificity of the exported protein for its secretion apparatus (*e.g.*, α-hemolysin, serine protease, exotoxin A).

Almost all of the Gram negative secretion mechanisms which have been characterized thus far involve more than one accessory gene (Pugsley, et al., 1990; Hirst and Welch, 1988), which suggests that these export systems are quite complex. In addition to their complexity, these specialized export mechanisms appeai to be specific for the virulence factors that they export (Hirst and Welch, 1988). Interestingly, DNA sequence comparisons of certain export loci between different genera have demonstrated

homologs, although the homologies between the excreted virulence factors themselves are not high (Highlander, et al., 1990). Consequently, in some cases, export systems from one genus have been shown to functionally complement export defects in another (*e.g.*, *Pasteurella haemolytica lktBD* and *E. coli hlyBD*). This observation suggests that in some systems specific common domains may exist among different excreted proteins which interact with components of the secretory apparatus. Additionally, excreted virulence factors from different genera which show high homology to one another may be efficiently exported by the secretion machinery of the other genus. *E. coli* heat-labile toxin (LT) is normally released from the bacteria at very low levels, while its homolog in *V. cholerae* (cholera toxin, CT) is excreted at high levels (Neill, et al., 1983). When structural genes for LT are transferred to *V. cholerae*, this toxin is excreted as efficiently as CT (Hirst, et al., 1984; Neill, et al., 1983). On the contrary, when CT is expressed in *E. coli*, it is not excreted. These data suggest that the secretory apparatus for toxin export in *Vibrio* may be absent in *E. coli*. A more provocative hypothesis, however, is that LT secretion components homologous to the CT export apparatus are present in *E. coli* but their expression or activity is under a selective form of control or regulation. Thus, efficient export of LT in *E. coli* may not occur under normal *in vitro* growth unless special conditions are imposed (*i.e.*, low pH, the presence of certain ions or nutrients, etc.). Since the genes for both of these toxin export pathways have not yet been identified, this hypothesis is merely speculative. While the genes which encode the components of specific export systems for virulence factors are quite different from the *sec* loci, functional similarities between some of the gene products can be seen. The *sec*-independent excretion of *E. coli* α-hemolysin is directed by the gene products of three loci, *hlyB*, *hyID* and *tolC* (Holland, et al., 1990; Wandersman and Delepelaire, 1990). One of these proteins, HylB, has been shown to possess ATP-binding activity (Holland, et al., 1990), which may suggest a function similar to SecA, possibly that of a protein "translocase". A family of ATP-dependent translocators has been identified based on

homologous amino acid domains within the proteins (Blight and Holland, 1990). This group of export accessory proteins encompasses not only HlyB but eukaryotic species as well.

Despite recent strong interest in identifying and characterizing excretion mechanisms of Gram negative virulence factors, the accessory export loci of many important pathogens remain undefined, and *Shigella* is no exception (Table 1). As will be described below, several virulence factors have been identified which are required to be excreted to confer *Shigella's* pathogenicity.

Excreted virulence determinants of *Shigella*.

Three chromosomal determinants of *Shigella* virulence have been identified as virulence factors required to be exported extracellularly to exert their effect (Table 2). Interestingly, the chromosomally-encoded excreted virulence determinants identified to date appear to play a role in the later stages of *Shigella* pathogenesis. Two of these determinants, lipopolysaccharide and aerobactin, are non-proteins which effect the virulence phenotype of *Shigella* beyond invasion and intercellular spread (Table 2). The third chromosomal locus of *Shigella* which specifies an exported virulence determinant is *stx* which encodes the polypeptide subunits of Shiga toxin. Synthesis of this powerful cytotoxin by *Shigella* spp. as well as by certain pathogenic strains of *E. coli*, has been well documented (reviewed by O'Brien and Holmes, 1987). The *stx* locus in *S. dysenteriae* has been mapped to a position close to *pyrF* (Sekizaki, et al., 1987). Studies directed toward clarifying the role of Shiga toxin in *Shigella* virulence suggest that it is not necessary for the initial steps in the pathogenesis of the organism (Sansonetti, et al., 1986; Hale and Formal, 1981). In a key series of experiments, Fontaine and coworkers (1988) generated a Tox$^-$ mutant of *S. dysenteriae* by *in vitro* mutagenesis and allelic exchange, and then tested the mutant for its virulence phenotype and ability to cause disease in an animal model. Although the Tox$^-$ strain effectively invaded and killed

Table 2. Virulence-Associated Loci of *Shigella*

Locus	Product(s)	Phenotype of Mutants	Role in Virulence
Chromosomal			
his/pro-linked	O-antigen biosynthesis	Sereny negative	inflammatory response
iuc	aerobactin	Sereny attenuated	extracellular growth in host (?)
kcpA	trans-activator of *virG* (?)	plaque negative	regulatory
stx	Shiga-toxin	virulent, reduced blood in stool of infected animals	destruction of vascular tissue
virR	thermoregulatory repressor	virulent at low temperatures (30°C)	regulatory
ompR	osmoregulatory activator	invasion attenuated	regulatory
Plasmid			
ipaA	70 kDa antigen	invasive	post-invasion (?)
ipaBCD	invasion mediators; cytolysin (IpaB)	non-invasive; adherent	invasion; lysis of phagosome
ipaH	58 kDa antigen	none isolated	?
virF	thermoregulatory activator of *virB*	non-invasive	regulatory
virB	thermoregulatory activator of *ipaBCDA*	non-invasive	regulatory
virG	actin polymerizing protein	plaque negative	intercellular spread
mxi	Ipa export apparatus	non-invasive	invasion; post-invasion (?)

cultured cells, it was markedly reduced in its ability to produce blood and polymorphonuclear cells in the stool of intragastrically infected monkeys. Additionally, unlike the toxin-producing strain, the Tox$^-$ construct did not evoke histopathological changes in monkey colonic tissues. This study correlated with the findings of others who demonstrated a direct cytotoxic effect by the toxin on vascular endothelium (Obrig, et al., 1988). Taken together, these data suggest that Shiga toxin induces colonic vascular damage during infection which ultimately influences the severity of the disease (Fontaine, et al., 1988).

The genes for Shiga toxin have been cloned and consist of two loci encoding an 8 kDa host receptor-binding B-subunit and an 32 kDa enzymatic A-subunit (Strockbine, et al., 1988). Sequencing and minicell analysis revealed that the toxin subunits possess N-terminal cleavable signal sequences. These data suggest that both subunits are secreted individually through the inner membrane of *Shigella* by the *sec*-dependent export pathway. Assembly of the subunits then presumably occurs in the periplasm. A problem, however, arises when one considers how the holotoxin is further exported across the outer membrane in order for it to exert its cytopathic effects. The oligomeric protein has been classically described as a cell-associated virulence factor which resides predominantly in the periplasm, and is apparently not excreted across the outer membrane at significant levels (Donohue-Rolfe and Keusch, 1983; Griffen, and Gemski, 1983). Consequently, the holotoxin can only be released from the bacteria in relatively large amounts by osmotic shock or polymyxin B treatment. Despite the apparant lack of an outer membrane export mechanism, low levels of toxin are still released into the culture supernatant under normal *in vitro* growth conditions (Donohue-Rolfe and Keusch, 1983). Furthermore, by use of a semi-continuous chemostat fermenter system, McIver and coworkers (McIver, et al., 1975) established that *S. dysenteriae* releases toxin at higher levels throughout stationary phase, following an intracellular accumulation of the protein during the first 8 hours of growth. Although the release of

the toxin has been presumed to be from autolysis or "leakage" from the bacteria, the actual mechanisms by which the toxin moves from the periplasm to the extracellular environment were not carefully examined in these early studies. One method that has been proposed for Shiga toxin excretion is the transport of the toxin within outer membrane blebs (Donohue-Rolfe and Keusch, 1983). It is also possible that a specific excretion apparatus exists for the toxin which is not active under normal *in vitro* culture conditions. The toxin export apparatus may, therefore, be "triggered" by some environmental signal which may be mimicked by stationary phase culture conditions.

A 220 kb plasmid has been demonstrated to be an absolute requirement for full virulence of both *S. sonnei* (Sansonetti, et al., 1981) and *S. flexneri* (Sansonetti, et al., 1982), in addition to enteroinvasive strains of *E. coli* (Hale, et al., 1983). Numerous proteins have been shown to be encoded by this large plasmid. At least seven polypeptides were identified in invasive *S. flexneri* which were absent in a non-invasive strain with a deletion in the plasmid (Hale, et al., 1983). Additionally, nine other plasmid-encoded proteins are localized to the outer membrane of invasive strains of *S. flexneri*, *S. sonnei*, and enteroinvasive *E. coli* (Hale, et al., 1983). In a later study, seven virulence-associated proteins (A through G) were identified by 2-dimensional gel electrophoresis, and are temperature-regulated for expression (Hale, et al., 1985). Four of these proteins, A through D (78, 62, 43, and 38 kDa, respectively), are highly immunoreactive on Western blots with antiserum from convalescent monkeys which suggests that they may be surface-expressed antigens. These proteins, designated Ipa for invasion plasmid antigens (Buysse, et al., 1987), were subsequently hypothesized to be "loosely" associated with the outer membrane of the bacteria, since incubating the bacteria in distilled water results in release of the proteins (Oaks, et al., 1986). Ipa A-D appear, therefore, to be excreted virulence factors (Table 2).

The *ipa* loci are encoded within a 37 kb cloned fragment (pHS4108) of the invasion plasmid, which is the minimum DNA sequence necessary to confer the invasive

phenotype onto a non-invasive, plasmidless *S. flexneri* strain (Maurelli, et al., 1985). This DNA alone, however, is not sufficient to confer a positive response in the Sereny test, which suggests that additional virulence loci lie outside this 37 kb region of the plasmid. Tn*5* insertions in the cloned 37 kb fragment (pHS4108) from *S. flexneri* define 5 non-contiguous regions within a 20 kb span necessary for the invasive phenotype (Baudry, et al., 1987). These regions encode Ipa A-D, and based on the ability of a single insertion in *ipaB* to alter expression of all four of these proteins, the genes encoding them were hypothesized to comprise an operon in the order *ipaBCDA*. Interestingly, loss of IpaA expression alone does not affect invasion (Baudry, et al., 1987).

Using a λgt11 expression system to generate protein fusions, Buysse, et al. (1987) were able to map the genes which encode IpaB, C, and D to two contiguous restriction fragments from the invasion plasmid. In contrast to previous findings (Baudry, et al., 1987), *ipaBCD* were found to be expressed from discrete transcriptional units, indicating that they probably did not form an operon (Buysse, et al., 1987). The nucleotide sequences for both *ipaB* and *ipaC* (Baudry, et al., 1988) reveal predicted polypeptides whose molecular weights are in agreement with the original proteins identified by Hale and coworkers (Hale, et al., 1985). Homology searches of both coding sequences show no similarity with any known prokaryotic genes. Also, the N-termini of the predicted amino acid sequences of both proteins do not possess cleavable signal leader sequences characteristic of *sec*-dependent exported proteins. Additionally, the hydropathy plots of both IpaB and IpaC reveal hydrophilic N- and C-termini with 3 central hydrophobic domains each of 10 to 40 amino acid residues, possibly representing membrane spanning regions. Analysis of additional upstream sequences disclosed the presence of an open reading frame, *ippI*, specifying a 17 kDa product (Baudry, et al., 1988), and part of an additional coding sequence, later identified as specifying a putative 24 kDa protein (Sasakawa, et al., 1989). Although a product with

a molecular weight predicted by the *ippI* locus was detected in minicells (Baudry, et al., 1988), no evidence exists that it is an exported protein. Additionally, examination of the predicted amino acid sequences for both IppI and the 24 kDa product does not reveal cleavable signal peptides. In an independent study (Venkatesan, et al., 1988), sequences were obtained for *ipaB, ipaC*, as well as *ipaD*. Although the sequencing data was in general agreement with that obtained previously (Baudry, et al., 1988), additional data obtained from Northern blot analyses revealed the presence of seven overlapping transcripts and suggested a complex system of *ipa* regulation (Venkatesan, et al., 1988). Multiple *ipa* transcripts were also predicted by Sasakawa's group (Sasakawa, et al., 1989). However, using S1 nuclease protection, their findings revealed the presence of only three transcripts spanning the entire *ipaBCD* region. Taken together these three studies suggest that, regardless of the regulatory scheme of these loci, the DNA sequencing data are consistent with a hypothesis that the *ipa* gene products may be translocated across the bacterial envelope by a unique export system. Finally, analysis of the N-terminal predicted amino acid sequence of IpaA demonstrated that this Ipa product also lacks a cleavable signal peptide (Venkatesan and Buysse, 1989).

Although at least three of the Ipa proteins have been strongly correlated with the ability of *Shigella* to enter its host cells, direct evidence for individual functions of these antigens has only recently been reported. High and coworkers (1992) used allelic exchange to replace the wild-type *ipaB* locus with a mutated allele so that downstream transcription of the remainder of the *ipa* genes was not disrupted. The mutant was found to be non-invasive on HeLa cells and was unable to elicit actin polymerization. Furthermore, the mutant failed to gain entry into the host cytoplasm when internalized by macrophages in addition to being contact hemolytic negative. These data represent the first demonstration that IpaB is responsible for lytic release from the vacuole and suggests that IpaB acts as the membrane-lysing toxin during infection. Additionally it has been reported that purified IpaB possesses hemolytic activity (High, et al., 1992).

Analysis of similar mutants of *ipaA*, *ipaC*, and *ipaD* by the techniques just described should prove extremely informative.

The *ipaH* locus is an additional virulence-associated gene unlinked to the *ipaBCD* cluster (Buysse, et al., 1987), but unlike the *ipaBCD* loci, it does not appear to be temperature-regulated (Hartman, et al., 1990). Southern hybridization analysis indicated that *ipaH* exists on the invasion plasmid in multiple copies (Hartman, et al., 1990). Subsequent mapping placed the locus 8 kb downstream from the *ipa* gene cluster on a 4.5 kb restriction fragment containing five copies of the gene which vary in sequence homology to one another (Venkatesan, et al., 1991). Like IpaA-D, an analysis of the predicted amino acid sequence of IpaH revealed the lack of a cleavable signal sequence (Hartman, et al., 1990). Unlike IpaB and IpaC, however, the predicted protein is almost entirely hydrophilic. Despite these findings, IpaH is reported to be present in water extracts and is immunoreactive with convalescent human serum (Hartman, et al., 1990), and thus may represent another *Shigella* virulence-associated protein which is excreted. Although the majority of the *ipaH* sequence does not match other bacterial genes or their products, a common leucine/proline-rich (LPX) repeat motif was found which shows homology to the *Yersinia pestis* outer membrane protein, Yop M. While this excreted *Y. pestis* antigen has been characterized as inhibiting thrombin-induced platelet aggregation in human plasma (Leung, et al., 1990), a similar role for IpaH has yet to be identified. Clarifying the role of this antigen in the pathogenesis of *Shigella* has been complicated by the presence of multiple copies of the gene which makes construction of an IpaH$^-$ mutant difficult.

Western blotting of whole cell lysates of wild-type *S. flexneri* with convalescent monkey and human serum identified a high molecular weight (140 kDa) antigen (Oaks, et al., 1986) which is surface-expressed (Lett, et al., 1989; Bernardini, et al., 1989). Expression of this protein is correlated with a region on the invasion plasmid located greater than 30 kb downstream from the *ipa* gene cluster (Lett, et al., 1989).

Mutations in this region render the bacteria Sereny test negative (Sasakawa, et al., 1986) and reflect an inability of the bacteria to spread from cell to cell after invasion (Pal, et al., 1989). Results obtained by Bernardini et al. (1989) suggest that the product of *virG* (*icsA*) may mediate condensation of F-actin within the host which then facilitates intracellular movement of the bacteria. The sequence of *virG* revealed a predicted polypeptide of approximately 120 kDa and the absence of a signal sequence (Lett, et al., 1989). Interestingly, two species of the protein have been detected using anti-serum raised against a synthetic peptide of VirG (Venkatesan, et al., 1992). A lower molecular weight species (100 kDa) is detected in cell-free supernatants of a wild-type *S. flexneri* strain, while a 120 kDa species is cell-associated. Together with the observation that the *virG* coding sequence does not predict a signal peptide, these findings suggest that VirG may be processed/modified and exported by a mechanism independent of the normal *sec* secretion pathway.

Although several chromosomal and plasmid-encoded regulatory genes which effect virulence have been identified in *Shigella* (Table 2), no experimental evidence exists which suggests that their gene products are exported. Only one known example of an exported (outer membrane) regulatory protein in Gram negative bacteria has been reported. The P6 outer membrane protein of *Haemophilus influenzae* directly interacts with DNA to control transcription (Sikkema, et al., 1992). This finding, however does not preclude the possibility that some regulatory protein(s) not directly involved in binding DNA, are exported into the outer membrane in *Shigella*. One could envision these polypeptides as functioning as part of a regulatory complex which could transduce modulatory signals from the exterior of the cell to the DNA binding component (protein) inside the cell. Thus, some *Shigella* regulatory proteins associated with virulence could, in fact, be exported products. A similar regulatory role has been proposed for the outer membrane protein YopN of *Yersinia pseudotuberculosis* (Forsberg, et al., 1991).

The *mxi* loci.

The existence of a novel *Shigella* export system for virulence-associated proteins was initially suggested by the findings of Hromockyj and Maurelli (1989) who reported the identification of new temperature-regulated virulence loci located across a 9 kb region, at least 6 kb upstream of the *ipa* genes and transcribed in the opposite direction. Mutants in this region are non-invasive on HeLa cell monolayers, although they synthesize wild-type levels of IpaA-D polypeptides. However, analysis of these mutants by whole-cell ELISA (Mills, et al., 1988) revealed a significant reduction in surface expression of IpaB and IpaC. Because it appeared that these loci are involved in surface presentation or export of the *ipa* proteins, they were designated *mxi* for membrane expression of invasion plasmid antigens. Similarly, Tn3 insertions generated in *S. sonnei* result in some non-invasive mutants which make wild-type levels of IpaA-D (Watanabe, et al., 1990). The loci defined by these inserts were designated *invAKJH* and appear to map within a region analogous to *mxi*, although they were not characterized for their Ipa secretion phenotype.

Project objectives.

Although many *Shigella* virulence determinants, both chromosomal and plasmid-encoded, have been characterized, it is clear that additional virulence-associated loci remain unidentified. With the exception of *virG* and *virF*, all the plasmid-encoded virulence genes thus far identified lie within the 37 kb *Sau*3A subclone of the invasion plasmid, pHS4108. Since this DNA spans only 20% of the invasion plasmid, it seems likely that other virulence genes are located on the remainder of the plasmid. Additionally, only five genes (*ipaBCDA*, and *virB*) on pHS4108 have been identified as encoding virulence factors. This total represents only about 25% of the cloned virulence region in pHS4108.

The initial purpose of these studies, therefore, was to identify and characterize novel virulence loci, both chromosomal and plasmid, in *S. flexneri.* This objective was addressed through the generation, isolation, and characterization of avirulent mutants by random mutagenesis. Temperature regulation was chosen as an initial screening criterion for identification of avirulent mutants, since this property is a common characteristic of expression of the *Shigella* virulence genes studied to date. Advances in gene fusion technology have brought about the routine use of stable mutagenizing DNA constructs which possess transposon-like properties to generate mutants with unique phenotypes (reviewed by Silhavy and Beckwith, 1985). Lambda/Mu bacteriophage hybrids, containing the *lacZ* coding sequence, are able to stably insert into target DNA and inactivate a gene of interest. Expression of a gene inactivated in this way is marked by expression of the cytoplasmic enzyme, β-galactosidase, which is driven by the insert gene's promoter. Using this "reporter" system, a temperature-regulated locus may be identified on the basis of differential expression of β-galactosidase at two different temperatures. Some hybrid phage constructs permit the translation of the fused gene such that a hybrid molecule is synthesized in which the N-terminal portion of the protein is specified by coding sequence from the insert gene, and the C-terminus is specified by the gene for β-galactosidase (Bremer, et al., 1984). Provided the mutated bacteria can tolerate the presence of the fusion protein, its production can be exploited to obtain useful information regarding the native gene product.

In the course of the preliminary experiments conducted in this study, an avirulent protein fusion mutant was isolated concurrently with the operon fusion mutants described by Hromockyj and Maurelli (1989). Although the mutant synthesized wild-type levels of both IpaB and IpaC intracellularly, it was depleted for the outer membrane expression of both antigens as determined by Western blot analysis. Ipa surface expression of four of the five operon fusion mutants also appeared to be altered, based on the results of a whole-cell ELISA (Hromockyj and Maurelli, 1989). It was

hypothesized, therefore, that the insert site of the protein fusion mutant is in a region near those of the transcriptional fusions. Collectively, the mutations defined a region on the invasion plasmid which controls export of Ipa proteins, and the locus was named accordingly (*mxi*). The expanse of DNA specifying the plasmid-encoded *mxi* locus suggests that multiple genes may be present in this region. Thus, a unique multi-component protein export system may exist in *Shigella* which has yet to be characterized. The primary objective of this thesis is to further characterize the *mxi* virulence-associated region on the invasion plasmid, specifically through the analysis of the *mxi* protein fusion mutant, designated BS260.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The *Shigella* strains used in this study are derivatives of the wild-type strain *S. flexneri* 2a, 2457T (Formal, et al., 1958) and are described in Table 3. For most experiments, bacteria were grown in tryptic soy broth (TSB, Difco Laboratories, Detroit, MI) at 37°C or 30°C. Solid media for routine culture growth and maintenance was made by adding Bacto agar (Difco) to TSB at 1.5% final concentration (tryptic soy agar, TSA). Alternatively, working stocks of strains were maintained on Bacto-MacConkey agar with lactose (Difco). Selective media was made by addition of one or several of the following antibiotics to TSB, TSA, or MacConkey: kanamycin, 50 μg/ml; ampicillin, 100 μg/ml; tetracycline, 5 μg/ml. Initial selection of *Shigella* mutagenized by fusion phage was carried out on M9 salts minimal media (Davis, et al., 1980) supplemented with 0.5% glucose as the carbon source and nicotinic acid at 10 μg/ml. Luria Bertani broth (LB) consisted of 1% Bacto-tryptone, 0.5% Bacto-yeast extract, and 0.5% NaCl (Davis et al., 1980). Low calcium induction experiments were performed in calcium-chelated TSB (T-OX) which was prepared following published methods (Higuchi and Smith, 1961). $C_2O_4Na_2$ (sodium oxalate, Sigma Chemicals, St. Louis, MO) was added to 5X TSB at 20 mM final concentration and a precipitate allowed to form overnight at 4°C over chloroform. The mixture was then filtered through #1 Whatman filter paper, brought to 1X with dH_20, and autoclaved. $MgCl_2$ and $C_2O_4Na_2$ were then aseptically added to 20 mM final concentration each.

All cloning vectors and recombinant plasmids used and constructed in this study are presented in Table 3.

Mutagenesis. Lysates of the protein fusion phage λp*lac*Mu9 and the helper phage, λpMu507 (Bremer, et al., 1984), were used to infect the wild-type strain of *S.*

TABLE 3. Bacterial strains and plasmids

Strain or Plasmid	Description	Reference or Source
Strains		
Shigella flexneri		
2457T	wild-type containing pSf2a140	Formal, et al., 1958
BS103	2457T cured of pSf2a140	Maurelli, et al., 1984
BS109	2457T *galU*::Tn*10*	This study
BS177	BS109 cured of pSf2a140	This study
BS181	2457T Mal+λ^s	Maurelli and Sansonetti, 1988
BS183	2457T pSf2a140 (*mxiC*::*lacZ*)	Maurelli and Sansonetti, 1988
BS189	2457T *virR*1::Tn*10*	Maurelli and Sansonetti, 1988
BS201	2457T *recA*56	Andrews, et al., 1991
BS226	2457T pSf2a140 (*mxiB*::*lacZ* 11.5)	Hromockyj and Maurelli, 1989
BS228	2457T pSf2a140 (*ipaB*::*lacZ* 17.6)	Hromockyj and Maurelli, 1989
BS230	2457T pSf2a140 (*mxi*::*lacZ* 17.7)	Hromockyj and Maurelli, 1989
BS232	2457T pSf2a140 (*mxiA*::*lacZ* 18.15)	Hromockyj and Maurelli, 1989
BS260	2457T pSf2a140 (*mxiA*::*lacZ* 1103)	Andrews, et al., 1991
BS281	2457T pSf2a140 (*lacZ* 2904, Inv+)	This study
BS282	2457T pSf2a140 (*lacZ* 3011, Inv+)	This study
BS283	2457T pSf2a140 (*lacZ* 3214, Inv+)	This study
BS441	*S. flexneri* 5, M90T pWR100 Δ(*icsA*::omega)	Bernardini, et al., 1989
E. coli K-12		
DH5α	ϕ80d Δ(*lacZYA-argF*) *hsd*R17	GIBCO BRL [a]

TABLE 3, continued.

Strain or Plasmid	Description	Reference or Source
Plasmids		
pUC19	cloning vector, Ap^r *lacZ*+	Yannish-Perron, et al., 1985
pBS	cloning vector, Ap^r lacZ+	Stratagene[b]
pKS	cloning vector, Ap^r *lacZ*+	Stratagene[b]
pMLB524	fusion cloning vector, Ap^r*lacZ* '	Berman, et al., 1984
pATM009	9 kb operon fusion end joint clone from BS183 in pMLB524	This study
pHS4108	38 kb *Sau*3a sub-clone of pSf2a140	Maurelli, et al., 1985
pHS4011	11 kb *Eco*RI fragment from pHS4108	Maurelli, et al., 1985
pHS5101	14 kb *Sal*I fragment from pHS4108	Maurelli, et al., 1985
pHS5102	11 kb *Sal*I fragment from pHS4108	Maurelli, et al., 1985
pHS5103	13 kb *Sal*I fragment from pHS4108	Maurelli, et al., 1985
pGPA001	9 kb protein fusion end joint clone from BS260 in pMLB524	Andrews, et al., 1991
pGPA002	22 kb protein fusion end joint clone from BS281 in pMLB524	This study
pAEH006	12 kb operon fusion end joint clone from BS226 in pMLB524	Andrews, et al., 1991
pAEH008	7 kb operon fusion end joint clone from BS230 in pMLB524	Hromockyj and Maurelli, 1989
pAEH009	10 kb operon fusion end joint clone from BS232 in pMLB524	Hromockyj, 1991
pGPA010	*mxlA*'; 1.8 kb *Hind*III fragment from pAEH006 in pUC19	Andrews and Maurelli, 1992
pGPA011	*mxlC*'; 2.5 kb HindIII fragment from pAEH006 in pUC19	Andrews and Maurelli, 1992
pGPA013	*mxlA*'; 1.3 kb *Hind*III fragment from pAEH006 in pUC19	Andrews and Maurelli, 1992
pGPA040	*mxlA*+; 3.8 kb *Xba*I-*Eco*RI fragment from pAEH006 in pUC19	Andrews and Maurelli, 1992
pGPA042	*mxlA*+; 11 kb *Eco*RI fragment from pHS4011 in pUC19	Andrews and Maurelli, 1992
pGPA043	*mxlA*+; pGPA042 insert in p*lac*-driven orientation	Andrews and Maurelli, 1992
pGPA045	*mxlA*+; 3.8 kb *Xba*I-*Eco*RI fragment from pGPA040 in pKS	Andrews and Maurelli, 1992

[a] GIBCO BRL, Life Technologies, Inc., Gaithersburg, MD

[b] Stratagene Cloning Systems, La Jolla, CA

flexneri 2a, 2457T. Fusion mutants were selected for resistance to kanamycin (Km^r) and screened for the lactose fermenting (Lac^+) phenotype at 37°C on minimal medium containing kanamycin and the colorometric β-galactosidase substrate 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal, Boehringer Mannheim Biochemicals, Indianapolis, IN). The Km^r Lac^+ lysogens obtained were further screened for the lactose non-fermenting (Lac^-) phenotype at 30°C to identify them as temperature-regulated mutants. The fusion mutations were then transferred to a "clean" (non-mutagenized) *Shigella* background by P1 transduction and selection for Km^r in order to segregate possible double insertions. The transductants were then retested for temperature-regulation by the quantitative assay for β-galactosidase (see below).

Bacteriophage P1 Transduction of *Shigella* Strains. P1 lysates were prepared as described (Silhavy et al., 1984). Subcultured donor strains were grown to log phase in LB + 5 mM $CaCl_2$. Fifty µl of a P1L4 lysate at 6-8 x 10^7 PFU/ml was then added to 1.0 ml of bacterial culture and allowed to adsorb for 20 minutes at 37°C. Two hundred µl of this mixture was then added to a tube containing 2.5 ml of molten L-soft agar (1% Bacto-tryptone, 0.5% NaCl, 0.5% yeast extract, 0.1% glucose, 5 mM $CaCl_2$, and 0.5% agar) and then spread over a P1 L-agar plate (1.2% agar). The plates were incubated right-side-up at 37°C for 6 hours until the bacterial lawn cleared. Overlays were then scraped from the plates, chloroform added, and the lysates centrifuged at 12,000 x g for 10 minutes to remove agar and cellular debris. The lysates were titered and stored at 4°C until use.

Recipient bacterial strains were grown in LB for 15 hours at 37°C with shaking. The bacteria were then subcultured 1:100 into LB + 5 mM $CaCl_2$ and grown to log phase. One ml of culture was infected with a P1L4 lysate at a multiplicity of infection from 1 to 3, and the phage allowed to adsorb for 20 minutes at 37°C. Infection mixtures were next centrifuged, suspended in 1.0 ml LB + 10 mM sodium citrate, and incubated for 90

minutes at 37°C. Transductants were plated on selection media at 10^{0} and 10^{-1} dilutions and plates incubated at 37°C.

Restriction Enzymes and Radionuclides. Restriction enzymes and reaction buffers used throughout the course of this study were obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN). Radionuclides used in Southern hybridizations (^{32}P) and DNA sequencing (^{35}S) were obtained from Amersham Corporation (Arlington Heights, IL).

DNA Preparation and Agarose Gel Electrophoresis. Plasmid DNA for routine use was prepared by a modification of the alkaline lysis method (Birnboim and Doly, 1979). One ml of an overnight culture was centrifuged and resuspended in 100 µl of 50 mM glucose + 10 mM EDTA + 25 mM Tris-HCl and incubated at 4°C for 10 minutes. Two hundred µl of 0.2 N NaOH + 1.0% SDS was next added and the mixture incubated for 5 minutes on ice. Chromosomal DNA was precipitated by the addition of 150 µl 3 N sodium acetate, pH 4.8, incubation on ice for 15 minutes, and centrifugation for 5 minutes at 10,000 x g. An equal volume of buffer-saturated phenol was next added to the supernatant which was then centrifuged to separate the aqueous layer from the phenol. An equal volume of chloroform was then added and the mixture centrifuged to removed the residual phenol. Plasmid DNA in the aqueous layer was precipitated overnight at -20°C with 3 volumes of 95% ethanol and washed once with 70% ethanol. The DNA pellet was then dried and resuspended in 20-40 µl dH_2O.

Recombinant plasmid DNA for sequencing was isolated and purified by the Qiagen column kit (Qiagen, Chatsworth, CA) following the manufacturer's procedure. TSB overnight cultures of bacteria were lysed using solutions provided in the kit and the clarified lysates passed through a QIAGEN-tip™. Bound purified plasmid DNA was released from the column by high salt elution. DNA was then precipitated with 0.6

volumes of isopropanol at room temperature and resuspended in dH_2O. Purity and yield was determined by measuring the A_{260}/A_{280} ratio of each preparation.

Qualitative assessment of plasmid DNA preparations was made by electrophoresis of samples through 0.7% agarose (Sigma) gels in TAE buffer (40 mM Tris-acetate, 1 mM EDTA) at 30-100 volts for 1-15 hours. Purification of linearized DNA for *in-agaro* ligations was performed in 0.8% SeaPlaque™ low melting point agarose (FMC Bioproducts, Rockland, ME) at 50 volts for 1/2 to 2 hours. Electrophoresed DNA was visualized by staining the gels from 2-30 minutes with a solution of 0.5 µg/ml ethidium bromide (Sigma) and illuminating the gels under ultraviolet light at 254 nm wavelength.

DNA Ligations. Linearized DNA was ligated into cloning vectors following the methods of Maniatis (1989). For the cloning of the fusion end joints, invasion plasmid preparations of *S. flexneri* fusion mutants were digested with *Eco*RI to completion and the restriction enzyme inactivated by heating to 60°C for 10 minutes. The fusion vector pMLB524 (Table 1) was also digested with *Eco*RI, and the ends dephosphorylated with calf intestinal alkaline phosphatase (Boehringer Mannheim). Both DNA preparations were mixed at a 2:1 insert to vector molar ratio. One tenth volume of ligation buffer (660 mM Tris-HCl, 50 mM $MgCl_2$, 10 mM DTE, 10mM ATP, pH 7.5) and 1 µl T4 -DNA ligase (1U/µl, Boehringer Mannheim) was then added to the mixture which was incubated at 15°C for 20 hours prior to inactivation of the ligase by heat. Ligation mixtures were stored at 4°C until they were used to transform *E. coli.* Alternatively, for cloning into pUC-derived vectors, ligations were performed *in agaro* (Maniatis, 1989). Endonuclease-restricted DNA was run through a low melt agarose gel and the appropriate DNA fragment excised. The agarose slice was then melted at 65°C for 10 minutes and placed at 37°C to maintain fluidity. Restricted vector DNA, 10x ligation buffer, and ligase were then added and the mixture shifted to a 25°C water bath. After a 90 minute

incubation period, the ligase was heat-inactivated and the mixture stored at 4°C until use.

Transformation of *Shigella* and *E. coli*. Competent cells of *S. flexneri* 2a were prepared by the method of Dagert and Erlich (1979). Overnight cultures of the strains to be transformed were subcultured 1:100 in fresh medium and grown to an A_{600} of 0.6-0.9. The bacteria were next heated for 10 minutes at 50°C and then chilled on ice for 1 hour prior to centrifugation at 6000 x g for 5 minutes. The cell pellets were resuspended in 1/2 the original culture volume with 0.1 M $MgCl_2$ and re-centrifuged. The bacteria were next suspended in 0.01 M $CaCl_2$ + 0.05 M $MgSO_4$ and incubated on ice for 10 minutes. Finally, the cells were centrifuged and resuspended in 0.1 M $CaCl_2$ at 1/10 the original volume and incubated on ice for 15 hours prior to transforming with DNA. Competent cells of *E. coli* were prepared following the method of Hanahan (1985). An overnight culture of *E. coli* DH5α was sub-cultured 1:100 into 100 ml of fresh media and grown to A_{550} of 0.5. After incubation for 10 minutes at 4°C, the culture was centrifuged and suspended in 1/4 the original volume with standard freezing buffer (SFB, 10 mM CH_3COOK, 100 mM KCl, 45 mM $MnCl_2 \cdot H_2O$, 10 mM $CaCl_2 \cdot H_2O$, 3 mM $HACoCl_3$, 10% glycerol, pH 6.4). After a 15 minute incubation on ice, the cells were pelleted and resuspended in 1/15 the original volume with SFB. Dimethyl sulfoxide (DMSO, Sigma) was next added to a final concentration of 4% and the mixture incubated on ice for 5 minutes. A second aliquot of DMSO was then added and, after an additional 5 minute incubation period, the cells were frozen in a dry ice/ethanol bath and stored in 1 ml aliquots at -70°C until use.

Competent *Shigella* and *E. coli* were transformed by adding plasmid DNA to 200 µl of cells and incubating the mixture on ice for 30 minutes. The mixtures were heat shocked at 42°C for 2 minutes then chilled on ice for 2 minutes prior to addition of 2 ml pre-warmed SOB medium (2% Bacto-tryptone, 0.5% Bacto-yeast extract, 0.05% NaCl, 10

mM $MgCl_2$, 2.5 mM KCl). The cells were next incubated without shaking at 37°C for 90 minutes. After concentrating the cells to 1/10 volume in SOB, 100 µl of each transformation mix was plated on the appropriate selection plates.

Quantitative β-galactosidase Assay. Assessment of β-galactosidase levels in Lac^+ strains was made by following the method of Miller (1972). Overnight cultures of the strains to be tested were subcultured and grown to an A_{600} of 0.3-0.7. After chilling samples on ice for 20 min, bacteria were lysed with 50 µl of 0.1% SDS and 100 µl of chloroform, and 0.5 or 0.1 ml of the lysate added to 0.5 or 0.9 ml of prewarmed Z buffer (60 mM $Na_2HPO_4 \cdot 7H_2O$, 40 mM $NaH_2PO_4 \cdot H_2O$, 10 mM KCl, 1 mM $MgSO_4 \cdot 7H_2O$, and 50 mM β-mercaptoethanol) to 1.0 ml final volume. Two tenths of a ml of O-nitrophenyl-β-D-galactopyranoside (ONPG, 4.0 mg/ml in 0.1 M phosphate buffer, pH 7.0; Sigma) was then added to each sample and after the appropriate time, the color reaction was stopped with 0.5 ml of 1 M Na_2CO_3. Units of β-galactosidase activity based on A_{420} values were standardized with the following formula:

$$\frac{A_{420}-(1.75 \times A_{550})}{\text{time} \times \text{volume} \times A_{600}} \times 1000 = \text{Units, enzyme activity}$$

Virulence assays:

a.) Quantitative Congo red binding. Since the ability of *Shigella* to bind the planar dye Congo red correlates with virulence (Maurelli, et al., 1984), a quantitative Congo red binding assay was developed and employed as one measurement of the virulence phenotype. Bacterial strains to be tested were grown overnight at 37°C and 30°C on TSA supplemented with 0.025% Congo red (Sigma). Ten to 20 colonies of bacteria were scraped from each plate, resuspended in 600 µl dH_2O, and the A_{660} measured to account for variations in cell density. Acetone was then added to the bacterial suspensions to 40%

final concentration and the dye extracted for 10 minutes at room temperature. The bacteria were centrifuged at 5000 x g and the supernatant containing the extracted dye read at A_{488} (absorbance maxima of Congo red). An arbitrary level of Congo red binding (CRB) was then calculated by dividing the A_{488} by the A_{660} value. CRB was standardized to % of wild-type dye binding. Variability between absolute dye binding values from multiple experiments for the wild-type control (2457T) was consistently less than 10%.

b.) HeLa cell invasion assay. Bacterial strains to be tested were grown overnight, subcultured and grown to log phase, and standardized to an A_{600} of 0.72 in Minimal Essential Medium (MEM, Cellgro™, Mediatech, Washington, D.C.) after two washes with phophate buffered saline (PBS). The bacterial samples were then applied to semi-confluent monolayers of HeLa cells which had been washed twice with fresh pre-warmed MEM. After centrifuging the tissue culture plates for 20 minutes at 3000 x g to allow the bacteria to come into close proximity to the HeLa cells, the infected monolayers were incubated at 37°C for 30 minutes. After this time, the plates were washed five times with PBS and overlayed with 1.0 ml of fresh MEM containing 20 μg/ml gentamicin (Sigma). After an additional 90 minutes incubation, the infected monolayers were washed five more times with PBS, fixed for 5 minutes with methanol, and stained with 0.4% Giemsa (stock solution: 20% w/v in buffered methanol, pH 6.8, Sigma) in PBS for 20-30 minutes. Bacterial invasion was enumerated as the number of HeLa cells invaded/number of total HeLa cells counted and standardized to % of wild-type invasion. Individual HeLa cells were considered invaded if they contained greater than five intracellular bacteria. A minimum of 100 HeLa cells were counted for each infected monolayer.

c.) Plaque assay. The initial steps of the plaque assay (Oaks et al., 1986), were performed essentially as described for the HeLa cell invasion assay except that absorbance-standardized bacteria were diluted 1:1000 and 1:10,000 in DMEM (MEM +

0.45% glucose) prior to addition to the monolayers. After 90 minutes incubation at 37°C, infected monolayers were overlayed with DMEM soft agar (DMEM + 0.25% agarose, 5.0% fetal calf serum, 20 μg/ml gentamicin). The plates were then incubated at 37°C under CO_2 for 24-48 hours. Plaques which formed from the destruction of the HeLa cell monolayer were scored relative to wild-type where -, no plaques; +, 5-25% of wild-type; ++, 25-50% of wild-type; +++ 50-80%; ++++, 80-100% of wild-type.

d.) Sereny test. The Sereny test was performed as an additional means to assay for intracellular bacterial multiplication, as well as the ability of the microorganisms to cause an inflammatory response in a mammalian host (Sereny, 1955). Bacterial strains to be tested were streaked for confluency on TSA and incubated at 37°C overnight. The right eye of a Hartley Guinea Pig (approximately 250 grams) was heavily inoculated by rolling a sterile cotton swab streaked from the overnight culture plate across the tissue surface. The left eye served as the uninoculated control. A positive test result was the development of a characteristic keratoconjunctivitis (inflammation and exudate) after 48 hours.

e.) Contact hemolytic activity. Contact hemolysis was assessed in λp*lac*Mu9 mutants as follows: Overnight cultures of the bacterial strains to be tested were subcultured, grown to late log phase, centrifuged, and washed once in PBS. The bacteria were next absorbance-standardized and added to PBS-washed sheep red blood cells (SRBs) which had been adjusted to 4 x 10^9 cells/ml. Fifty μl each of the bacteria/SRB mixes were next added to wells of a 96 well flat-bottom microtiter plate and agitated on a plate shaker. The plates were then centrifuged at 2200 x g for 10 minutes at 4°C to bring the bacteria into close contact with the erythrocytes. The plates were next incubated for 2 hours at 37°C after which the pellets in each well were resuspended in 150 μl of cold PBS and centrifuged. The supernatants were then read at A_{540} to measure

the degree of lysis of the RBCs, and the values were expressed as the percent of wild-type.

Radiolabelling of DNA and Southern Hybridization. DNA restriction fragments used as probes in Southern blots were isolated from gels by excision and electroelution (Elutrap™, Schleicher and Schuell Inc., Keene, NH). Electroeluted DNA was then labelled with [α-^{32}P] CTP by a random primer labelling kit (Pharmacia LKB Biotechnology Inc., Piscataway, NJ) following the manufacturer's protocol. Probe DNA was denatured at 100°C for 3 minutes, then chilled to 4°C for 2 minutes. The radionuclide and 5-10 units of Klenow fragment were then added to the DNA and the mixture incubated for 60 minutes at 37°C. The DNA was then chromatographed through a Sephadex G-50 column (Bio-Rad). Level of activity (cpm) of the radiolabelled probe was assessed in a Beckman LS 7500 scintillation counter. Prior to blotting, the probe DNA was base-denatured with 0.1 M NaOH for 10 minutes and neutralized with 180 mM Tris-HCl + 20 mM Tris-base.

Hybridization of radiolabelled probes to immobilized DNA was carried out following the method of Southern (1975). DNA preparations to be probed were first electrophoresed through agarose gels as above. The gels were washed twice with 0.25 M HCl for 10 minutes each wash, and then neutralized with 3 washes of 0.5 M Tris-HCl + 1.0 M NaCl. After two washes with dH_2O, DNA was base-denatured with two washes in 0.5 M NaOH + 1.0 M NaCl for 15 minutes each. The gels were again washed with dH_2O and the DNA transferred in 1.5 M NaCl + 0.15 M sodium citrate (10x SSC) by capillary action onto 0.45 µm nitrocellulose sheets (Bio-Rad Laboratories, Richmond, CA). After two washes in 2x SSC, the blots were baked for 2-3 hours at 80°C. The blots were then rinsed for 2 minutes in 6x SSC and pre-hybridized with 6x SSC + 0.5% SDS + 5x Denhardt's solution (0.2% Ficoll, 0.2% polyvinylpyrrolidone, 0.2% BSA) + 100 µg/ml salmon sperm DNA (Sigma). After pre-hybridization for 15 hours at 56°C, the

blots were hybridized with approximately 3 x 10^6 cpm of radiolabelled DNA in 6x SSC + 0.5% SDS + 10 mM EDTA + 5x Denhardt's + 100 µg/ml salmon sperm DNA. The blots were then washed three times with 2x SSC + 0.2% SDS, warmed to 50°C, air dried, and autoradiographed for 20 hours at -70°C with XAR-5 film (Eastman Kodak, Rochester, NY).

DNA Sequencing and Sequence Analysis. DNA sequencing was performed using plasmids pGPA010, pGPA011, pGPA013, and pGPA040 (Table 3) as double-stranded templates with the Sequenase Version 2.1 DNA sequencing kit (U.S. Biochemical Corp., Cleveland, OH). The protocol was followed as essentially described by the manufacturer. Briefly, 10 to 20 µg of Qiagen-tip™ purified plasmid DNA was base-denatured with 0.2 N NaOH for 5 to 10 minutes at room temperature and neutralized with 0.4 volumes of 5 M ammonium acetate (pH 7.5). The DNA was next precipitated with 4 volumes of ice cold ethanol and the pellet washed with 70% ethanol. The DNA was then resuspended in dH_2O, 10x reaction buffer, and 1-2 µl of primer. Annealing was performed by heating the mixture to 65°C for 2-4 minutes and gradually reducing the temperature to 37°C over 30 minutes. dNTP's, [α-^{35}S] ATP, 0.1 M DTT, and Sequenase™ were then added to the primed DNA template, and the reaction allowed to proceed for 3 minutes at 20°C until chain-terminated by aliquoting the mixture into each of four tubes containing ddNTP's. Reactions were run on 7% polyacrylamide + 46% urea gels for 2-6 hours at 80 watts, constant, using an IBI model STS 45 vertical gel apparatus. Gels were then fixed in 10% methanol + 10% glacial acetic acid prior to vacuum drying for 75 minutes at 80°C. The dried gels were then autoradiographed with XAR-5 film (Eastman Kodak) at room temperature for 1-5 days.

The complete DNA sequences and derived amino acid sequences of *mxi* open reading frames were analyzed with the Genetics Computer Group (GCG) Version 6.2 software (Devereux, et al., 1984). Isoelectric point determinations were made using the

ISOELECTRIC program. Homology searches were performed at both DNA and amino acid levels using the FASTA and TFASTA programs to scan GenBank (Release 70.0) and EMBL (Release 29.0) databases.

Western blotting. Detection of *Shigella* antigens (Ipa and MxiA) in some experiments was performed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis in a discontinuous buffer system (SDS-PAGE, Laemmli, 1970). Samples consisting of whole bacterial cells or protein preparations were standardized to culture density or total protein by BCA-Lowry (Pierce Chemical Co., Rockford, IL). Bacterial cells were lysed and/or proteins denatured by addition of sample buffer (phosphate buffer [50 mM K_2HPO_4 + 50 mM Na_2HPO_4, pH 7.2], 2.0% SDS, 12.0% glycerol, 0.01% bromophenol blue, 5% β-mercaptoethanol) and heating in a water bath for 10 minutes at 100°C. Eight to 12% gels were run for 5-15 hours at 50-200 volts, constant, at 15°C. Electrophoresed proteins were then electroblotted onto nitrocellulose at 500 mA for 1.5 hours, 15°C, in transfer buffer (39 mM glycine [free base, Sigma], 48 mM Tris-Base, 20% methanol, pH 8.3). Blots were blocked with 3% casein hydrolysate (Sigma) in PBS for 30 minutes and immunolabelled overnight with a 1:1000-1:5000 dilution of anti-IpaB, anti-IpaC monoclonal antibodies (MAbs, kindly provided by Jerry Buysse and John Mills) or polyclonal rabbit hyperimmune serum (1:50-1:2000, prepared in this laboratory - see p. 39). After three washes with PBS + 0.05% Tween-20 (PBST), alkaline phophatase-conjugated goat anti-mouse (Boehringer Mannheim) or goat anti-rabbit IgG (Bio-Rad) at a 1:1000 dilution was added as the secondary label. The blots were then developed for 5-15 minutes with fast red TR-salt and naphthol AS MX-phosphate (Sigma) in 50 mM Tris buffer, pH 8.0 following two washes with PBST.

IpaB and IpaC signals on blots of cellular fractions and proteinase K digestions were quantitated using a Shimadzu CS-930 laser scanning densitometer at 550 nm and the area under the absorbance peaks for the bands corresponding to the *ipa* polypeptides

determined with a CCS-1 integration program cassette and DR-2 data recorder. Alternatively, Western blots were scanned by a Datacopy model 230 digital scanner and the mean density of each band calculated by Image v1.30 digital image analysis software on a Macintosh II computer.

Assays To Detect Exported Bacterial Antigens:

a.) Suspension-labelling immunoassay (SLIM). To analyze the amount of surface-associated antigens on wild-type *S. flexneri* and selected fusion mutants, bacteria were grown to log phase (A_{600}= 0.3-0.9), washed once in PBS, and incubated at room temperature for 2 hours with a 1:2500 dilution of MAbs in PBS + 3% casein in Eppendorf tubes. After three washes with PBS, the cells were then labelled with a 1:1000 dilution of a goat anti-mouse IgG [$F(ab')_2$] alkaline phosphatase conjugate (Boehringer Mannheim) for 1 hour at room temperature. Just prior to addition of enzyme substrate, the antibody-bound cells were washed twice with PBS and transferred to clean plastic tubes. Substrate (Sigma 104™, Sigma) was added and after 30 minutes the reactions were stopped by the addition of 300 µl 3 N NaOH and the cells pelleted by centrifugation. The supernatants containing cleaved substrate were then read at A_{405} in an LKB model 4050 Ultraspec II spectrophotometer.

b.) Cell-free ELISA. Excreted bacterial antigens were measured by a modified ELISA as follows: wild-type *Shigella* and fusion mutants were grown to log phase, centrifuged, and resuspended in PBS to an A_{600} of 5.0. The resuspended cells were then incubated at room temperature for 2 hours, centrifuged, and the supernatant passed through a 0.22 µm low protein binding membrane filter (Millipore Corporation, Bedford, MA). The undiluted cell-free PBS supernatants (100 µl) were then added to wells of a 96-well microtiter plate in quadruplicate (Costar, Cambridge, MA) and incubated for 2 hours at room temperature. Three percent casein blocker in PBS was then applied after the wells

were emptied of the supernatants. After washing twice with PBST, MAbs were added to the wells at a 1:2500 dilution and incubated for 2 hours at 37°C. Following three washes with PBST, a 1:500 dilution of alkaline phosphatase-conjugated anti-mouse IgG was added for 1 hour at room temperature. Following two washes with PBST, the wells were washed once with DEA buffer (8% diethanolamine, 500 mM $MgCl_2$, 3 mM NaN_3; pH 9.2) and developed for 30 minutes with alkaline phosphatase substrate at 1 mg/ml in DEA buffer. Reactions in the wells were stopped with 3 N NaOH and the A_{405} recorded using a Bio-Tek Microplate ELISA Reader.

For the *mxi* complementation experiments, an alternate method was utilized to detect excreted antigen using a cell-free "tube" ELISA. This assay was performed essentially as an extension of the SLIM assay. After transfer of antibody-labelled bacteria to clean Eppendorf tubes as above, the old tubes were washed once with PBS to remove residual cells. Cell-free antigen/antibody complexes adhering to the tubes were detected by the addition of enzyme substrate, and the color reaction stopped after 5 minutes. The solution in each tube was then read at A_{405} and the values expressed as the percent reactivity of wild-type. Reactivity from antibody alone adhering to the inside surface of the tubes was consistently less than 10% of the positive control (2457T). Variability between absolute A_{405} values from multiple experiments for 2457T was less than 17% for excreted IpaB and less than 8% for excreted IpaC.

c.) Precipitation and detection of excreted bacterial proteins in culture supernatants. Bacteria were grown overnight in TSB at 37°C and subcultured 1:100 into fresh TSB. After approximately 2 hours incubation at 37°C with agitation, supernatants from 20 ml of the log phase cultures were obtained by pelleting the bacteria at 10,000 x g for 10 minutes. Total protein was precipitated from the supernatants with ammonium sulfate added to 70% saturation at 4°C and incubating the mixture for approximately one hour. After centrifugation for 30 minutes at 10,000 x g, the protein pellets were resuspended in 1 ml of distilled water containing 1 mM

phenylmethyl-sulfonylfluoride (PMSF, Sigma) to inhibit proteolysis. Samples were standardized to the A_{600} of the least dense culture or to total protein prior to application onto a 12% SDS-PAGE gel. The gels were then electroblotted and the blots immunolabelled and developed as described above. Alternatively, total protein profiles were visualized by silver staining gels immediately after electrophoresis with the Rapid-Ag-Stain kit (ICN Radiochemicals, Irvine, CA).

d.) Proteinase K digestion. The amount of surface-exposed, cell-associated antigen was assessed by proteolytic susceptibility with the serine peptidase, proteinase K. A log phase culture of the wild-type strain, 2457T, was washed once and suspended in 50 mM Tris + 100 mM $CaCl_2$. The cells were then either sonicated on ice for 4- 30 second bursts or left intact. Nine hundred µl of lysate or whole cells was then aliquoted into each of 4 tubes and proteinase K (Boehringer Mannheim) added to one tube each of whole cells and lysate at 250 µg/ml concentration. One tube each of lysate and cells were left untreated. After 1 hour incubation at 4°C, PMSF was added to all the tubes at 5 mM final concentration and the mixtures incubated on ice for an additional 10 minutes. Following this, SDS-PAGE sample buffer was added to the samples which were then heated to 100°C for 10 minutes. After SDS-PAGE and electroblotting. the samples were probed with anti-IpaB and IpaC MAbs. Western blots were quantitated as described above.

e.) Immunogold Electron Microscopy. Analysis by electron microscopy of surface expression of Ipa B and IpaC on *Shigella* strains was performed by air drying PBS-washed log phase whole bacteria on formvar/carbon 200 mesh copper grids (Ted Pella, Inc., Redding, CA). Grids were labelled with a 1:10 to 1:10,000 dilution of IgG fractions from anti-Ipa B and IpaC Mabs or polyclonal, monospecific anti-Ipa serum in PBS + 1% BSA and 1% Tween-20. After three washes with 1% BSA/Tween, grids were labelled with a 1:25 dilution of 10 nm gold-conjugated anti-mouse or anti-rabbit IgG (Auroprobe™, Amersham) for 1/2 to 2 hours. After additional washing, the immunolabelled grids were negatively stained with 0.5 to 1% ammonium molybdate

(Sigma) for 30-60 seconds followed by washing twice with distilled water and air-drying. Grids were observed under a Jeol model 100CX transmission electron microscope at an acceleration voltage of 80,000.

Some samples were prepared following a procedure similar to the suspension-labelling immunoassay. Bacteria were washed once and standardized to the A_{600} of the least dense culture. Cells were then either left untreated or pretreated with 0.01% Glutaraldehyde (grade 1, Sigma) or 2 mg/ml of the protein cross-linking agent dimethyl suberimidate·2HCl (DMS, Pierce) prior to resuspension in PBS + 3% casein. The bacteria were then labelled directly in suspension with primary and secondary immunological reagents for the above stated incubation times and temperatures. After this step, the labelled cells were applied to coated grids and stained as above.

Cellular fractionation. Wild-type *Shigella* and selected fusion mutants were fractionated into cytoplasmic, periplasmic, and total membrane components by a modification of reported methods (Deidrich, et al., 1977; Flanagan, et al., 1989). One hundred ml of late log phase bacterial cultures were centrifuged and suspended in one tenth volume of TEP (100 mM Tris, 1.0 mM PMSF and 0.02% NaN_3), then centrifuged and suspended in an equal volume of 20% sucrose. After a rapid freeze/thaw in dry ice-alcohol, lysozyme (Sigma) was added to 0.4 mg/ml and the cells incubated at 37°C for 30 minutes with gentle agitation. Spheroplast formation was confirmed by phase contrast microscopy and the periplasmic fraction was removed from the spheroplasts by centrifugation. Spheroplasts were then resuspended in ice cold TEP and subjected to sonication for 4- 15 second bursts. The total membrane fraction was separated from the cytoplasm by removing debris and unlysed cells from the sonicate by first centrifuging for 10 minutes at 10,000 x g, then at 50,000 x g for 2 hours at 4°C. The membrane pellet was resuspended in TEP with Triton X-100 added to 1.0% and incubated at 37°C for 10 minutes. The outer membrane (Triton-insoluble fraction) was then separated

from the inner membrane by pelleting at 50,000 x g for 2 hours. Fractions were assayed for total protein by BCA-Lowry (Pierce) and stored at -20°C. Membrane and periplasmic fractions were checked for cytoplasmic cross-contamination by assessing glucose-6-phosphate dehydrogenase activity in each following a standard assay procedure for this enzyme (Scott, 1975).

Gel filtration chromatography. Enrichment for high molecular weight Ipa complexes was performed by running a 0.5 ml sample of a sonic lysate of either 2457T or BS260 (prepared as described above) through approximately 100 ml of Sephadex G-100 (Pharmacia) resin packed in a 120 x 1 cm glass column. A flow rate of 2.0 mls/hour was maintained with 0.1M Tris (pH 7.0) elution buffer. The void volume was measured with both Dextran blue 2000 (> 2 mDa) and aldolase (150 kDa) as the high molecular weight markers which are excluded from the gel matrix. Bovine serum albumin (66 kDa), which is retained in the gel, was also used as a reference marker, and was mixed in equal volumes with either one of the two high molecular weight markers during void volume measurements. Sixty- 1 ml fractions were collected for each sample run and the total protein elution profile determined by measuring A_{280} of each fraction. The presence of Ipa A through D in the G-100 fractions was determined by immunoblotting 20 µl of each sample with anti-Ipa rabbit hyperimmune serum prepared against water extracts of 2457T (see below). Ipa levels from each fraction were individually quantitated by densitometric scanning of Western blots as described above.

Purification of β-galactosidase hybrid protein. The *mxi* hybrid protein (MxiHP) produced by BS260 was isolated by growing 1 to 7 liters of BS260 overnight, centrifuging, and resuspending the pellet in cold TEP at 1/20 the original volume. The cells were then disrupted by sonic lysis with 8- 15 second bursts at 4°C. Alternatively,

TEP-suspended cells were sonicated in TEP supplemented with the non-ionic detergent Nonidet P-40 (NP-40, Sigma) at 1% final concentration. Scale-up preparations of the hybrid protein were also made by lysis of the harvested bacteria in a French pressure cell at 1000 psi in TEP + 1% NP-40. The lysates prepared by any of these three methods were next centrifuged at 10,000 x g for 10 minutes to remove cell debris, and then centrifuged at 50,000 x g for 2 hours. The protein in the clarified lysates was concentrated by ammonium sulfate precipitation and the pellets resuspended in cold TEP to 20 mg/ml total protein. The protein concentration was then further adjusted to 4 mg/ml with TBSN (50 mM Tris, pH 7.3, 150 mM NaCl, 0.2% NP-40). The lysates were next passed over a β-galactosidase affinity column (Promega Corporation, Madison, WI) to bind the hybrid protein. After washing the column with several volumes of TBSN, the bound MxiHP was eluted with bicarbonate buffer (0.1 M $NaHCO_3/NaCO_3$, pH 10.8). Samples were assayed for recovery and purity at various steps throughout the purification procedure by BCA-Lowry, SDS-PAGE, Western blot, and the β-galactosidase assay.

Generation of hyperimmune rabbit serum. Monospecific antiserum to MxiA was generated by parenteral immunization with affinity-purified MxiHP which had been concentrated with a Centricon™ microconcentrator (Amicon, Danvers, MA). Two 6 week old male New Zealand White rabbits were pre-bled and injected subcutaneously in each hind quarter with 1 ml each of 100-300 μg of MxiHP mixed 1:1 with Freund's complete adjuvant and boosted with antigen in incomplete adjuvant at 2-4 week intervals. One rabbit was administered the purified protein in its native conformation. A second rabbit was immunized with MxiHP which was heat-denatured and reduced with β-mercaptoethanol, electrophoresed through an 8% polyacrylamide gel and excised as a 5 mm gel slice. The gel slice was crushed and mixed 1:1 with adjuvant just prior to immunization. The IgG fraction was purified from the rabbit hyperimmune serum by

passage over a Staph-A Sepharose column (Pierce), de-salted through G-25 exocellulose (Pierce), and adsorbed 3-5 times with acetone-fixed plasmidless *S. flexneri* strain BS103, grown at 37°C and once with wild-type 2457T grown at 30°C.

Anti-Ipa rabbit serum was generated in a similar fashion except that the immunizing antigen consisted of a distilled water extract (Hale, et al., 1985) of an overnight culture of 2457T. Whole serum was then adsorbed extensively with acetone-fixed BS103 and used directly on electroblots at a 1:1000-1:2000 dilution. When this anti-Ipa hyperimmune serum was used for suspension-labelling of bacteria for electron microscopy, Ipa B and IpaC-specific antibody fractions were isolated by adsorbing a 1:500 dilution of the total serum with nitrocellulose on which had been electroblotted a whole cell lysate of 2457T. After incubation at room temperature for 6 hours, the blot was washed several times with PBS and nitrocellulose strips which corresponded to the positions of IpaB and IpaC were excised. The bound monospecific polyclonal antibodies were then eluted from the blot strips with 0.2 M glycine (pH 2.8) + 1 mM EGTA and neutralized with 10x PBS (Maniatis, 1989). The antibody preparations were then assayed for IpaB and IpaC specificity by Western blotting whole cell lysates of wild-type *S. flexneri.*

RESULTS

Part I. Identification of the *mxiA* locus and characterization of IpaB and IpaC as excreted virulence factors.

Isolation and virulence phenotypes of temperature-regulated protein fusion mutants. Wild type *S. flexneri* 2a, strain BS181, was infected with the protein fusion phage λp*lac*Mu9 and the mixture plated on selective media containing kanamycin and X-gal. Over 4200 Km^r Lac^+ lysogens were isolated and 9 were found to be temperature-regulated for β-galactosidase. Bacteriophage P1 lysates were next generated on these 9 isolates and the lesions transduced into a clean *Shigella* background (strain 2457T) to segregate possible double insertions. The resulting Lac^+ transductants maintained their temperature-regulated phenotype (Table 4). Four of the protein fusion mutants demonstrated high 37°/30°C ratios (>30) characteristic of the pattern of temperature-regulated expression of *Shigella* virulence. Plasmid DNA from each of the fusions was next isolated and analyzed on agarose gels. Eight of the nine fusions appeared to possess inserts localized to the 220 kb invasion plasmid based on a shift in mobility between the plasmid DNA isolated from wild-type and the plasmids from the mutated strains (Table 4). When each of the fusion mutants was tested in the various *in vitro* assays for virulence, only one, BS260, was found to be avirulent. This mutant was negative in the Sereny test, very weakly invasive for HeLa cells, and only partially contact hemolytic (Table 4). Two of the mutants, BS259 and BS270, were found to be invasive at intermediate levels relative to wild-type, although they were fully capable of forming plaques and eliciting an inflammatory response in the Sereny test. The remaining 6 protein fusion mutants were fully invasive and behaved similarly to the wild-type parent in the other virulence assays. The non-invasive fusion, BS260,

TABLE 4. Characterization of Protein Fusion Mutants of *Shigella flexneri* 2457T

MUTANT	37°C/30°C ß-GAL RATIO	HeLa INVASION [a]	PLAQUE FORMATION [b]	CONTACT HEMOLYSIS [c]	SERENY TEST [d]	INSERT SITE [e]
BS259	5	43	++++	0.63	POS	Plasmid
BS260	33	5	ND	0.12	NEG	Plasmid
BS270	6	40	++++	ND	POS	Chromosome
BS271	8	63	++++	ND	POS	Plasmid
BS276	6	85	ND	0.75	POS	Plasmid
BS281	117	63	++++	0.57	POS	Plasmid
BS282	31	77	++++	0.45	POS	Plasmid
BS283	30	74	++++	0.62	ND	Plasmid
BS284	15	77	++++	0.68	POS	Plasmid

[a] Values for wild-type 2457T ranged from 58 to 86%.

[b] - = no plaques; + = 5-25% of wild-type; ++ = 25-50%; +++ = 50-80% of wild-type; ++++ = 80-100% of wild-type

[c] Values for wild type ranged from A_{540} 0.54 to 0.70

[d] POS = keratoconjunctivitis after 48 hours.

[e] Determination based on the presence or absence of a detectable shift in mobility of the 220 kb invasion plasmid on an agarose gel.

ND = not done

as well as the two invasive fusions with the highest β-galactosidase ratios, BS281 and BS282, were selected for further analysis.

Detection of hybrid proteins in λp*lac*Mu protein fusion mutants. In order to identify the LacZ hybrid proteins in selected fusion mutants, whole cell lysates of 2457T (wild-type), BS260, BS281, and BS282 were run on SDS-PAGE, electroblotted to nitrocellulose, and probed with MAb to β-galactosidase. As shown in Figure 1, the LacZ/Vir hybrid protein of BS260 migrated as a single band at a slightly higher molecular weight (123 kDa, lane 2) than native β-galactosidase (116 kDa, lane 6). Doublets were detected in lysates of both BS281 and BS282. The upper band of BS281 migrated to approximately 141 kDa (lane 3), while the higher molecular weight band in BS282 was positioned at 127 kDa (lane 4). The lower bands of both of these mutants corresponded to the same position as native β-galactosidase.

Cellular localization of the hybrid protein products made in the protein fusion mutants was next examined on electroblots of cytoplasmic, inner, and outer membrane fractions probed with anti-β-galactosidase MAb. The transcriptional fusion mutant, BS226, was also fractionated and run as a control for localization of native β-galactosidase (a cytoplasmic enzyme). As shown in Figure 2, lane 2, the cytoplasmic fraction of the control strain, BS226 contained a high level of the native enzyme while a low level was detected in the inner membrane (lane 3) and none in the outer membrane fraction (lane 4). In contrast, the non-invasive protein fusion mutant, BS260, possessed higher levels of the β-gal/Vir hybrid protein in the membrane fractions (lanes 6 and 7). The remainder of the mutants presented a variable distribution of their hybrid proteins in the cellular fractions (lanes 8-16). It was concluded from this experiment that the virulence protein defined by BS260 may normally be localized in the cell envelope of wild-type *Shigella*.

Figure 1. Detection of hybrid proteins in selected translational gene fusions of *S. flexneri.* Western blot of whole cell lysates probed with anti-β-galactosidase MAb. Lane 2, BS260 (37°C); lane 3, BS281 (37°C); lane 4, BS282 (37°C); lane 5, 2457T (37°C). Lanes 1 and 6 are pre-stained and unstained molecular weight standards (respectively) which contain native β-galactosidase. Immunolabelled whole cell lysates of the same mutants grown at 30°C were negative (not shown).

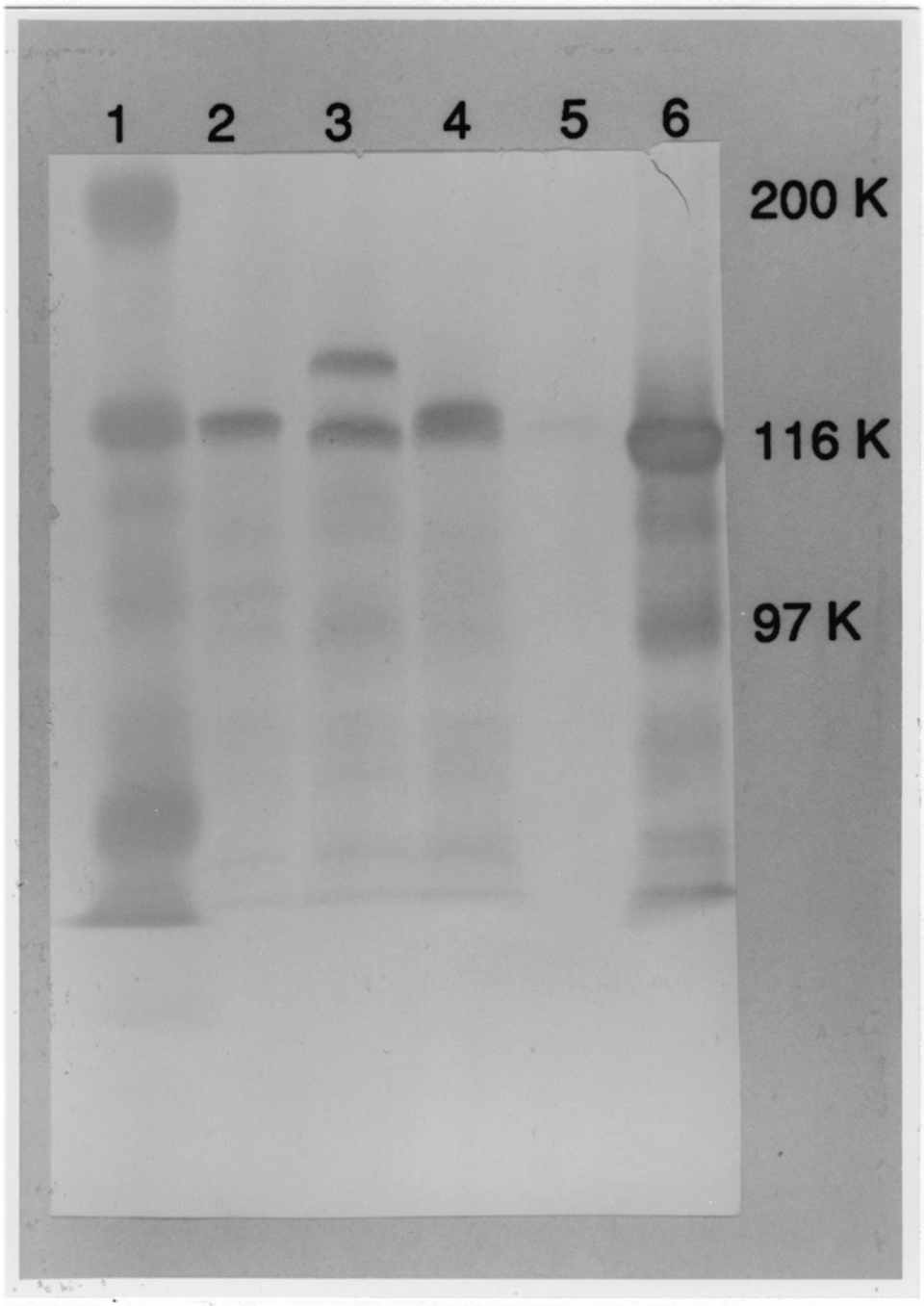
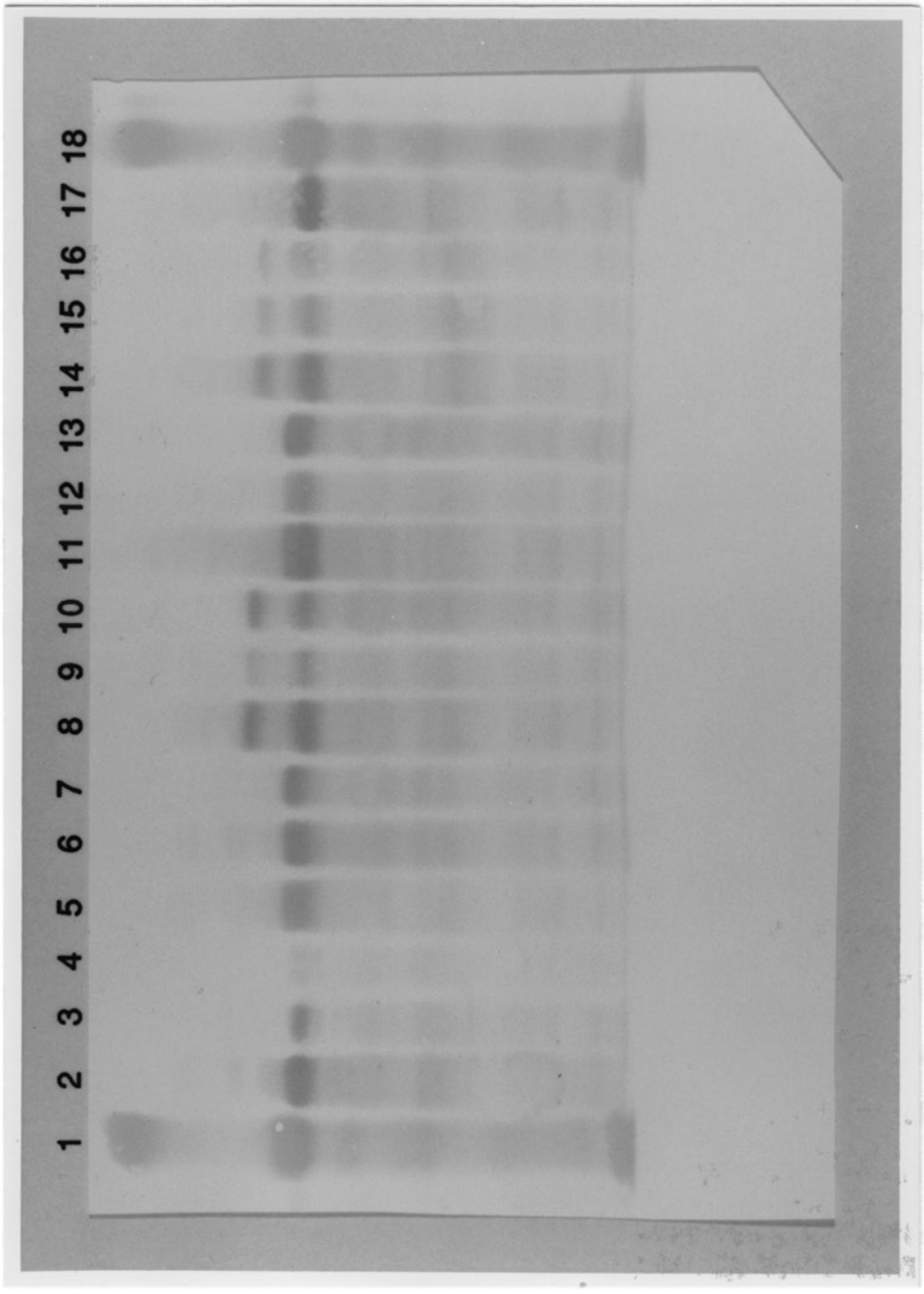

1
2
3
4
5
6
200 K
116 K
97 K

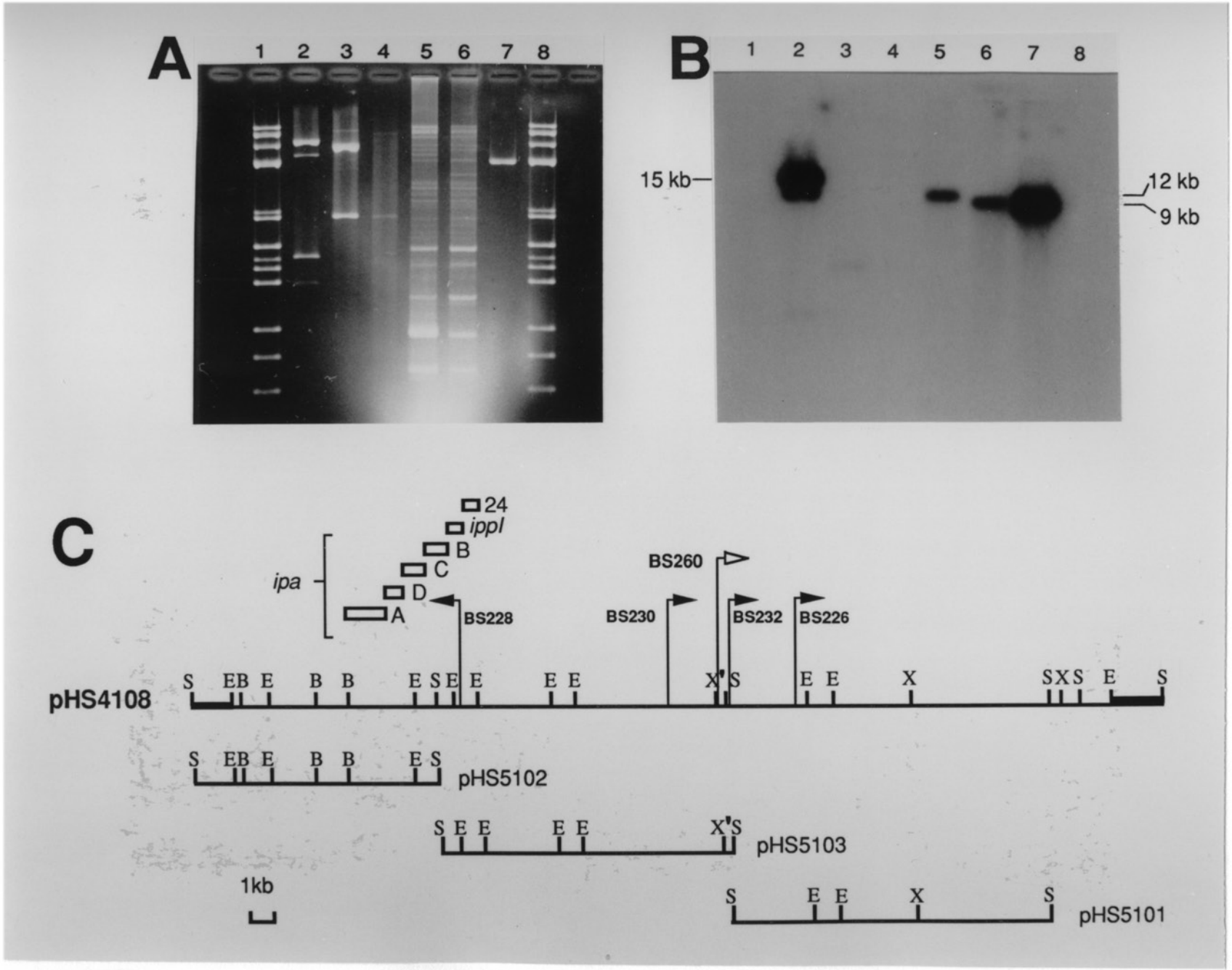
Figure 2. Localization of hybrid proteins in *S. flexneri* fusion mutants. Western blot analysis of the cytoplasmic fraction, inner, and outer membrane fractions of BS260 (lanes 5-7), BS281 (lanes 8-10), BS282 (lanes 11-13), and BS283 (lanes 14-16). Lanes 2-4, represent cellular fractions of the operon fusion mutant, BS226 which produces native β-galactosidase (cytoplasmic). Lane 17, cytoplasmic fraction of BS260. Lanes 1 and 18 contain pre-stained molecular weight standards. Samples were standardized to total protein prior to their application on the SDS-PAGE gel.


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

Cloning, mapping, and expression of protein fusion end joints. The cloning and mapping of the protein fusion end joints was done using a procedure previously employed for cloning the operon fusion end joint of BS226 (Hromockyj and Maurelli, 1989). Plasmid DNA from the BS260 and BS281 protein fusion mutants were digested with *EcoR*I, ligated into the *EcoR*I-digested fusion vector pMLB524 (Table 3), and the resulting recombinant DNA transformed into *E. coli* DH5α. Clones were selected for vector-encoded ampicillin resistance and screened for the Lac$^+$ phenotype of the fusion. To determine if the fusion end joints of the protein fusion mutants mapped within the 37 kb cosmid clone of the invasion plasmid (pHS4108, Figure 3c), the recombinant plasmids were purified, radiolabelled, and used as probes in a Southern hybridization analysis of the three *Sal*I subclones spanning pHS4108 (Table 3).

The size of the *Eco*RI fragment from BS260 in the resulting recombinant plasmid, pGPA001, was calculated to be 9 kb (Figure 3a). This represented 6 kb of DNA upstream from the actual end joint, with the *lacZ* coding sequence comprising the remaining 3 kb. Using this fragment to probe a Southern blot revealed that the BS260 mutation was within the pHS5103 sub-clone of pHS4108 (Figure 3b, lane 2). Moreover, hybridization of this fragment to a restriction digest of the 2457T invasion plasmid placed the fusion phage insertion site in BS260 in a 12 kb *EcoR*I fragment (Figure 3b, lane 5). A restriction analysis of pGPA001 further localized the fusion phage insert precisely between an *Xba*I and a *Sal*I site on this fragment. Based on the map of pHS4108, the locus defined by BS260 was therefore located 11 kb downstream of the *ipa* operon, and 4 kb upstream from the insertion in the non-invasive operon fusion, BS226 (Figure 3c). The *EcoR*I restriction fragment from the cloned fusion end joint of the invasive Lac$^+$ mutant BS281 (pGPA002, Table 3) was determined to be approximately 20 kb (not shown). Thus, the clone consisted of at least 17 kb of invasion plasmid DNA upstream of *lacZ* sequence. When this insert fragment was used as a probe

Figure 3. Mapping of the fusion phage insertion in BS260. (A) Representative agarose gel of *Sal*I-digested DNA from the three *Sal*I sub-clones of the 37 kb cloned region (pHS4108) of the invasion plasmid: pHS5103 (lane 2), pHS5102 (lane 3), and pHS5101 (lane 4). Lanes 5 and 6 are *EcoR*I digests of 2457T and the parent mutant, respectively. Lane 7 represents the homologous probe DNA as a positive control. Lanes 1 and 8 contain *BamHI*/*EcoR*I digested Adenovirus-2 DNA as a negative control and size marker. Sizes of marker DNA bands from top to bottom (kb): 35.9, 21.3, 14.3, 11.7, 6.2, 5.9, 4.7, 4.3, 4.0, 3.7, 2.7, 2.2, 1.7. (B) The same gel as in A, blotted and probed with the ^{32}P-labelled cloned insert fragment from BS260. (C) Map of the *Sau*3A sub-clone, pHS4108, showing the position of the insert in BS260 in relationship to other *mxi* mutants and the *ipa* operon. Also shown are the regions of pHS4108 covered by the *Sal*I sub-clones used in the Southern blotting.


A
1 2 3 4 5 6 7 8
B
1 2 3 4 5 6 7 8
15 kb
12 kb
9 kb
C
24
ippI
B
C
D
A
ipa
BS228
BS260
BS230
BS232
BS226
pHS4108
S EB E B B E S E E E E X' S E E X SXS E S
pHS5102
S EB E B B E S
pHS5103
S E E E E X'S
pHS5101
S E E X S
1kb

against a digest of the 2457T invasion plasmid, it hybridized to a 22 kb *EcoR*I fragment. Unlike BS260 however, the locus defined by BS281 mapped outside pHS4108, since the cloned end joint from BS281 failed to hybridize with any of the three *Sal*I sub-clones (not shown).

Since pGPA001 contained 6 kb of DNA upstream of the fusion end joint, it seemed likely that sufficient DNA was present to include the temperature-regulated promoter which was driving expression of the fusion. An analysis of β-galactosidase activity from pGPA001 in an *E. coli* host (DH5α) however, exhibited low levels of expression of the fusion at both 30°C and 37°C (Table 5). To test the possibility that these low enzyme levels were due to the absence of chromosomal regulatory genes present only in *Shigella,* pGPA001 was transformed into the plasmidless *S. flexneri* derivative BS103. This manipulation failed to raise β-galactosidase levels to those of the parent mutant (Table 5). β-galactosidase levels also remained low when plasmid-encoded activators were restored by transforming pGPA001 into an invasion plasmid-containing *recA Shigella* background (BS201, Table 3). These results are in contrast to the findings with a previously described non-invasive operon fusion mutant, BS226 (Hromockyj and Maurelli, 1989). When the cloned end joint from this mutant was placed in a wild-type *Shigella* background, β-galactosidase levels were restored (Table 5). Similarly, when the cloned end joint (pGPA002) from the Lac^+ invasive protein fusion mutant, BS281, was transformed into BS201, enzyme levels were also elevated (Table 5). The above results suggested that the promoter for the gene fusion in BS260 was not contained on the 9 kb end joint fragment cloned in pGPA001. The promoters for the loci in BS226 and BS281 however, did appear to be present on the *EcoR*I fragments containing their respective fusion end joints. Thus, BS260 and BS226 represent mutations in independent transcription units. Additionally, the loci defined by BS226, as well as BS281, appear to require plasmid-encoded trans-activators for full expression.

TABLE 5. β-galactosidase levels of the cloned BS260, BS226, and BS281 fusion end joints.

	Units β-galactosidase [a]		
	37°C	30°C	37°/30°
BS260	330	12	28
DH5-α/pGPA001	30	14	2
BS103/pGPA001	24	21	1
BS201/pGPA001	25	8	3
BS226	447	15	30
DH5-α/pAEH006 [b]	55	69	<1
BS103/pAEH006 [b]	63	67	<1
BS201/pAEH006 [b]	456	51	9
BS281	377	12	33
DH5-α/pGPA002	16	11	1
BS103/pGPA002	45	17	3
BS201/pGPA002	865	15	59

[a] As defined by Miller (1972).

[b] From Hromockyj (1991); Andrews, et al., (1991)

Control of the BS260 *vir* locus by the chromosomal regulatory gene *virR*.

To determine if the virulence locus defined by the mutant BS260 was under the control of the *Shigella* chromosomal thermoregulatory locus, *virR*, a P1 lysate made on the *virR*::Tn*10* strain, BS189 (Table 3) was used to infect BS260. The Km^r Tet^r Lac^+ transductants isolated were then screened for derepression of the β-galactosidase temperature-regulated phenotype. Interestingly, although the gene fusion in BS260 was constitutive for temperature-regulated β-galactosidase expression (Table 6), deregulation was caused by a reduction of enzyme levels at the permissive temperature (37°C) and not from an increase of enzyme levels at 30°C as was observed with the control BS281 (Table 6) and with previously described *vir*::*lacZ* transcriptional fusions (Hromockyj and Maurelli, 1989). This observation led to the hypothesis that a secondary mutation may have been selected as a result of inactivating the repressor locus (*virR*) in BS260. Because inactivation of *virR* should cause constitutive expression of the β-gal/Vir fusion protein, an unfavorable condition for bacterial survival may arise since high level expression of certain membrane-driven hybrid proteins can be lethal (Silhavy and Beckwith, 1985). Therefore, to determine if a second-site mutation was generated during the construction of BS260 *virR*::Tn*10*, P1 lysates were made on one of these transductants and used to reinfect wild-type *Shigella.* This was done to segregate the putative secondary mutation from the Tn*10* insertion in *virR* and to establish an approximate linkage distance between the second site lesion and the λp*lac*Mu9 insertion site in the invasion plasmid. When the phage insert was transduced into a "clean" wild-type background, two distinct Lac phenotypes were isolated. A temperature deregulated $Lac^{+/-}$ phenotype characteristic of the original BS260 *virR*::Tn*10* transductant was isolated at a frequency of 20%, while the remainder of the transductants were of a Lac^+ phenotype characteristic of the parent mutant, BS260. These results indicated that a secondary mutation was linked to the

Table 6. Regulation of *S. flexneri* protein fusions by the thermoregulatory locus *virR* [a]

STRAINS [b]	Units, β-galactosidase 37°C	30°C	37°/30°
BS260	158	7	23
BS260 *virR*::Tn*10* #1	13	6	2
BS260 *virR*::Tn*10* #2	13	5	3
BS281	216	3	72
BS281 *virR*::Tn*10*	828	131	6

[a] Values are from a single representative experiment.

[b] *virR*::Tn*10* transductants #1 and #2 of BS260 represent independent isolates.

phage insertion site in BS260 *virR*::Tn*10* which caused a significant reduction in β-galactosidase expression.

Ipa export phenotype of BS260. To determine if the non-invasive protein fusion mutant, BS260, was defective or altered in its expression of the *ipa* gene products, a Western blot of whole cell lysates of 2457T, BS260, BS281, and BS282 was performed using anti-Ipa convalescent monkey serum. Ipa A through D were present at the same levels in all of the strains grown at 37°C (Figure 4, lanes 1, 3, 5, and 7) and their expression was temperature-regulated, since lysates from cultures of the same strains grown at 30°C possessed low levels of the antigens (Figure 4, lanes 2, 4, 6, and 8).

Two previously isolated non-invasive operon fusion mutants, BS226 and BS232, make normal intracellular levels of Ipa proteins relative to wild-type, but appear to be defective in surface expression or presentation of IpaB and IpaC (Hromockyj and Maurelli, 1989). It was based on this secretion-defective phenotype that the loci involved in facilitating export of Ipa proteins were designated *mxi* for membrane expression of invasion plasmid antigens. The detection of Ipa secretion defects was based on the results obtained by a whole cell ELISA designed to measure surface-associated antigen (Mills, et al., 1988). A caveat of this assay, however, is that it does not distinguish between cell-associated versus cell-free antigen. Therefore, to assay more directly for surface-exposed, cell-bound antigen on the *mxi* mutants, an anti-Ipa MAb suspension labelling immunoassay (SLIM) was developed. Unlike the whole cell ELISA, the SLIM assay was devised to measure relative amounts of surface-associated antigen independent of released or excreted antigen. When the assay was employed on log phase cultures of BS260, the strain was found to bind significantly lower levels of anti-IpaB MAb (Figure 5a) relative to wild-type. A similar phenotype was seen with the *mxi* operon fusion mutant, BS226. All three strains (2457T, BS260, BS226), however, bound very low levels of anti-IpaC MAb (Figure 5b). This finding was in contrast to the

Figure 4. Expression of IpaA-D in selected protein fusion mutants of *S. flexneri*. Whole cell lysates of BS260 (lanes 1, 2), BS281 (lanes 3, 4), BS282 (lanes 5, 6), and 2457T (lanes 7, 8) were run on SDS-PAGE, electroblotted, and probed with anti-*Shigella* convalescent monkey serum. Cultures were grown at 37°C (odd lanes) and 30°C (even lanes). The bands corresponding to IpaA (78 kDa), IpaB (62 kDa), IpaC (42 kDa), and IpaD (37 kDa) are indicated.

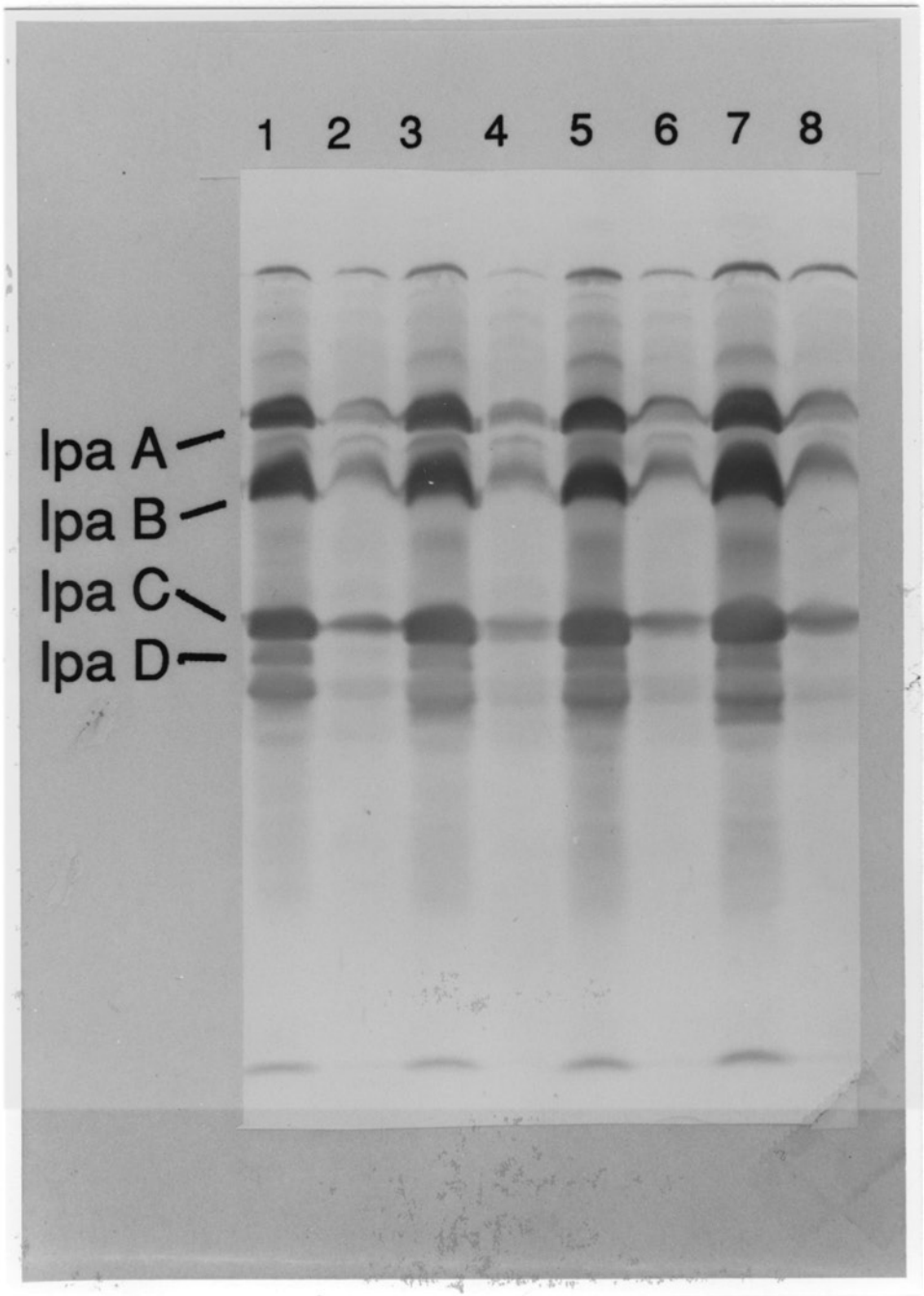
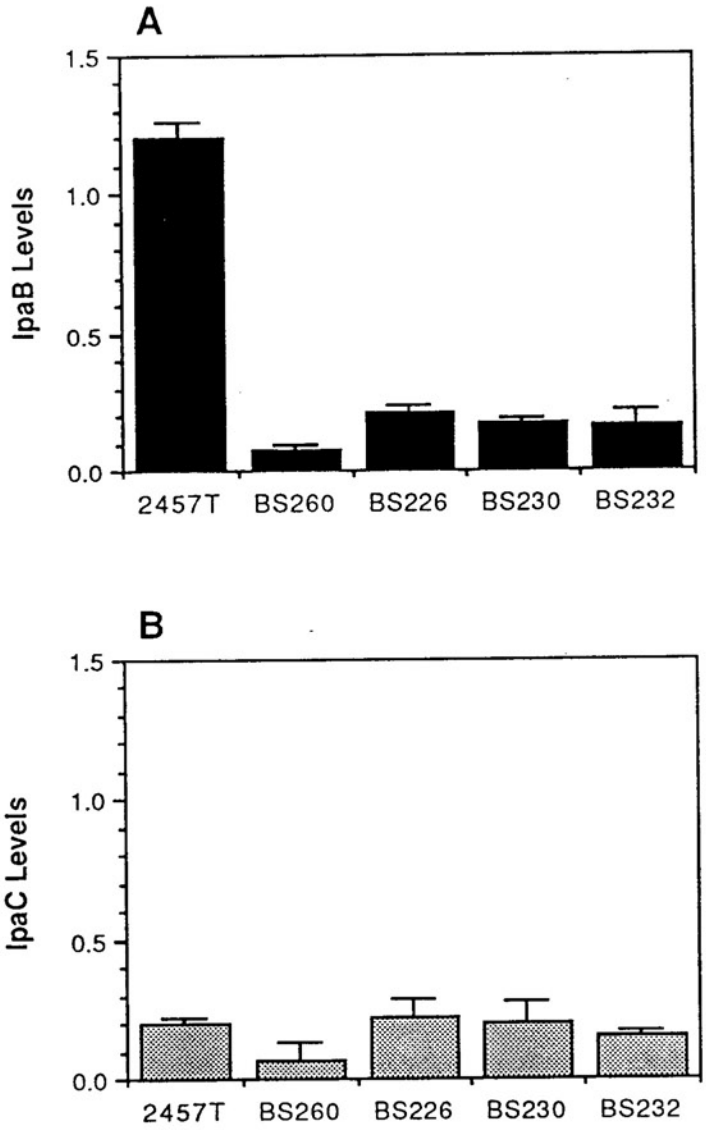

1 2 3 4 5 6 7 8
Ipa A
Ipa B
Ipa C
Ipa D

Figure 5. Detection of cell surface-associated IpaB and IpaC on wild-type cells and fusion mutants by SLIM. Log phase bacteria were immunolabelled in suspension with MAb to the Ipa proteins and the amount of surface-associated antigen detected with an alkaline phosphatase-conjugated secondary antibody. (A) IpaB levels represented by reactivity of cells with anti-IpaB MAb. (B) IpaC levels represented by reactivity of cells with anti-IpaC MAb. Samples were standardized by adjusting volume against culture adsorbance. MAb reactivities are expressed as A_{405} values. Absorbance values were corrected for background by subtracting the reactivity obtained for the plasmidless derivative BS103 (<5.0% of WT). Error bars represent two standard deviations from the mean.


A
IpaB Levels
1.5
1.0
0.5
0.0
2457T
BS260
BS226
BS230
BS232
B
IpaC Levels
1.5
1.0
0.5
0.0
2457T
BS260
BS226
BS230
BS232

results obtained by others using the whole cell ELISA which indicated that a significant amount of IpaC is surface-associated on wild-type cells (Mills, et al., 1988). Two other *mxi* operon fusion mutants, BS230 and BS232 (Hromockyj and Maurelli, 1989), also tested in the SLIM assay, behaved similarly to the protein fusion mutant (Figure 5a, b). These data suggest that BS260 was deficient in surface expression of IpaB. Since these results are in agreement with the phenotype assigned to the *mxi* operon fusion mutants, the mutated locus in BS260 was designated *mxiA*. Because the mutations in BS260 and BS226 appeared to be localized to two separate transcription units, the locus in BS226 was designated *mxiB*.

Detection of released/excreted virulence-associated proteins. The possibility was considered that IpaB was being exported normally to the surface in the *mxi* mutants but was unstable or weakly associated with the outer membrane. If this hypothesis was correct, IpaB should have been found in culture supernatants of *mxi* mutants. Therefore, a cell-free ELISA was performed using the anti-IpaB (and IpaC) MAbs to detect any released antigen. As shown in Figure 6, microtiter plate wells coated with cell-free PBS supernatant from the two *mxi* mutants (BS260 and BS226) were only weakly reactive to both monoclonals. Wells exposed to supernatants from wild-type *Shigella,* however, reacted strongly with the monoclonals suggesting that excretion of the Ipa proteins was in fact a wild-type characteristic.

To confirm the hypothesis that Ipa B and IpaC are, in fact, excreted from actively growing cultures of wild-type *Shigella*, ammonium sulfate-precipitated culture supernatants from log phase 2457T, BS260, and BS226 were reacted with rabbit anti-Ipa serum on a Western blot. While all the bacterial strains possessed comparable levels of intracellular IpaB and IpaC, the culture supernatants of the *mxi* mutants failed to yield significant levels of both antigens (Figure 7).

Figure 6. Detection of released/excreted IpaB and IpaC by wild-type *Shigella* and fusion mutants BS260 and BS226 in a cell-free ELISA.

(A) IpaB levels represented by reactivity of PBS supernatants with anti-IpaB MAb.

(B) IpaC levels represented by reactivity of PBS supernatants with anti-Ipa C MAb. Reactivity of MAbs are expressed as A_{405} values which were corrected for background by subtracting reactivity of the plasmidless derivative, BS103. Error bars represent two standard deviations from the mean.

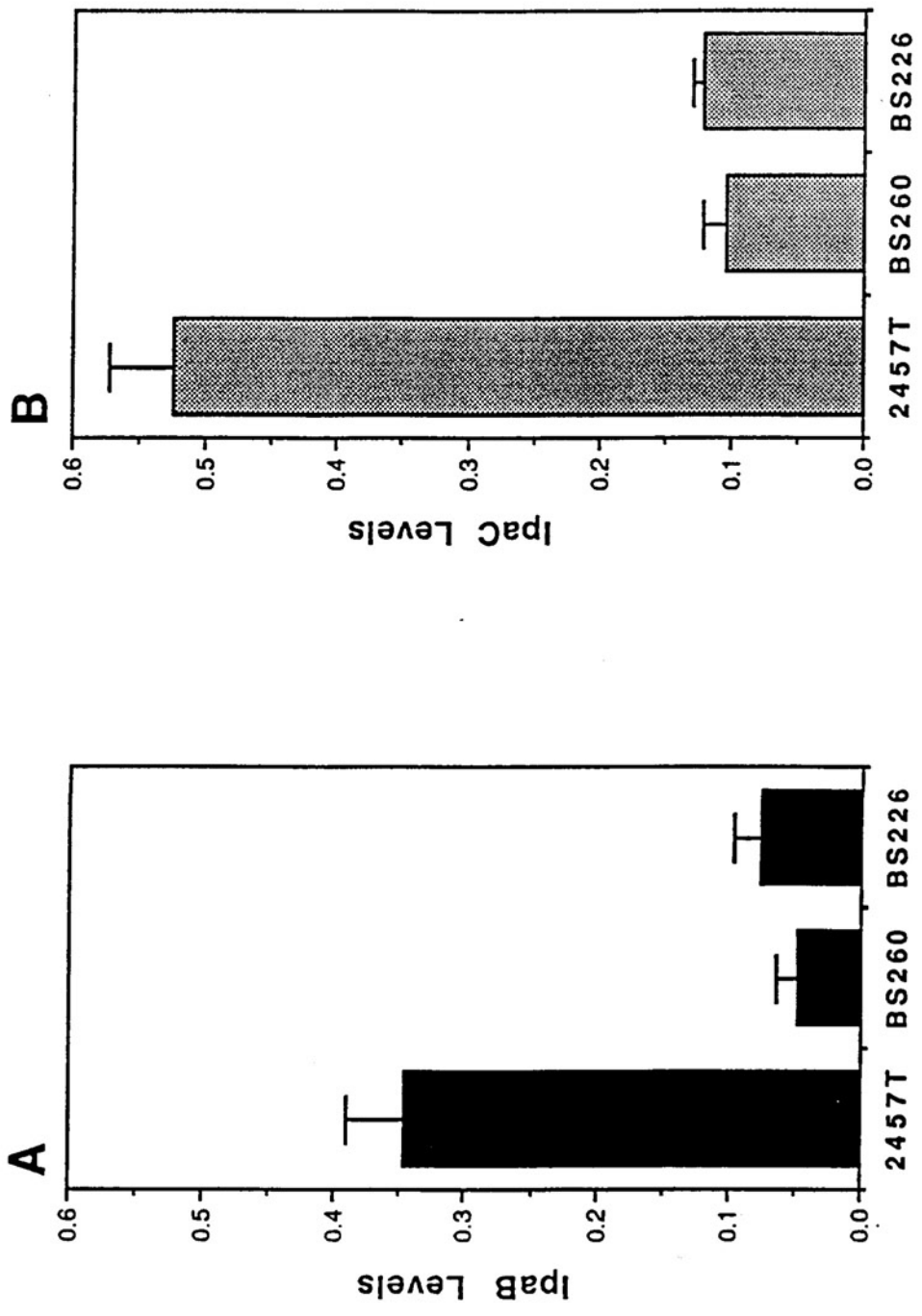
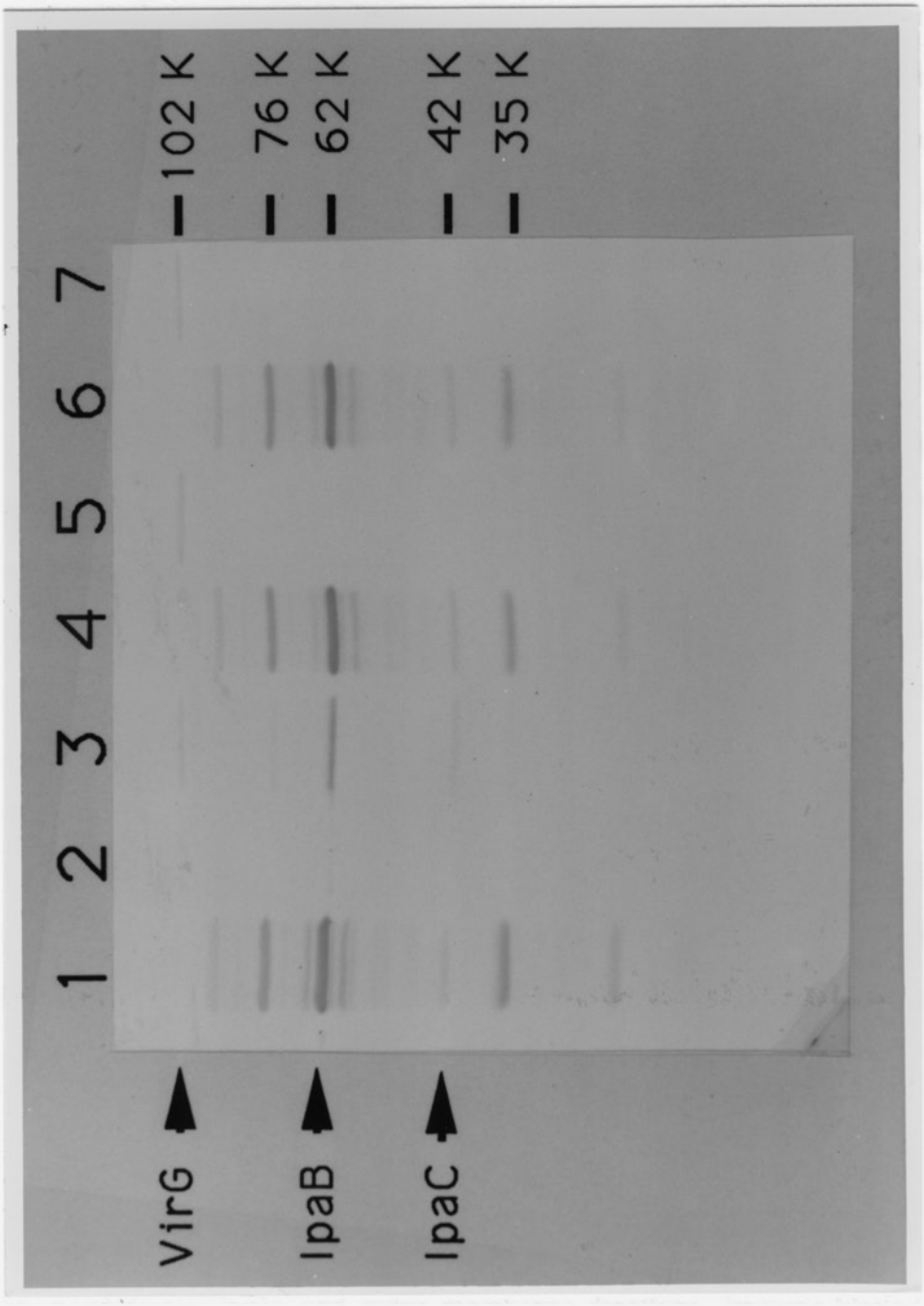

A
IpaB Levels
0.0
0.1
0.2
0.3
0.4
0.5
0.6
2457T
BS260
BS226
B
IpaC Levels
0.0
0.1
0.2
0.3
0.4
0.5
0.6
2457T
BS260
BS226

Figure 7. Release/excretion of IpaB, IpaC, VirG, and other proteins from log phase cultures of wild-type *S. flexneri* and *mxi* mutants. Supernatant proteins were precipitated with ammonium sulfate, separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with rabbit hyperimmune serum to exported *Shigella* proteins. Lanes 1, 4, and 6: whole cell lysates of 2457T, BS260, and BS226, respectively. Lanes 3, 5, and 7: concentrated culture supernatants of 2457T, BS260, and BS226, respectively. Lane 2, whole cell lysate from 10^6 cells of 2457T, representing Ipa contribution of bacteria remaining in the culture supernatant after centrifugation. Lanes 1, 4, and 6 were standardized by absorbance. Lanes 3, 5, and 7 were standardized by total protein. Indicated are the bands corresponding to excreted IpaB, IpaC, and VirG whose identity were confirmed by probing similar blots with the appropriate monospecific antibodies.


1 2 3 4 5 6 7
102 K
76 K
62 K
42 K
35 K
VirG
IpaB
IpaC

In addition to excreted IpaB and IpaC, the wild-type culture supernatant was found to contain three other proteins corresponding to 102 kDa, 76 kDa, and 35 kDa (Figure 7, lane 3). Both the 76 kDa and 35 kDa polypeptides, although only weakly detected in the wild-type supernatant, were absent in the culture supernatants of the *mxi* mutants. Levels of the 102 kDa excreted protein in the mutants, however, appeared comparable to wild-type and, unlike the Ipa proteins, this polypeptide was efficiently excreted since no intracellular pool of this species was detected (Figure 7, lane 1). The previously characterized *virG* polypeptide, which is implicated in intra- and intercellular spread of *Shigella*, has been reported to be an exported protein between 100-120 kDa (Lett, et al., 1989; Venkatesan, et al., 1992). To determine if the 102 kDa species detected in the above blotting experiment was identical to the *virG* gene product, a similar blot of supernatants from log phase cultures was probed with a rabbit anti-VirG serum (kindly provided by Dr. P. Sansonetti). Wild-type as well as the *mxi* mutants excreted a similar 102 kDa species which was reactive with the anti-serum, while this protein was completely absent in a *virG* deletion strain, BS441 (Figure 8). This confirmed that the 102 kDa protein detected with the rabbit polyclonal serum was VirG (Figure 7).

To visualize other plasmid-specific excreted products which may have not been detected by immunoblotting, SDS-PAGE gels of culture supernatants of wild-type, BS103, and the *mxi* mutants were silver-stained to obtain total exported protein profiles. No additional plasmid-encoded polypeptides were detected in the culture supernatants of either wild-type or the *mxi* mutants (Table 7).

Cellular fractionation of *mxi* mutants. To further characterize the Ipa export defect in the *mxi* mutants, BS260 and BS226, log phase cells were separated into cytoplasmic, periplasmic, inner and outer membrane fractions, immuno-blotted, and the distribution of IpaB and IpaC quantitated by laser densitometry. As shown in Figure 9, although high levels of IpaB were detected in the cytoplasm of all strains, analysis of

Figure 8. Identification of excreted VirG in culture supernatants of *S. flexneri*. Western blot of whole cell lysates and culture supernatants of *S. flexneri* immunolabelled with rabbit polyclonal anti-VirG serum. Lane 1, 2457T lysate; lane 2, 2457T supernatant; lane 3, BS260 lysate; Lane 4, BS260 supernatant; Lane 5, BS226 lysate; Lane 6, BS226 supernatant; lane 7, BS441 ($VirG^-$) lysate; lane 8, BS441 supernatant. The high molecular weight cell-associated species and the lower molecular weight excreted form are indicated.

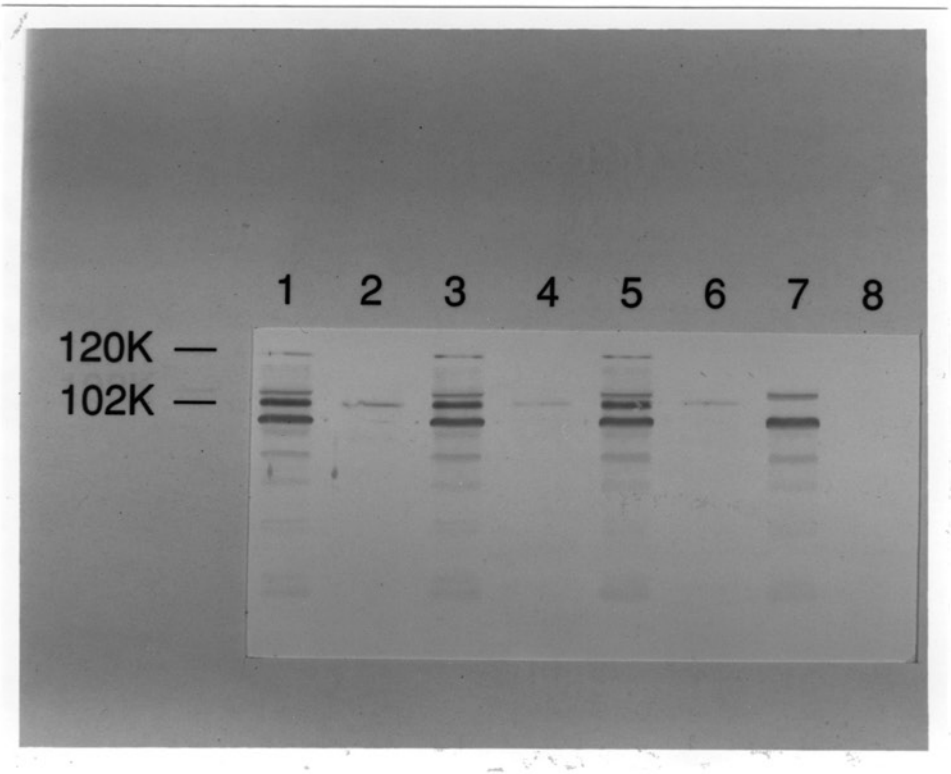

1 2 3 4 5 6 7 8
120K —
102K —

Table 7. Proteins detected in Supernatants from Selected Strains of *Shigella flexneri* 2a

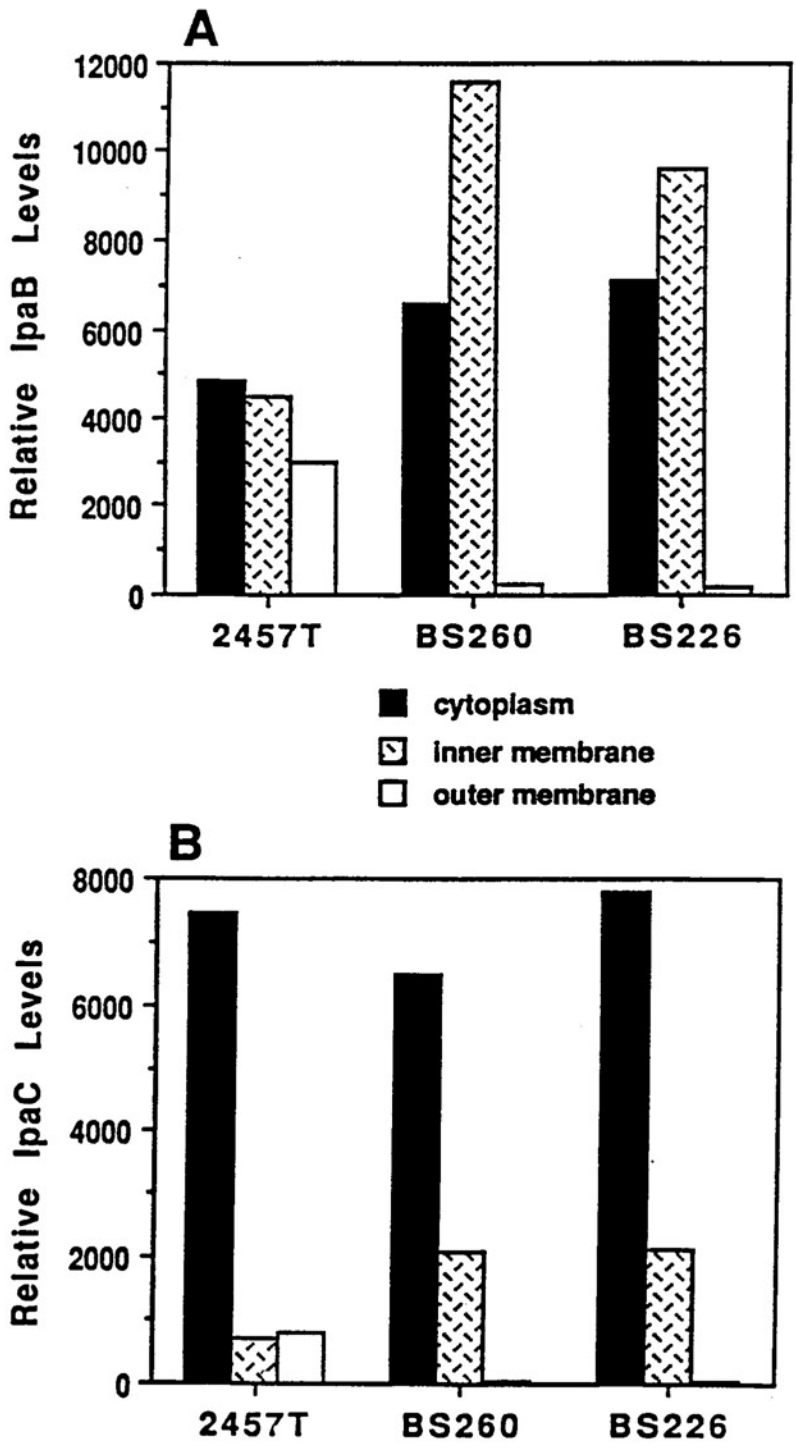
STRAINS	2457T	BS103	BS260	BS226	2457T + CR	BS103 + CR
Excreted Species (kDa)	115		115	115	115	
	108	108	108	108	108	108
	96	96	96	96	96	96
	87		87	87	87	
	79	79	79	79	79	79
	76				+ 76 +	
	74	74	74	74	74	74
	60				+ 60 +	
	48				+ 48 +	
	32	32	32	32	32	32
	30				+ 30 +	
					[22]	
					[17]	

"+ +" designates elevated levels relative to wild type.

"[ ]" indicates new species.

CR = Congo red

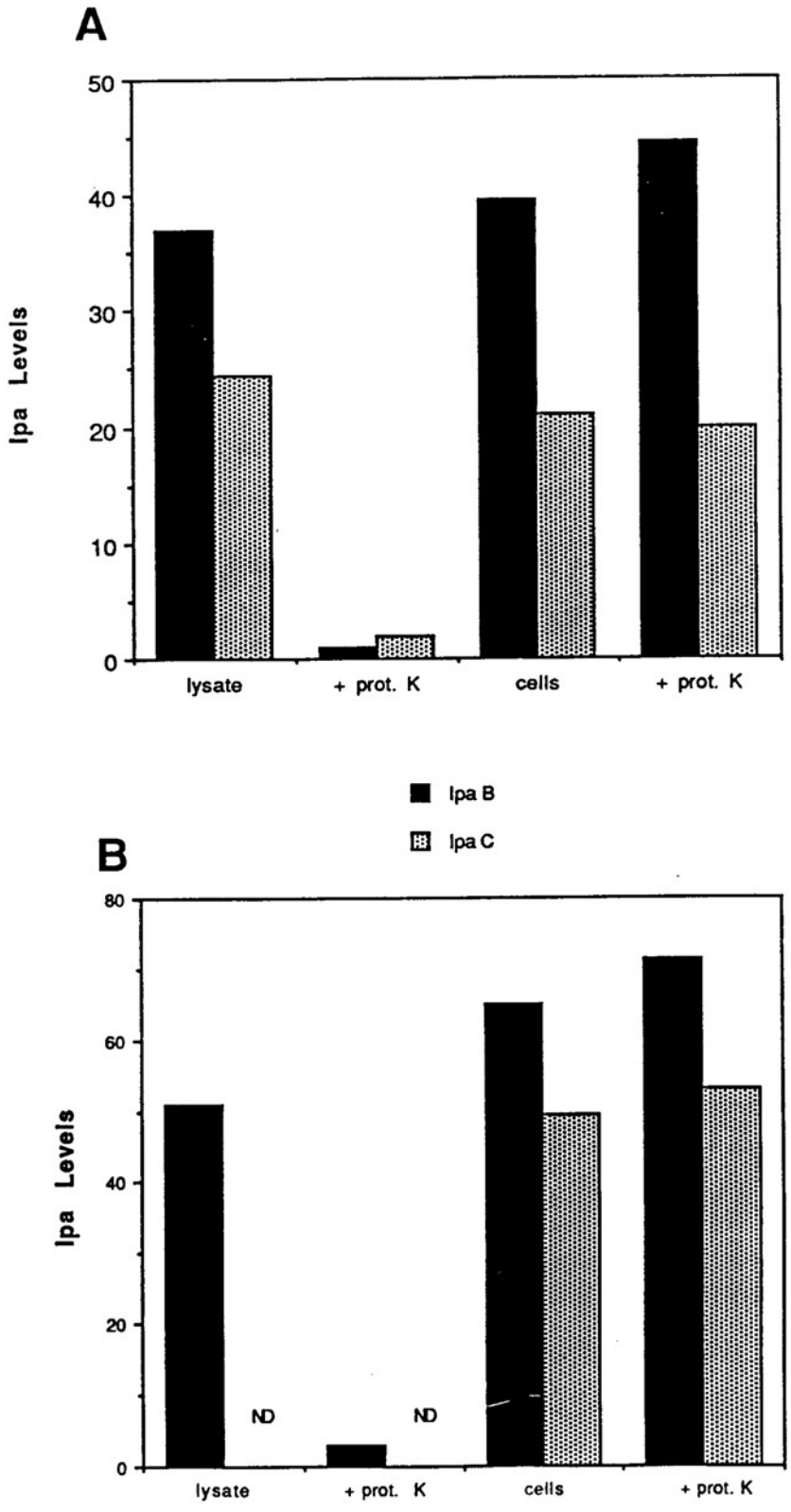
Figure 9. Distribution of IpaB and IpaC in wild-type *Shigella* and fusion mutants BS260 and BS226. Cells were fractionated and immunoblots of the electrophoresed fractions probed with MAb to IpaB and IpaC. Blots were analyzed by laser densitometry and the values standardized to total protein. Black bars, cytoplasmic fraction; Stippled bars, inner membrane; White bars, outer membrane.
(A) Intracellular distribution of IpaB. (B) Intracellular distribution of IpaC.


A
Relative IpaB Levels
12000
10000
8000
6000
4000
2000
0
2457T
BS260
BS226
cytoplasm
inner membrane
outer membrane
B
Relative IpaC Levels
8000
6000
4000
2000
0
2457T
BS260
BS226

the distribution of IpaB and IpaC in the *mxi* mutants revealed a 2 to 3-fold accumulation of these antigens in the inner membrane and little or no IpaB or IpaC in the outer membrane. Also, in all strains, greater levels of IpaC were detected in the cytoplasm relative to IpaC levels in the other cellular fractions. Although low levels of the antigens were also detected in the periplasmic fraction of all the strains, there was no difference in periplasmic levels between the mutants and wild-type (data not shown).

Surface expression of IpaB and IpaC in wild-type *S. flexneri.* Based on the above data, it was concluded that while high level intracellular pools of both antigens were evident in wild-type cells, extracellular IpaB was both cell surface-associated and excreted. In contrast, extracellular IpaC appeared to be predominantly excreted. To confirm this conclusion, the amount of cell-surface IpaB and IpaC on wild-type cells was assessed by measuring susceptibility of the total cell-associated antigen to proteolytic digestion. To determine if IpaB and IpaC were degradable by the serine peptidase Proteinase K, the enzyme was first added to sonic lysates of strain 2457T. Enzyme-treated lysates were probed with anti-IpaB and IpaC MAbs following SDS-PAGE and electroblotting. Quantitative Western blotting revealed exquisite sensitivity of both the antigens to the enzyme (Figure 10). Since surface-exposed cell-associated Ipa should therefore be susceptible to the enzyme, wild-type whole cells were next treated with the protease. As shown in Figure 10a, nearly all of the cell-associated IpaB and IpaC was completely protected from degradation suggesting that the bulk of these proteins in wild-type *S. flexneri* is intracellular and/or cell-free and not predominantly cell surface-associated. To determine if the protection from degradation was due to a steric effect of oligosaccharide side chains of bacterial lipopolysaccharide (LPS), whole cells of a rough derivative of 2457T, BS109, was treated in an identical manner to 2457T. The same results were seen as with the wild-type strain which indicated that LPS did not interfere with the ability of the protease to attack surface-exposed antigen (Figure 10b).

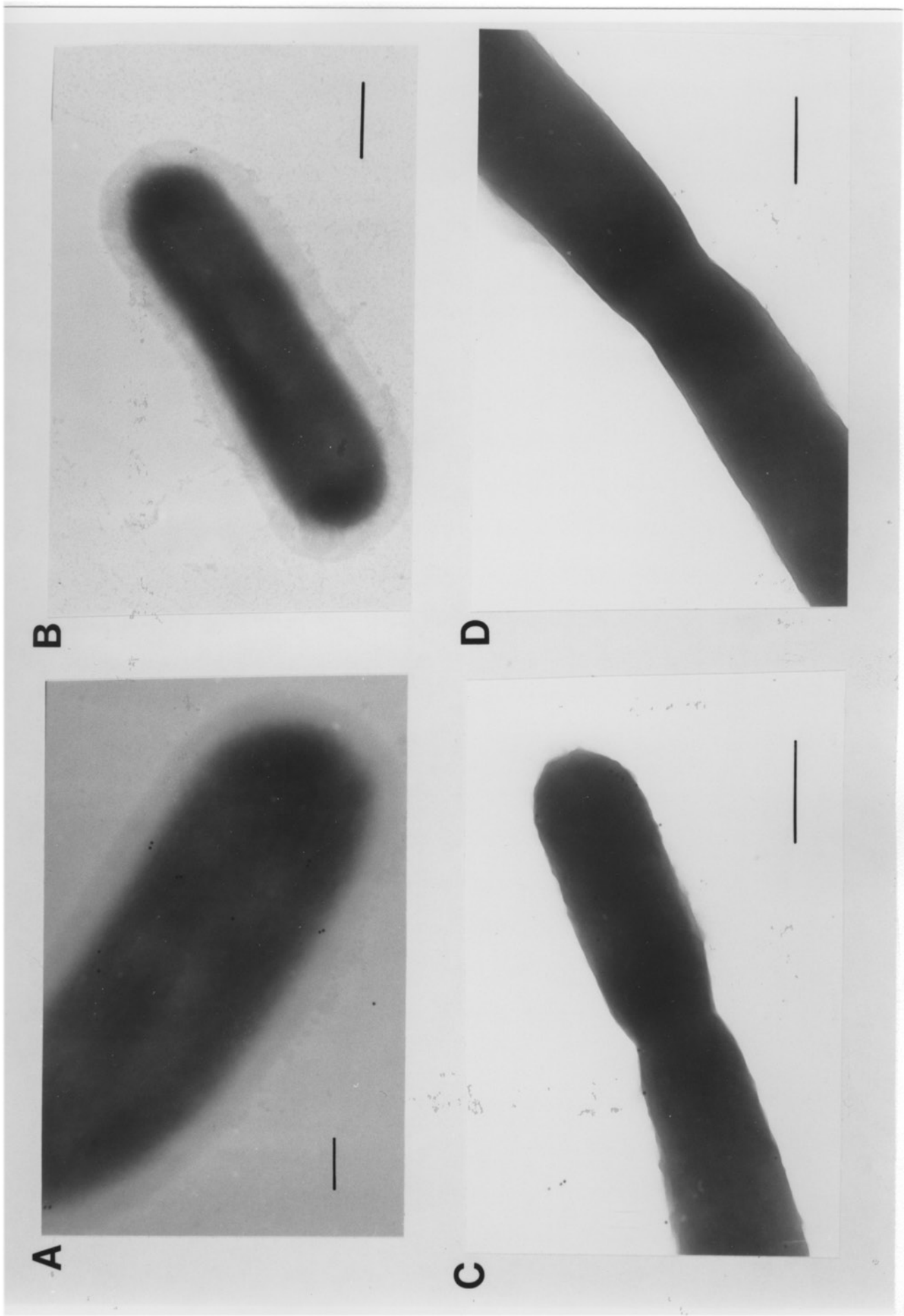
Figure 10. Susceptibility of cell-associated IpaB and IpaC to digestion by proteinase K. Proteinase K-treated sonic lysates and whole cells of *S. flexneri* 2a were analyzed by quantitative Western blotting with anti-IpaB and IpaC MAbs to assess the degree of surface-exposed antigen. (A) wild-type strain 2457T. (B) Rough derivative strain BS109. All samples were from the same culture of 2457T and BS109. ND = not determined.


A
50
40
30
20
10
0
Ipa Levels
lysate
+ prot. K
cells
+ prot. K
Ipa B
Ipa C
B
80
60
40
20
0
Ipa Levels
ND
ND
lysate
+ prot. K
cells
+ prot. K

Surface-association of IpaB and IpaC on wild-type *Shigella* was also examined by immunoelectron microscopy. Cells of 37°C-grown log phase cultures of *S. flexneri* 2457T were first labelled with monoclonal antibody to IpaB followed by a secondary label of gold-conjugated anti-mouse IgG and negatively stained with ammonium molybdate. Anti-IpaB labelled wild-type cells consistently bound 5-20 gold particles to their surface while cells of the plasmidless strain BS103 always possessed < 5 particles with most bacteria completely devoid of immunogold label (Figure 11). Additionally, when wild-type cells were labelled with a polyclonal monospecific rabbit anti-IpaB or IpaC serum fraction, no gold particles were detected. In contrast, a total IgG fraction of anti-*Shigella* (wild-type) whole cell serum, used in the same labelling technique heavily labelled the bacteria (>100 gold particles/cell). To rule out the possibility that polysaccharide side chains of LPS may be sterically blocking antibody/antigen interactions, the amount of surface-associated IpaB was evaluated on a rough derivative of *S. flexneri*, BS109. Although BS109 was able to export IpaB, no gold particles were detected on the surface of these bacteria labelled with anti-IpaB (not shown). Wild-type cells pretreated with either glutaraldehyde (not shown) or DMS (Figure 11) prior to labelling with the primary antibody showed no difference in their ability to bind gold-conjugated particles relative to untreated bacteria. These data support the hypothesis that only very low levels of IpaB and/or IpaC are cell surface-associated/exposed during log phase growth of wild-type *Shigella flexneri*.

Inability to restore the invasive phenotype to *mxi* mutants by addition of IpaB/C-enriched culture supernatants. One possible function for excreted IpaB and IpaC may be to facilitate uptake of the bacteria into host cells. To test this hypothesis, a culture supernatant (concentrated 20x) from Congo red-induced cultures of wild-type *S. flexneri* was diluted 1:20 into a suspension of log phase cells of BS226. Supernatants from such Congo red-induced cultures possess levels of IpaB and IpaC

Figure 11. Determination of surface-associated Ipa on wild-type *S. flexneri* by immunogold electron microscopy. (A) Immunogold labelling of wild-type strain 2457T coated with anti-IpaB MAb. (B) Immunogold labelling of BS103 with anti-IpaB MAb. (C) Labelling of DMS-pretreated 2457T with anti-IpaB MAb. (D) DMS-pretreated BS103 labelled with anti-IpaB MAb. Bars represent 0.2 μm.


A
B
C
D

between 7 and 38-fold greater than bacteria grown in the absence of the dye (see Part III of Results section below). BS226 mixed with this conditioned supernatant was then used to infect semi-confluent HeLa cell monolayers following the standard protocol for the invasion assay. No difference in levels of invasion were seen between BS226 alone and BS226 pretreated with the Ipa-enriched supernatant (<5% invasion).

Absence of homology between the Ipa secretory system and the *E. coli hly* operon. One interesting characteristic of the *mxiA* mutant, as well as the *mxi* operon fusion mutants, was their low level of contact hemolytic activity (Table 4; Hromockyj and Maurelli, 1989). The possibility that genetic homology may exist between the *mxi* loci of *S. flexneri* and the hemolysin structural and accessory genes of hemolytic *E. coli* was considered, since *E. coli* hemolysin A has been demonstrated to be exported by a unique multi-component apparatus (Holland, et al., 1990). A Southern blot analysis was performed using the cloned *E. coli hly* operon, pSF4000 (Welch, et al., 1983), as a probe against the *Sal*I subclones of pHS4108. None of the *Sal*I subclones, however, showed any homology with the *E. coli hly* genes. Additionally, an attempt was made to functionally complement the *mxi* secretion defect by transforming BS260 with the entire cloned *hly* operon of *E. coli*. This experiment seemed reasonable since secretion defects in some Gram negative organisms can be functionally complemented by the accessory gene products from different genera (Highlander, et al., 1990). However, while the transformed *mxi* mutants secreted functional hemolysin at wild-type *E. coli* levels (i.e., β-hemolysis on blood agar), cell-free ELISA analysis showed that the Ipa secretion defects failed to be complemented by the *hly* operon (Table 8).

Detection of Ipa complexes in wild-type *S. flexneri* and the *mxi* fusion mutant BS260. One hypothesis concerning the nature of the lesion in BS260 was that

Table 8. Phenotypes of *mxi* mutants transformed with the *hly* operon (pSF4000).

STRAINS [a]	HEMOLYTIC ACTIVITY [b]	EXPORTED [c] IpaB	EXPORTED [c] IpaC	CELLULAR [d] IpaB	CELLULAR [d] IpaC
2457T	NEG	0.569	0.782	ND	ND
BS103	NEG	-	-	ND	ND
BS260	NEG	0.093	0.152	138.0	113.7
BS260 (pACYC184)	NEG	0.179	0.155	ND	ND
BS260 (pSF4000)	POS	0.123	0.124	137.9	113.5
BS232	NEG	0.080	0.133	146.6	134.3
BS232 (pACYC184)	NEG	0.178	0.217	ND	ND
BS232 (pSF4000)	POS	0.206	0.243	149.7	123.0

[a] pACYC184 transformants represent vector-only negative controls.

[b] Positive hemolytic activity measured as ß-hemolysis on blood agar.

[c] Exported Ipa values determined by cell-free ELISA.

[d] Cellular Ipa values determined by quantitative Western blots.

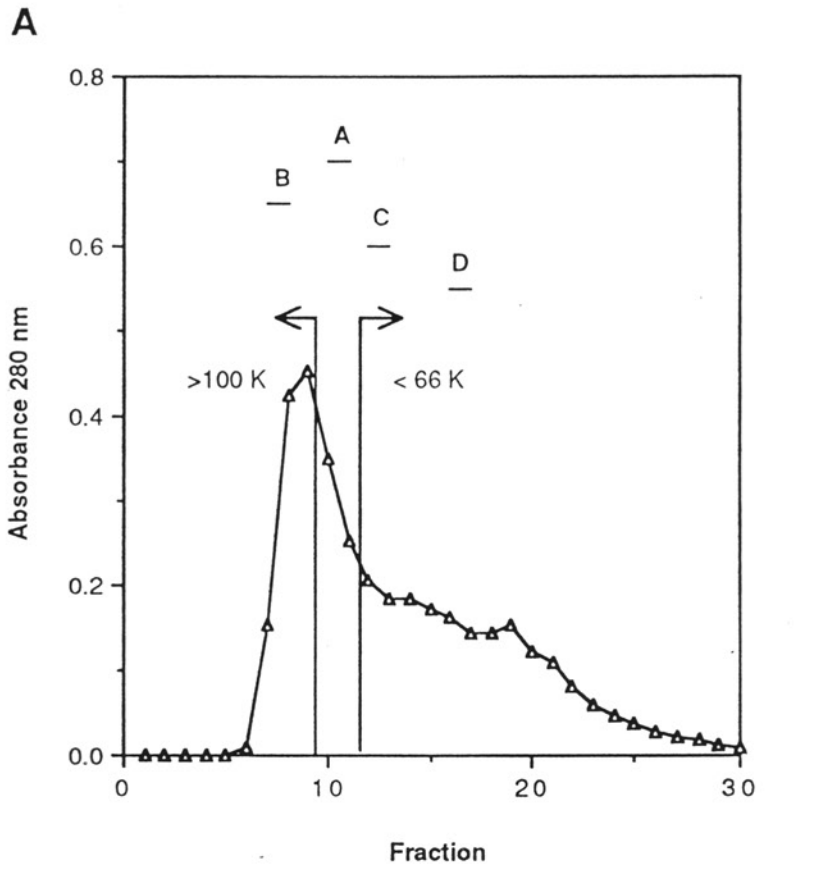
ND = not determined.

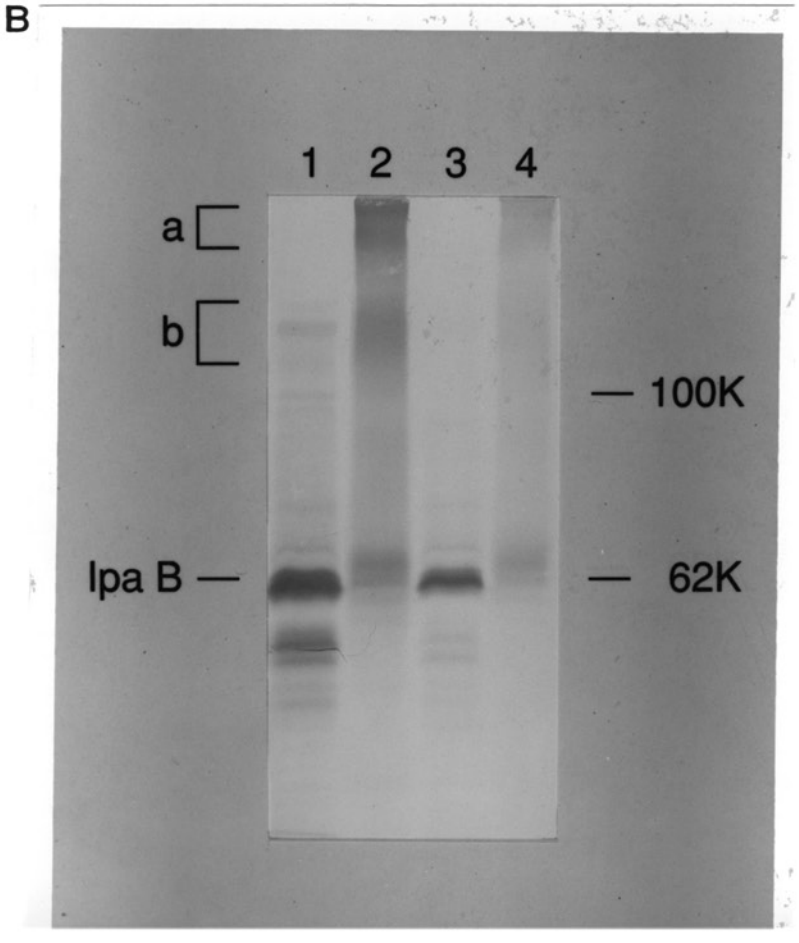
the mutated locus represented a gene which encoded a product which facilitates Ipa export by complexing with pre-excreted Ipa proteins. Alternatively, the accessory protein defined by BS260 may catalyze the formation of an export-competent Ipa complex. To address this hypothesis, the existance of Ipa complexes in wild-type S. flexneri 2a was first assessed. Lysates of log phase cells of strain 2457T or the *mxi* mutant BS260 were passed through a G-100 gel filtration column to isolate high molecular weight species (>100 kDa) from void volume fractions. The amounts of IpaA-D in the void volume fractions were determined by immunoblotting with rabbit anti-Ipa serum. As shown in Figure 12a, IpaA, C, and D were retained in the sieving column and eluted in the order of the calculated molecular weights of their monomeric forms (based on SDS-PAGE). In contrast, IpaB was not retained in the column and was found almost entirely in the void volume. The Ipa elution profile for BS260 was identical to wild-type with the bulk of IpaB detected in the void volume fractions. To confirm that IpaB in the void volume fractions from 2457T and BS260 existed as a high molecular weight complex, the void volume fraction from both strains with the highest quantity of IpaB were split into each of two aliquots. The protein cross-linking agent DMS was added to one pair of samples while the remainder of the selected fractions were left untreated. All the samples were then analysed by SDS-PAGE under reducing conditions. As shown in Figure 12b, while the uncross-linked IpaB was reduced to a monomeric form in both strains (lanes 1 and 3), IpaB from both strains was covalently cross-linked in the void volume samples treated with DMS into at least two species greater than 100 kDa (lanes 2 and 4).

Part II. Characterization of the *mxiA* locus and its product.

Identification of wild-type MxiA. The *mxiA* fusion mutant, BS260, produced a hybrid protein of approximately 123 kDa as determined in whole cell lysates of BS260 immunoblotted with monoclonal antibody to β-galactosidase (Figure 1). Because the size

Figure 12. Detection of Ipa complexes in wild-type *S. flexneri* and the mxi fusion mutant BS260. A) Sephadex G-100 elution profile of total protein from a clarified lysate of wild-type 2457T. Void volume fractions are indicated by a right angle arrow marking the position of elution of the molecular weight marker aldolase (150 kDa). Also, indicated by an arrow, is the elution position of bovine serum albumin (60 kDa). Positions of elution peaks for IpaA-D are shown as detected by Western blotting of fractions with anti-Ipa rabbit hyperimmune serum. BS260 profiles were identical to the wild-type. B) Anti-IpaB western blot of G-100 fraction 1 (void volume) from lysates of wild-type 2457T and the *mxiA* mutant BS260. Lane1, 2457T, untreated; lane 2, 2457T cross-linked with 2mg/ml DMS (see materials and methods); lane 3, BS260 untreated; lane 4, BS260 DMS cross-linked. Areas of the blot labelled "a" and "b" mark the presence of high molecular weight complexes. Also indicated, is the location of uncomplexed IpaB (62 kDa) and the position on the gel corresponding to 100 kDa (based on the migration of molecular weight standards).


A
0.8
0.6
0.4
0.2
0.0
Absorbance 280 nm
A
B
C
D
>100 K
< 66 K
0
10
20
30
Fraction


B
1
2
3
4
a
b
— 100K
lpa B —
— 62K

of the native enzyme is 116 kDa, the extreme N-terminus of the hybrid polypeptide possessed amino acid residues specified by the *mxiA* coding sequence. The fusion protein, designated MxiHP, was therefore used to generate monospecific polyclonal rabbit anti-serum to the N-terminal epitope(s) of MxiA, and the IgG fraction was purified to use as an immunological reagent in the preliminary identification of the native *mxiA* gene product. To optimize the yield of MxiHP, a purification scheme was developed utilizing high concentrations of the non-ionic detergent NP-40. Bacteria were first either sonicated or lysed by a French Pressure cell in the presence of the detergent. French pressure lysis was found to yield 8-fold greater amounts of total protein than sonication. The addition of the detergent also greatly improved the yield of hybrid protein by solubilizing the membrane-associated species as evidenced by the bulk of the β-galactosidase activity localizing in the clarified bacterial lysate (total β-gal activity in soluble fraction with no NP-40: 23%; with NP-40: 85%). By direct affinity chromatography of this clarified lysate, about 50% of the hybrid protein bound to the anti-β-galactosidase column, and the final product eluted from the column was greater than 99% pure by analysis of Coomasie blue-stained SDS-PAGE gels and Western blots (Figure 13). In addition to the fusion protein, some of the preparations yielded a 55 kDa coeluant from the affinity column which was present in varying amounts among different column fractions and/or preparations (Figure 13). Two New Zealand white rabbits were next immunized with the purified MxiHP. One rabbit was administered preparations which had been reduced, denatured, electrophoresed, and excised from polyacrylamide gels. Western blot analysis, however, showed that the serum response of this animal was primarily to β-galactosidase (not shown). The second rabbit was administered the antigen in its native state immediately after elution from the column. When a 1:100 dilution of the anti-MxiHP IgG fraction from this serum was used, 5 μg of the purified fusion protein could be weakly detected on a Western blot strip (Figure 14, lane 5). A 76 kDa polypeptide was weakly detected in lysates of 2457T grown at 37°C (lane 1) and

Figure 13. Affinity purification of the MxiA hybrid protein (MxiHP) from BS260 lysates. SDS-PAGE and Western blot analysis of affinity purified MxiHP from clarified lysates of the *mxiA* protein fusion mutant BS260. A) Coomasie blue-stained SDS-PAGE gel of pre-affinity chromatographed lysate, lane 1, and five fractions (lanes 2-6) eluted from an anti-β-galactosidase affinity column after passage of the lysate through the resin. The location of a 55 kDa coeluting species is indicated. B) Anti-β-galactosidase immunoblot of the above samples showing the location of the 123 kDa MxiA hybrid protein.

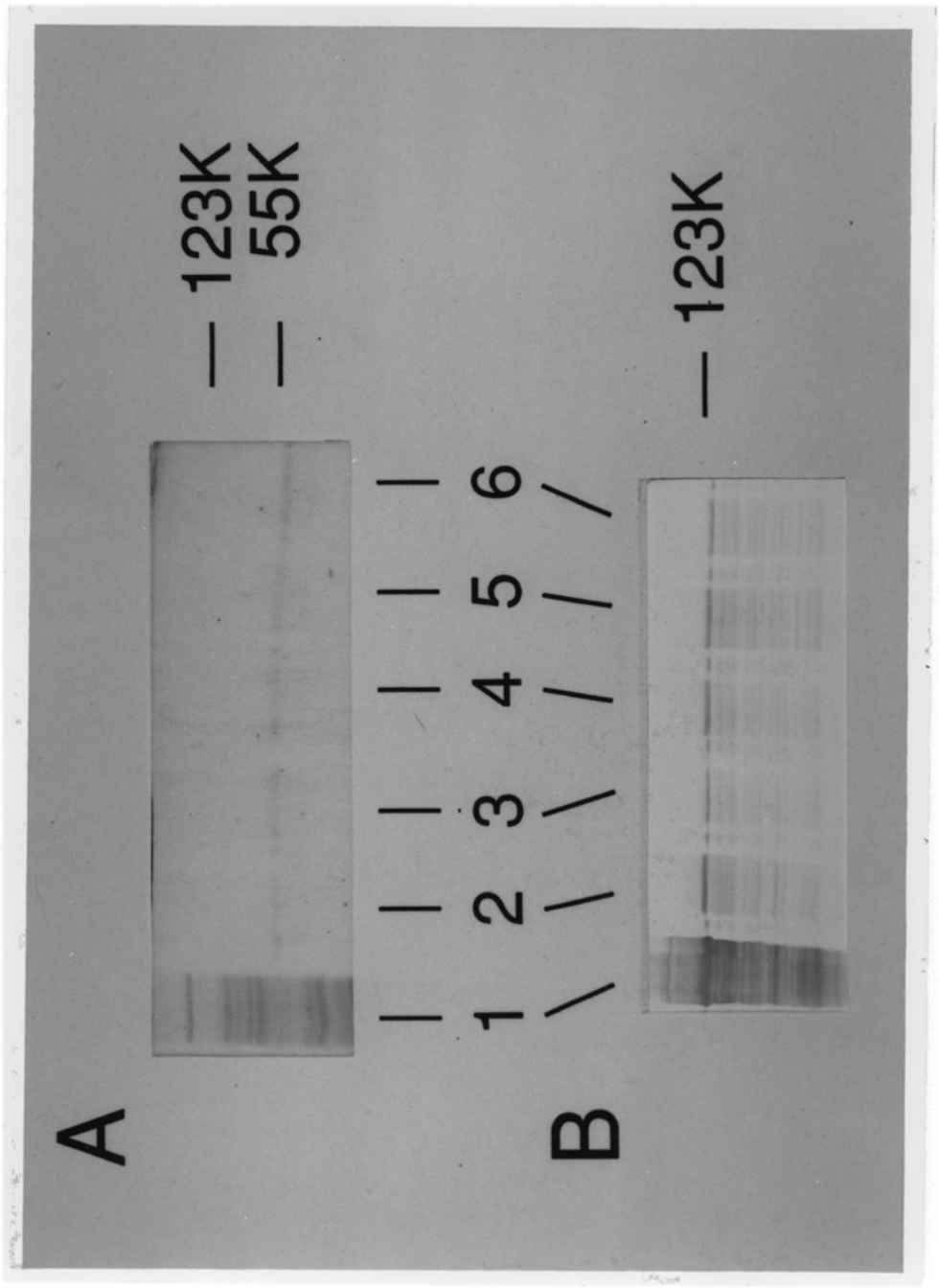
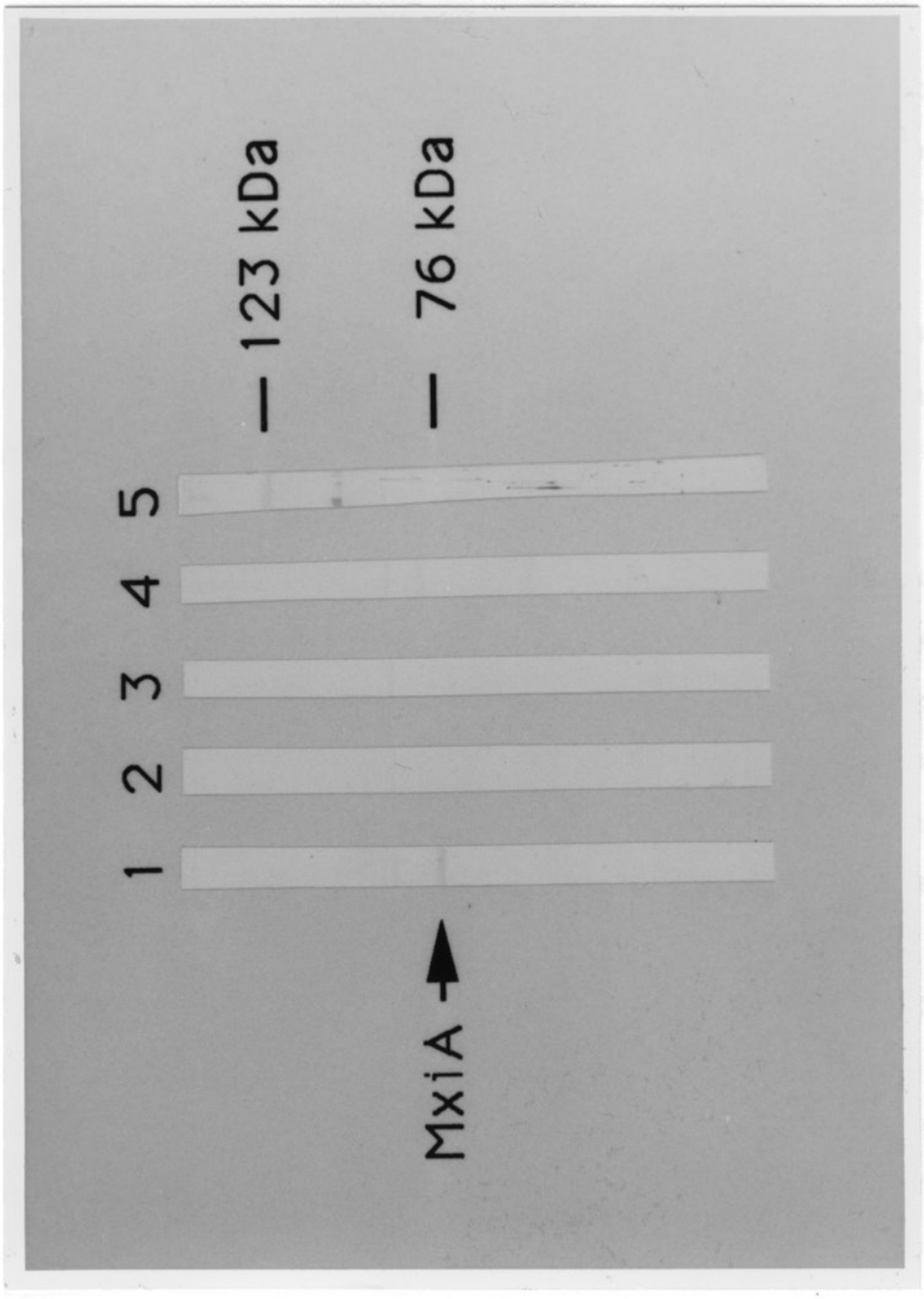

A
— 123K
— 55K
B
1 2 3 4 5 6
— 123K

Figure 14. Detection of the temperature-regulated *mxiA* gene product in *Shigella flexneri*. Electroblot strips of absorbance-standardized lysates of *mxi* mutants probed with anti-MxiHP anti-serum. Lane 1, 2457T (37°C); Lane 2, 2457T (30°C); Lane 3, BS103 (37°C); Lane 4, BS260 (37°C); Lane 5, purified MxiHP, 5 μg.


1 2 3 4 5
— 123 kDa
— 76 kDa
MxiA

was absent in cells grown at 30°C (lane 2). Further, BS103 grown at 37°C, as well as the *mxiA* fusion mutant, BS260, did not appear to possess this band (lanes 3 and 4, respectively). Therefore this protein was believed to be the putative *mxiA* gene product. A 90 kDa cross-reactive species was also detected in all the strains at both temperatures. Surprisingly, the fusion protein was not detected in lysates of BS260 (Figure 14, lane 4). The anti-MxiHP IgG fraction was next used to probe other *mxi* mutants. Whole cell lysates from BS230, BS232, and BS226 grown at 37°C, were absorbance-standardized, electrophoresed, electroblotted, and probed with a 1:250 dilution of anti-MxiHP. Only one *mxi* mutant, BS226, expressed the 76 kDa polypeptide (Figure 15). Additionally, BS226 appeared to express a 55 kDa species which was not detected in whole cell lysates of the other strains.

DNA sequencing strategy and analysis of the *mxiA* coding region. The putative *mxiA* coding region was subcloned on two contiguous *Hind*III restriction fragments (1.8 kb and 1.3 kb) into pUC19 and the resulting recombinant plasmids, pGPA010 and pGPA013 (Figure 16), were transformed into *E. coli* DH5α. The *Hind*III fragments were derived from DNA upstream of the previously reported cloned *mxiB* operon fusion end joint in pAEH006 (Table 3). These restriction fragments were believed to span the *mxiA* open reading frame based on the overlap occurring between pAEH006 and the cloned BS260 (*mxiA*) fusion end joint, pGPA001 (Table 3, Figure 16). A third construct, pGPA040, contained the 3.8 kb *Xba*I-*Eco*RI restriction fragment which overlaps the two *Hind*III fragments (Figure 16). This insert originated from a 9 kb *Eco*RI fragment of the 37 kb cosmid clone, pHS4108 from *S. flexneri* 5 which possesses all the virulence loci necessary to confer the invasive phenotype (Figure 16, Maurelli, et al., 1985).

The cloned fusion end joint from the *mxiA*::*lacZ* protein fusion mutant, BS260, was initially used as a template for sequencing. This fragment, contained in pGPA001, was

Figure 15. Detection of MxiA in other *mxi* fusion mutants of *S. flexneri*. Western blot of whole cell lysates of mxi operon fusion mutants immunolabelled with rabbit hyperimmune anti-MxiHP IgG fraction. Lane 1, wild-type 2457T (37^{o}C); lane 2, 2457T (30^{o}C); lane 3, BS103; lane 4, BS260; lane 5, BS230; lane 6, BS232; lane 7, BS226. An arrow indicates the location of MxiA (123 kDa).

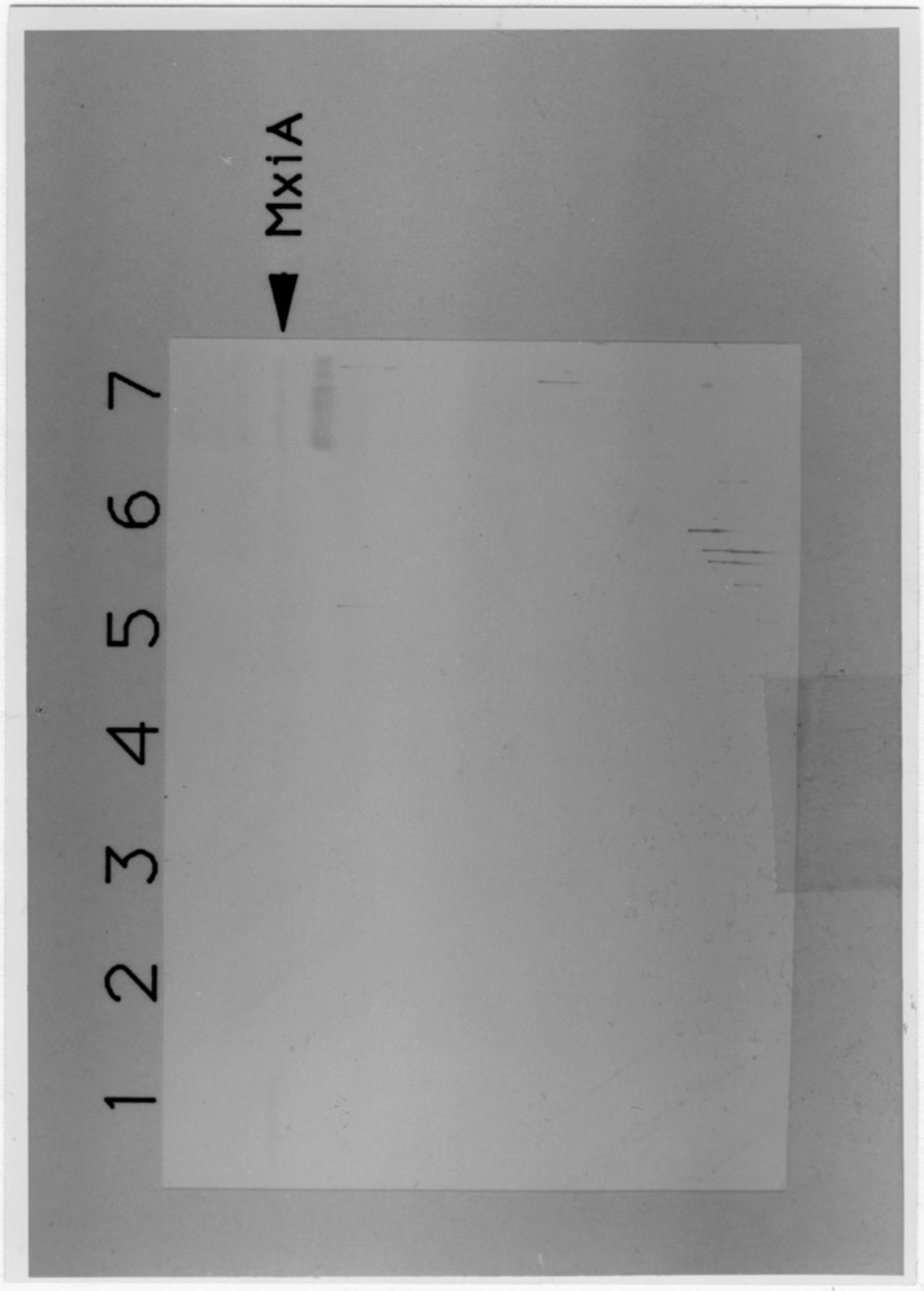
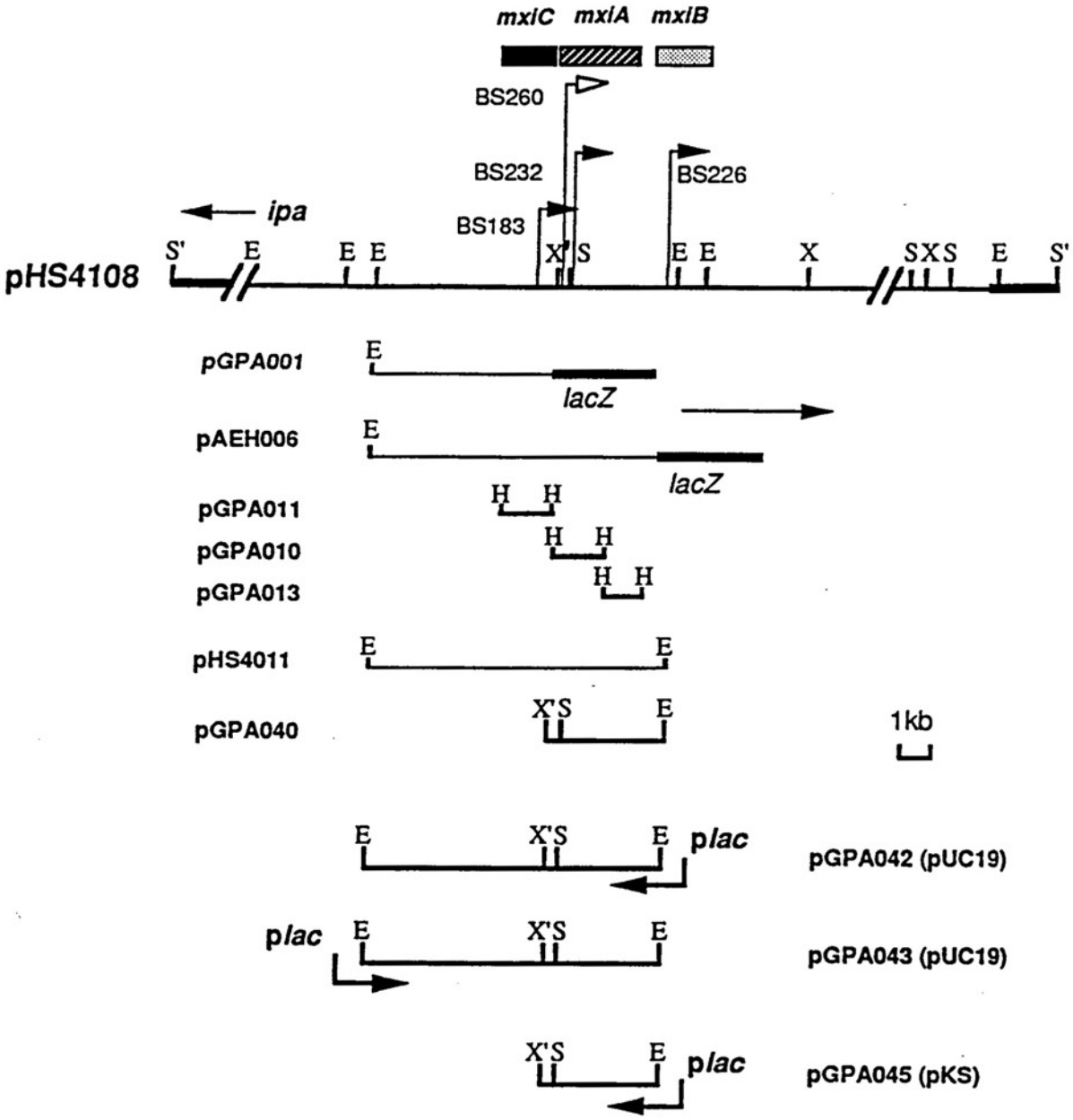

MxiA
1 2 3 4 5 6 7

Figure 16. Cloning and complementation strategy for the *mxiAC* region of *S. flexneri* 2a. Shown at the top of the figure are pHS4108, the 37 kb cosmid sub-clone of the 220 kb invasion plasmid, and the positions of the *mxi* loci relative to the *ipa* operon. Also indicated are the positions of the fusion phage inserts within the *mxiA* locus in BS260 and BS232, the *mxiB* mutation in BS226, and the *mxiC* insert in BS183. Arrows indicate the direction of transcription. The open arrow represents a protein fusion while closed arrows indicate operon (transcriptional) fusions. Below pHS4108 are *mxi* fusion end joint clones pGPA001 from BS260 (*mxiA*), and pAEH006 from BS226 (*mxiB*). Plasmids pGPA010 and pGPA013 (sub-cloned from pAEH006) as well as pGPA040 (sub-cloned from pHS4011) were used as double stranded templates to sequence through the *mxiA* open reading frame. pGPA011 was used as the template to obtain the *mxiC* sequence.

Clones used in the complementation experiments consisted of a 9 kb *Eco*RI fragment from pHS4011 ligated into the vector pUC19 in both orientations relative to the *lac* promoter (pGPA042, pGPA043). Additionally, an internal *Xba*I-*Eco*RI fragment was placed into the vector pKS in reverse orientation to p*lac* (pGPA045).

Abbreviations for restriction sites are as follows: E= *Eco*RI, H= *Hind*III, X'= *Xba*I, X=*Xho*I, S=*Sal*I, S'=*Sau*3A.

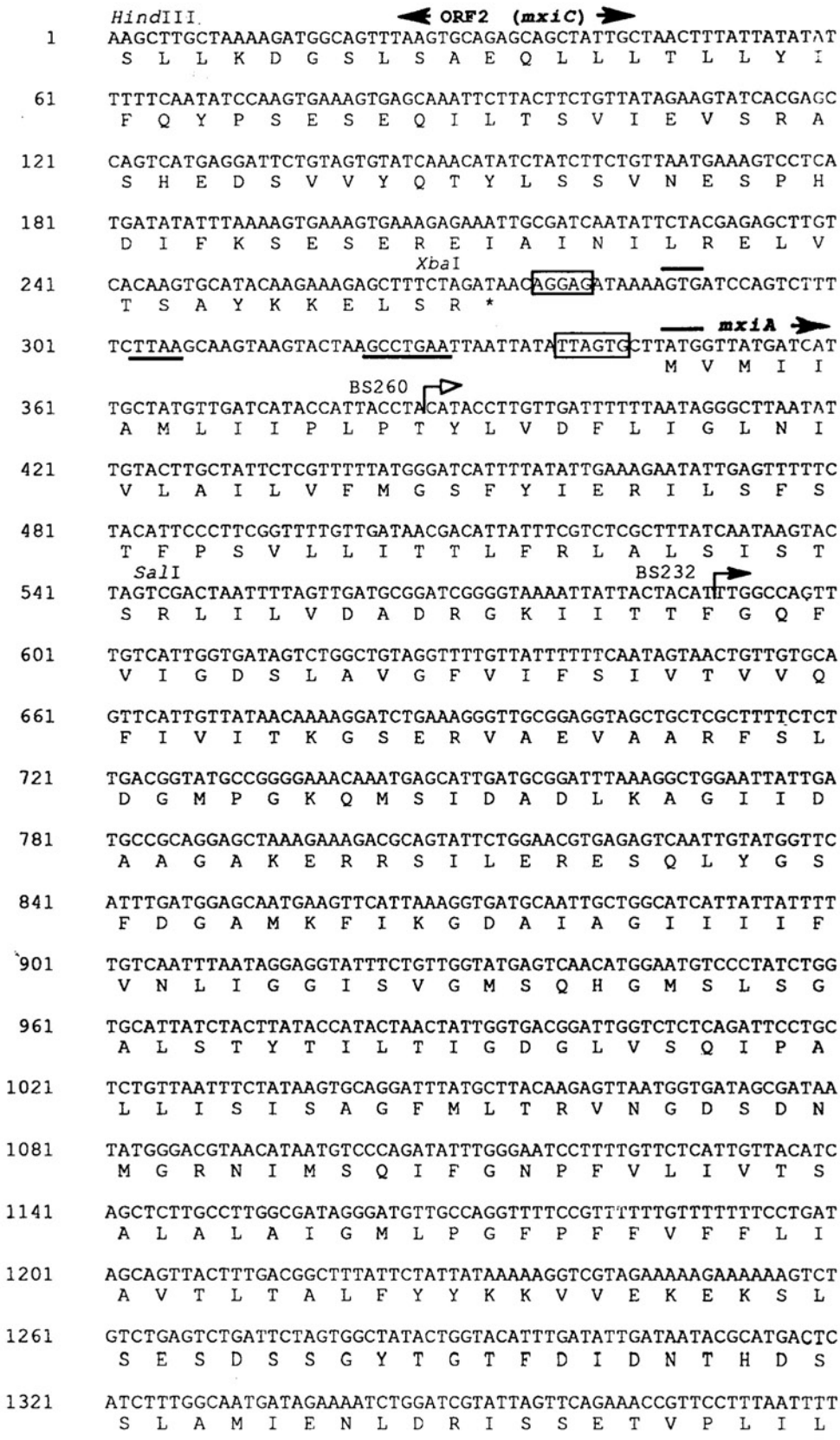

mxiC mxiA mxiB
BS260
BS232
BS226
BS183
ipa
pHS4108
S' E E E X' S E E X SXS E S'
pGPA001
E
lacZ
pAEH006
E
lacZ
pGPA011
H H
pGPA010
H H
pGPA013
H H
pHS4011
E E
pGPA040
X'S E
1kb
E X'S E plac
pGPA042 (pUC19)
plac E X'S E
pGPA043 (pUC19)
X'S E plac
pGPA045 (pKS)

primed with a *lacZ*-specific oligonucleotide to obtain the sequence through the fusion junction and into the extreme N-terminus of the *mxiA* coding region. This sequence was then used to define the reading frame, as well as to obtain a *mxiA*-specific primer to begin sequencing of the native gene. The complete nucleotide sequence of *mxiA* was determined from the *Hind*III fragment of pGPA010 and part of the *Hind*III fragment of pGPA013. A single large open reading frame (ORF) of 1998 nucleotides was found in frame with the *lacZ* protein fusion which most likely represented *mxiA* (Figure 17). The phage insertion in BS260 mapped precisely at nucleotide 387, only 13 codons downstream from the putative start of the *mxiA* ORF (Figures 16 and 17). A second ORF (ORF2) was found on the coding strand upstream and in frame to *mxiA* which continued past the 5' *Hind*III restriction site of pGPA010. A third open reading frame was also identified in frame to and at the end of the *mxiA* ORF.

To determine the precise location of the λp*lac*Mu fusion phage insert in the *mxi* mutant BS232, the cloned fusion end joint from this mutant (pAEH009, Table 3) was used as a template and primed with *lacZ* and *trpB*-specific oligomers to obtain sequence through the fusion junction. This sequence was aligned with the sequence of the *mxiA* coding region, and the operon fusion phage insertion in BS232 was found to map at nucleotide 590, within the *mxiA* coding region downstream of the *Sal*I restriction site (Figures 16 and 17). Thus, BS232 was confirmed as a *mxiA* transcriptional fusion mutant.

A possible ATG translational start site for the *mxiA* ORF was found at nucleotide 347 with a weak putative ribosomal binding site (Shine-Dalgarno; Kozak, 1983) located upstream (beginning at nucleotide 338) from this designated start codon. A second possible start site which utilized the alternate start codon GTG (nucleotide 287), but possessed a "more favorable" Shine-Dalgarno sequence, was also identified (Figure 17). Although analysis of further upstream sequence did not reveal a transcriptional start site strongly matching the *E. coli* promoter consensus (Hawley and McClure, 1983), a

Figure 17. Nucleotide sequence of *mxiA* and the 3' coding region of open reading frame 2 (ORF2, *mxiC*). The putative Shine-Dalgarno sequence (boxed) and ATG start codon for *mxiA* (overlined) are indicated. An alternate GTG start site with putative Shine-Dalgarno is also shown. A σ^{28}-like promoter is indicated by lines under the -10 and -35 sites. One letter codes for the predicted amino acid sequences are shown for both ORF2 and *mxiA*. Relevant restriction sites are also indicated above the sequence as well as the positions of the λp*lac*Mu fusion phage insertions in the *mxiA* mutants BS260 and BS232. The stop codons of ORF2 and the *mxiA* ORF are indicated below the sequence by asterisks. The ATG start site of *spa15* (Venkatesan, et al., 1992) is also indicated at the end of the *mxiA* sequence.


HindIII
ORF2 (mxiC)
XbaI
mxiA
BS260
SalI
BS232

```
1381    ATTATTTGCCGAAAATAAGATAAATGCAAATGATATGGAAGGTCTTATTGAAAGGATAAG
          L  F  A  E  N  K  I  N  A  N  D  M  E  G  L  I  E  R  I  R

1441    AAGTCAGTTCTTTATTGATTATGGTGTCAGGCTTCCAACTATTTTATATAGAACAAGCAA
          S  Q  F  F  I  D  Y  G  V  R  L  P  T  I  L  Y  R  T  S  N

1501    TGAGCTTAAGGTCGATGATATTGTTTTGTTAATAAATGAGGTGCGTGCCGATAGTTTTAA
          E  L  K  V  D  D  I  V  L  L  I  N  E  V  R  A  D  S  F  N

1561    TATATATTTTGACAAAGTGTGTATTACAGATGAAAATGGAGATATAGATGCTCTAGGTAT
          I  Y  F  D  K  V  C  I  T  D  E  N  G  D  I  D  A  L  G  I

1621    TCCTGTTGTTTCAACTTCATATAATGAACGTGTTATTTCTTGGGTAGACGTTTCATATAC
          P  V  V  S  T  S  Y  N  E  R  V  I  S  W  V  D  V  S  Y  T

1681    AGAAAATCTAACTAATATTGATGCTAAAATTAAAAGTGCTCAAGATGAATTTTATCACCA
          E  N  L  T  N  I  D  A  K  I  K  S  A  Q  D  E  F  Y  H  Q

1741    GTTGTCACAAGCTTTATTAAACAACATAAATGAGATTTTTGGTATACAAGAAACAAAAAA
          L  S  Q  A  L  L  N  N  I  N  E  I  F  G  I  Q  E  T  K  N

1801    TATGTTAGATCAGTTTGAAAATCGGTATCCTGATCTATTAAAGGAAGTCTTCCGACATGT
          M  L  D  Q  F  E  N  R  Y  P  D  L  L  K  E  V  F  R  H  V

1861    GACTATACAGAGAATTTCTGAGGTATTACAAAGATTGCTTGGAGAAAATATTTCTGTTCG
          T  I  Q  R  I  S  E  V  L  Q  R  L  L  G  E  N  I  S  V  R

1921    CAATTTAAAACTTATTATGGAGTCTTTGGCGCTTTGGGCTCCCAGAGAAAAAGATGTCAT
          N  L  K  L  I  M  E  S  L  A  L  W  A  P  R  E  K  D  V  I

1981    AACATTAGTTGAACATGTCCGTGCATCACTTTCTAGGTATATTTGTAGTAAAATAGCTGT
          T  L  V  E  H  V  R  A  S  L  S  R  Y  I  C  S  K  I  A  V

2041    TTCTGGTGAGATTAAAGTTGTGATGCTTTCCGGATATATTGAGGATGCAATAAGAAAAGG
          S  G  E  I  K  V  V  M  L  S  G  Y  I  E  D  A  I  R  K  G

2101    GATAAGGCAAACCTCTGGTGGCTCTTTCTTGAATATGGATATAGAGGTTTCGGATGAGGT
          I  R  Q  T  S  G  G  S  F  L  N  M  D  I  E  V  S  D  E  V

2161    AATGGAAACTTTAGCACATGCTTTGAGAGAATTGAGAAATGCAAAAAAAAAATTTCGTTCT
          M  E  T  L  A  H  A  L  R  E  L  R  N  A  K  K  N  F  V  L

2221    TTTGGTATCAGTAGATATACGTAGGTTTGTTAAAAGACTTATAGATAACAGATTTAAGAG
          L  V  S  V  D  I  R  R  F  V  K  R  L  I  D  N  R  F  K  S

2281    TATACTCGTTATATCGTATGCTGAGATTGATGAAGCATATACCATTAATGTATTAAAGAC
          I  L  V  I  S  Y  A  E  I  D  E  A  Y  T  I  N  V  L  K  T
                                  spa15 ➔
2341    TATTTAGTGAGGTTTAAATATGAGTAACATTAATTTAGTTCAATTAGTTAG
          I  *  *           M  S  N  I  N  L  V  Q  L  V
```

Figure 17, continued.

sequence which weakly resembled the σ^{28} promoter of *E. coli* (Helmann and Chamberlin, 1987) was identified between 18 and 44 bases upstream of the *mxiA* ATG translational start site. Similarity with the σ^{28} promoter occurred as a 3 of 4 base alignment at the -35 site and a 4 of 8 base alignment at the -10 site with the number of nucleotides in the spacer region (15 bases) matching the consensus.

The *mxiA* ORF specified a predicted polypeptide of 666 amino acids with a molecular weight of 74.03 kDa and pI of 5.02. A hydropathy plot of the predicted *mxiA* gene product demonstrated an extremely hydrophobic N-terminus with 6 putative transmembrane regions of 10-20 amino acids each (Figure 18a), possibly representing a membrane anchor. The C-terminus, approximately from residue 300 to the end of the protein, was essentially hydrophilic which suggested either a cytoplasmic or periplasmic location for this part of the protein.

A DNA homology search of the GenBank and EMBL databases revealed no significant similarities to any known prokaryotic genes. However, when the derived amino acid sequence of the *mxiA* polypeptide was compared, greater than 40% identity was found with the predicted translation product of ORF 5 of the virulence plasmid, pYVO3, of *Yersinia enterocolitica* (Viitanen, et al., 1990). This ORF was recently shown to be identical to *lcrD* of *Y. pestis* which encodes a protein involved in the low calcium response (Plano, et al., 1991). When the deduced amino acid sequence of the *mxiA* locus was compared with LcrD, 41% overall identity and 69% similarity was seen (Figure 19). The greatest homology occurred at the amino termini of the two proteins (Figures 18b and 19). Amino-terminal homology in the first two hundred residues represented greater than 85% similarity and 60% identity. High similarity between the amino acid sequences of LcrD and FlbF, a high level regulatory protein of the aquatic bacterium, *Caulobacter crescentus,* has also been reported (Plano, et al., 1991; Ramakrishnan, et al., 1991). The overall homology, however, between LcrD and MxiA appeared slightly greater than that demonstrated between LcrD and FlbF, although the degree of N-

Figure 18. Hydropathy analysis of the MxiA amino acid sequence and regions of homology with LcrD. (A) Hydrophobicity/hydrophilicity plot of MxiA. Analysis was performed by DNA Stryder version 1.1 software for the Macintosh based on the algorithm of Kyte and Doolittle (1982). Positive values represent more hydrophobic residues while negative values indicate more hydrophilic residues. Hatched bars above N-terminal hydrophobic peaks indicate putative membrane spanning domains. (B) Regions of amino acid homology between MxiA and LcrD in relationship to the hydropathy plot of MxiA. The amino acid alignment was initially performed using the GCG BESTFIT program with an assigned gap weight of 3.0 and a length weight of 0.1. The alignment was then broken down into groups of 20 residues and, based on the degree of identity within each group, assigned one of three gray-scale shades. These gray shades were then plotted on a horizontal bar representing the entire length of the two proteins.

☐ <25% amino acid identity ▩ 25-50% identity ■ >50% identity

N-terminal alignment of LcrD begins at residue 24 and ends at residue 702 of 705 total residues.

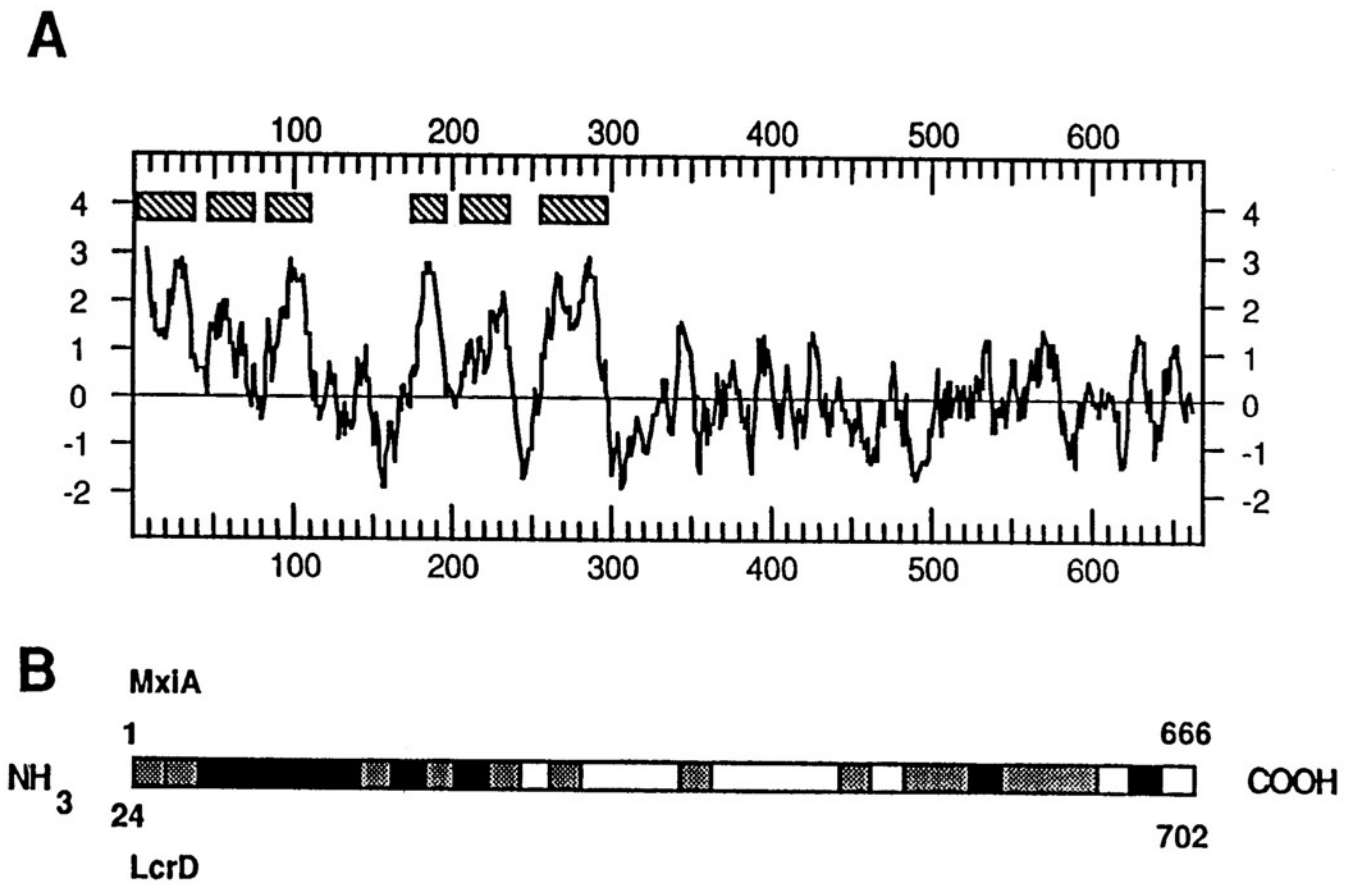
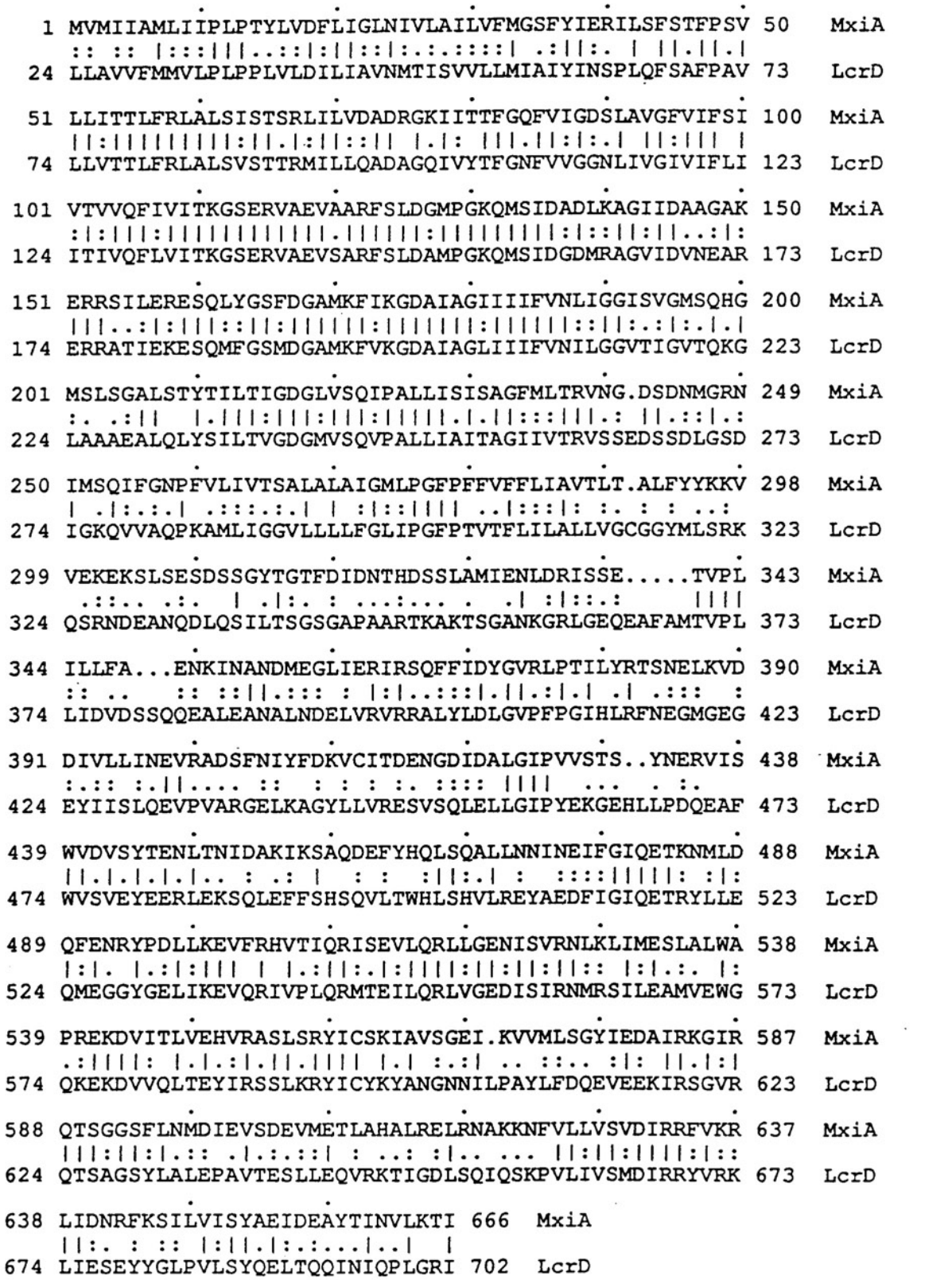

A
100
200
300
400
500
600
4
3
2
1
0
-1
-2
B
MxiA
1
666
NH3
COOH
24
702
LcrD

Figure 19. Amino acid alignment of the predicted proteins of *mxiA* of *Shigella* and *lcrD* of *Yersinia*. Lines between residues indicate identity while colons represent similarity between amino acids and periods indicate less similar residues. Amino acid alignment was performed as indicated in Figure 14. The predicted size of LcrD is 705 residues.


1 MVMIIAMLIIPLPTYLVDFLIGLNIVLAILVFMGSFYIERILSFSTFPSV 50 MxiA
24 LLAVVFMMVLPLPPLVLDILIAVNMTISVVLLMIAIYINSPLQFSAFPAV 73 LcrD
51 LLITTLFRLALSISTSRLILVDADRGKIITTFGQFVIGDSLAVGFVIFSI 100 MxiA
74 LLVTTLFRLALSVSTTRMILLQADAGQIVYTFGNFVVGGNLIVGIVIFLI 123 LcrD
101 VTVVQFIVITKGSERVAEVAARFSLDGMPGKQMSIDADLKAGIIDAAGAK 150 MxiA
124 ITIVQFLVITKGSERVAEVSARFSLDAMPGKQMSIDGDMRAGVIDVNEAR 173 LcrD
151 ERRSILERESQLYGSFDGAMKFIKGDAIAGIIIIFVNLIGGISVGMSQHG 200 MxiA
174 ERRATIEKESQMFGSMDGAMKFVKGDAIAGLIIIFVNILGGVTIGVTQKG 223 LcrD
201 MSLSGALSTYTILTIGDGLVSQIPALLISISAGFMLTRVNG.DSDNMGRN 249 MxiA
224 LAAAEALQLYSILTVGDGMVSQVPALLIAITAGIIVTRVSSEDSSDLGSD 273 LcrD
250 IMSQIFGNPFVLIVTSALALAIGMLPGFPFFVFFLIAVTLT.ALFYYKKV 298 MxiA
274 IGKQVVAQPKAMLIGGVLLLLFGLIPGFPTVTFLILALLVGCGGYMLSRK 323 LcrD
299 VEKEKSLSESDSSGYTGTFDIDNTHDSSLAMIENLDRISSE.....TVPL 343 MxiA
324 QSRNDEANQDLQSILTSGSGAPAARTKAKTSGANKGRLGEQEAFAMTVPL 373 LcrD
344 ILLFA...ENKINANDMEGLIERIRSQFFIDYGVRLPTILYRTSNELKVD 390 MxiA
374 LIDVDSSQQEALEANALNDELVRVRRALYLDLGVPFPGIHLRFNEGMGEG 423 LcrD
391 DIVLLINEVRADSFNIYFDKVCITDENGDIDALGIPVVSTS..YNERVIS 438 MxiA
424 EYIISLQEVPVARGELKAGYLLVRESVSQLELLGIPYEKGEHLLPDQEAF 473 LcrD
439 WVDVSYTENLTNIDAKIKSAQDEFYHQLSQALLNNINEIFGIQETKNMLD 488 MxiA
474 WVSVEYEERLEKSQLEFFSHSQVLTWHLSHVLREYAEDFIGIQETRYLLE 523 LcrD
489 QFENRYPDLLKEVFRHVTIQRISEVLQRLLGENISVRNLKLIMESLALWA 538 MxiA
524 QMEGGYGELIKEVQRIVPLQRMTEILQRLVGEDISIRNMRSILEAMVEWG 573 LcrD
539 PREKDVITLVEHVRASLSRYICSKIAVSGEI.KVVMLSGYIEDAIRKGIR 587 MxiA
574 QKEKDVVQLTEYIRSSLKRYICYKYANGNNILPAYLFDQEVEEKIRSGVR 623 LcrD
588 QTSGGSFLNMDIEVSDEVMETLAHALRELRNAKKNFVLLVSVDIRRFVKR 637 MxiA
624 QTSAGSYLALEPAVTESLLEQVRKTIGDLSQIQSKPVLIVSMDIRRYVRK 673 LcrD
638 LIDNRFKSILVISYAEIDEAYTINVLKTI 666 MxiA
674 LIESEYYGLPVLSYQELTQQINIQPLGRI 702 LcrD

terminal homology was the same (Ramakrishnan, et al., 1991). The reason was apparent after a more detailed analysis of the regions of homology between the proteins was made. When amino acid identity between MxiA and LcrD was analyzed across 20 residue stretches in conjunction with the hydropathy plot, an additional region of moderate to high homology was found in the C-terminal third of the two proteins. This domain (Figure 18b, amino acid 500-600) consisted of 100 amino acids separated from the N-terminal hydrophobic regions by a 200 residue spacer of lower homology (Figure 18b, amino acid 300-500). In contrast, homology between LcrD and FlbF in this region does not appear as strong (Ramakrishnan, et al., 1991). It was also of interest that small regions of lower homology were seen between some of the hydrophobic peaks in the N-terminus of MxiA and LcrD (Figures 18b and 19).

Given the homology between MxiA and LcrD, antiserum to a primary epitope of LcrD was obtained from the laboratory of Dr. Sue Straley which was specific for a 12 amino acid stretch in the N-terminus of the protein (amino acid 168 to 179). This antiserum was used to probe whole cell lysates of 2457T to determine if the *mxiA* gene product could be detected. The anti-LcrD peptide serum failed to react to a 76 kDa gene product across several dilutions while anti-MxiHP serum reacted with the putative MxiA protein in the same blots (data not shown).

Cloning *mxiA* and complementation of the *mxiAB* defects in protein and operon fusion mutants of *S. flexneri*. The 11 kb *EcoR*I sub-clone (pHS4011) from pHS4108, containing the intact *mxiA* coding region, was ligated into the cloning vector pUC19 in both orientations relative to the vector-specified *lac* promoter (pGPA042, pGPA043, Figure 16). A third construct, pGPA045 (Table 3), was generated which consisted of an *Xba*I to *EcoR*I fragment in the vector pKS in reverse orientation to p*lac* (Figure 16). These recombinant plasmids were then used to transform *mxi* mutants BS226, BS232, and BS260 to test for complementation of the

avirulent phenotype by assessing Congo red binding, Ipa B and IpaC export, and HeLa cell invasion.

Since the ability of *Shigella* to bind the planar dye Congo red correlates with virulence (Maurelli, et al., 1984) and the *mxi* mutants are all unable to bind the dye, a quantitative Congo red binding assay was developed and employed as an initial screen for complementation of the transformed *mxi* mutants. As shown in Table 9, although transformants of both *mxiA* mutants bound significantly higher levels of the dye compared to the parent mutants and the plasmidless derivative BS103, dye binding levels were intermediate to wild-type. However, when these transformants were tested in a HeLa cell invasion assay, all were able to enter the monolayers at wild-type levels. Moreover, when the smaller, non-p*lac* driven clone of the *mxiA* coding region, pGPA045, was introduced into the *mxiA* mutants, HeLa cell invasion was restored to a level near wild-type (Table 9). When the smaller *Xba*I-*Eco*RI fragment from pGPA045 was introduced into the vector pBS (Table 3) to drive expression of *mxiA* from p*lac*, the resultant transformants of *E coli* were extremely slow growing and appeared to readily lyse in liquid culture. Consequently, transformants of *mxi* mutants with this DNA were never isolated.

In contrast to the complementation of the *mxiA* mutants, a pGPA045 transformant of the *mxiB* mutant, BS226, was neither Congo red binding nor invasive (Table 9). Conversely, when the *Sal*I sub-clone, pHS5101 (Figure 3c) was introduced into BS226, the wild-type phenotype was restored to these transformants. However this clone, which contains the *mxiB* locus and a 3' truncate of *mxiA*, failed to restore Congo red binding and the invasive phenotype to either *mxiA* mutants BS260 or BS232 (Table 10).

The *mxiA* transformants were next assayed for their ability to export IpaB and IpaC. Table 9 shows that, although the *mxiA* mutants transformed with the three *mxiA* clones excreted the antigens at significant levels compared to the parent mutants, IpaB levels

TABLE 9. Complementation of *S. flexneri mxi* mutants with the wild-type *mxiA* locus

STRAINS	CONGO RED BINDING[a]	HeLa INVASION[b]	Ipa EXCRETION[c] IpaB	IpaC	SURFACE IpaB[c]
2457T	3.14	59	1.37	2.56	1.80
BS103	0.16	2	-	-	-
BS260 *mxiA*	0.44	0	0.04	0.28	0.18
BS260 (pGPA042)	2.00	72	0.64	2.38	1.04
BS260 (pGPA043)	1.41	66	1.04	2.48	0.76
BS260 (pGPA045)	2.17	65	0.55	2.48	0.81
BS232 *mxiA*	0.41	0	0.05	0.44	0.18
BS232 (pGPA042)	1.76	68	0.66	2.48	0.95
BS232 (pGPA043)	1.04	61	0.78	2.30	0.70
BS232 (pGPA045)	1.63	58	0.51	2.41	0.83
BS226 mxiB	0.63	0	ND	ND	ND
BS226 (pGPA045)	0.66	2	ND	ND	ND

[a] Congo red binding (CRB) values represent an average from three experiments. All values differ significantly from wild-type (2457T) within two standard deviations. Values for BS226 are from a single experiment (CRB for 2457T= 4.32)

[b] HeLa cell invasion values are from a single representative experiment.

[c] Levels of Ipa export represent an average from three experiments. IpaB export values for the *mxiA* transformants are significantly different from the positive (2457T) and negative (BS103) controls within two standard deviations.

ND= not determined.

TABLE 10. Complementation of *S. flexneri mxi* mutants with the wild type *mxiB* locus

STRAINS	CONGO RED BINDING	% HeLa INVASION
2457T	2.45	57
BS103	0.12	4
BS226 *mxiB*	0.27	0
BS226 (pHS5101)	1.91	39
BS260 *mxiA*	0.23	0
BS260 (pHS5101)	0.27	1
BS232 *mxiA*	ND	0
BS232 (pHS5101)	ND	3

ND = not determined.

were still less than 100% of wild type. In contrast, wild-type levels of excreted IpaC were restored in all the *mxiA* transformants (Table 9). The SLIM assay was next performed on the transformants to assess the degree of surface-associated IpaB. The *mxiA* transformants also showed intermediate levels of surface IpaB relative to wild-type, even though they were significantly higher than the control strains. The reduced levels of exported IpaB were not found to be due to excessive intracellular proteolysis of the antigen, since Western blots of absorbance-standardized whole cell lysates of the transformants revealed that levels of intracellular IpaB were normal (Figure 20). This result, however did not rule out the possibility that the extracellular antigen pool may be labile to proteolysis. To determine if proteolytic activity was present in supernatants of the transformed mutants, a cell-free ELISA was performed on PBS extracts of wild-type *Shigella* mixed in equal volumes (50 µl) with an extract of one of the *mxiA* transformants, BS260 (pGPA045) and allowed to incubate overnight at room temperature in microtiter plate wells. IpaB was detected at comparable levels in wells incubated with either 2457T supernatant alone or mixed with that of the *mxiA* transformant (Table 11). These experiments suggested that no significant proteolysis was occurring which could account for the reduced level of excretion in the *mxiA* transformants.

A plaque assay was next performed on BS260 and BS232 transformed with pGPA042 and pGPA043. As shown in Table 12, none of the *mxiA* transformants were able to plaque on HeLa cell monolayers after 24 hours. Only after a full 48 hours did plaques appear on monolayers infected with the *mxiA* transformants. Additionally, the plaques formed by the transformed mutants appeared smaller than wild-type *S. flexneri.*

Figure 20. Absence of proteolysis of IpaB in MxiA transformants. Cells of wild-type 2457T, *mxiA* transformants and the parent *mxiA* mutants were grown to log phase and standardized to the A_{600} of the least dense culture prior to harvest for quantitative analysis of IpaB. Electroblot of whole cell lysates probed with anti-IpaB MAb. Lane 1, 2457T; lane 2, BS260; lane 3, BS260(pGPA042); lane 4, BS260(pGPA043); lane 5, BS232; lane 6, BS232(pGPA042); lane 7, BS232(pGPA043).

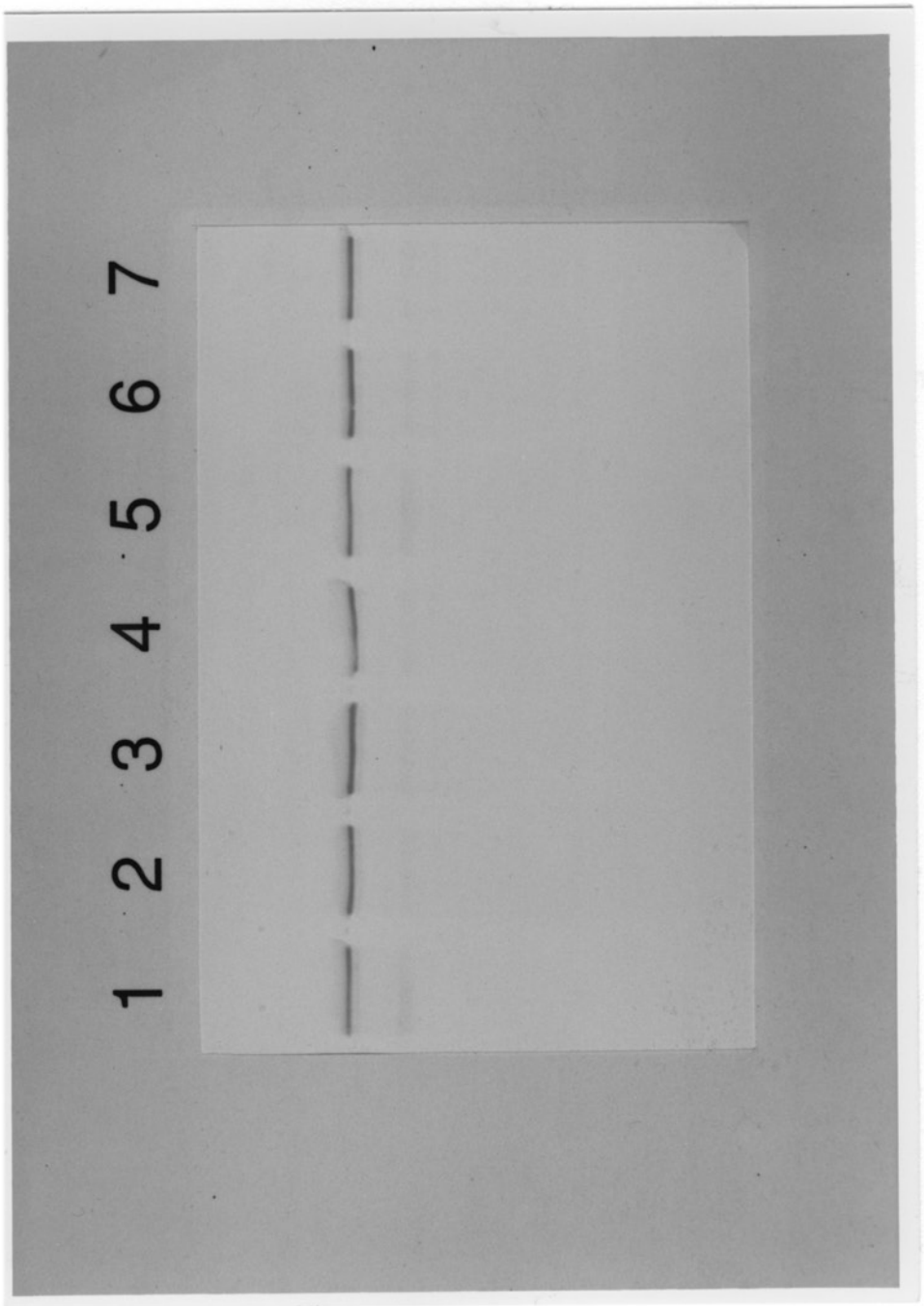

1 2 3 4 5 6 7

Table 11. Absence of proteolytic activity in culture supernatants of BS260 and a mxiA transformant.

Supernatants	Cell-free IpaB	% W.T.
2457T only	0.225	100
BS260 only	0.047	21
BS260 (pGPA045) only	0.121	54
2457T + BS260	0.224	99
2457T + BS260 (pGPA045)	0.283	126

TABLE 12. Plaque Forming Ability of *mxiA* Transformants

STRAINS	PLAQUE FORMATION [a] 24 Hrs.	48 Hrs.	PLAQUE SIZE
2457T	++++	++++	Large
BS103	-	-	-
BS260 (pGPA042)	-	++	small
BS260 (pGPA043)	-	++	small
BS232 (pGPA042)	-	+++	intermediate
BS232 (pGPA043)	-	+	small

[a] -, no plaques; +, 5-25% of wild-type; ++, 25-50%; +++, 50-80% of wild-type; ++++, 80-100% of wild-type

Part III. Characterization of additional *mxi* mutants.

Identification and analysis of the *mxiC* locus. In an attempt to further elucidate the extent of the multi-component *mxi* export system, another temperature-regulated avirulent *lacZ* fusion mutant, BS183 (Table 3), was examined in more detail. This mutant was previously characterized as expressing wild-type intracellular levels of IpaA-D (A. T. Maurelli, unpublished observations) but was completely non-invasive on HeLa cells (Maurelli and Sansonetti, 1988). It was therefore hypothesized that BS183 may possess a lesion in one of the *mxi* loci. The SLIM assay and cell-free ELISA were performed on this strain to determine its ability to excrete IpaB and IpaC. BS183 was, in fact, found to be deficient in export of both these antigens (Table 13), thus confirming the fact that the mutation in this strain affected the *mxi* phenotype.

The fusion end joint was next sub-cloned into pMLB524, the DNA sequence determined through the junction, and an alignment performed on the *mxiA* coding region as well as on upstream flanking DNA. The mutation in BS183 was found to lie within the 3' end of ORF2, precisely 89 bases upstream of the stop codon (Figure 21). DNA sequencing was then performed using pGPA011 (Figure 16) as a template to obtain additional upstream sequence. Based on an analysis of this DNA, the 5' boundary of ORF2 was defined (Figure 21). Since this sequencing data was derived from a single strand, it was compared with the DNA sequence of the homologous region from the invasion plasmid in the *S. flexneri* serotype 5 strain M90T (P. J. Sansonetti, unpublished data). A single base change was noted between the two sequences at base position 46 (from the ATG start site) which effected a conservative codon change from an arginine (serotype 5) to a lysine (ORF2, serotype 2). The complete coding region specified an ATG start site with a consensus Shine-Dalgarno sequence and an open reading frame of 1068 bases in frame with and upstream of *mxiA* (Figure 21). As seen in *mxiA*, analysis of DNA 5' to the ATG translational start site of ORF2 revealed a sequence with similarity to a σ^{28} promoter.

TABLE 13. Complementation of BS183 and BS230 with *mxiA* (pGPA045)

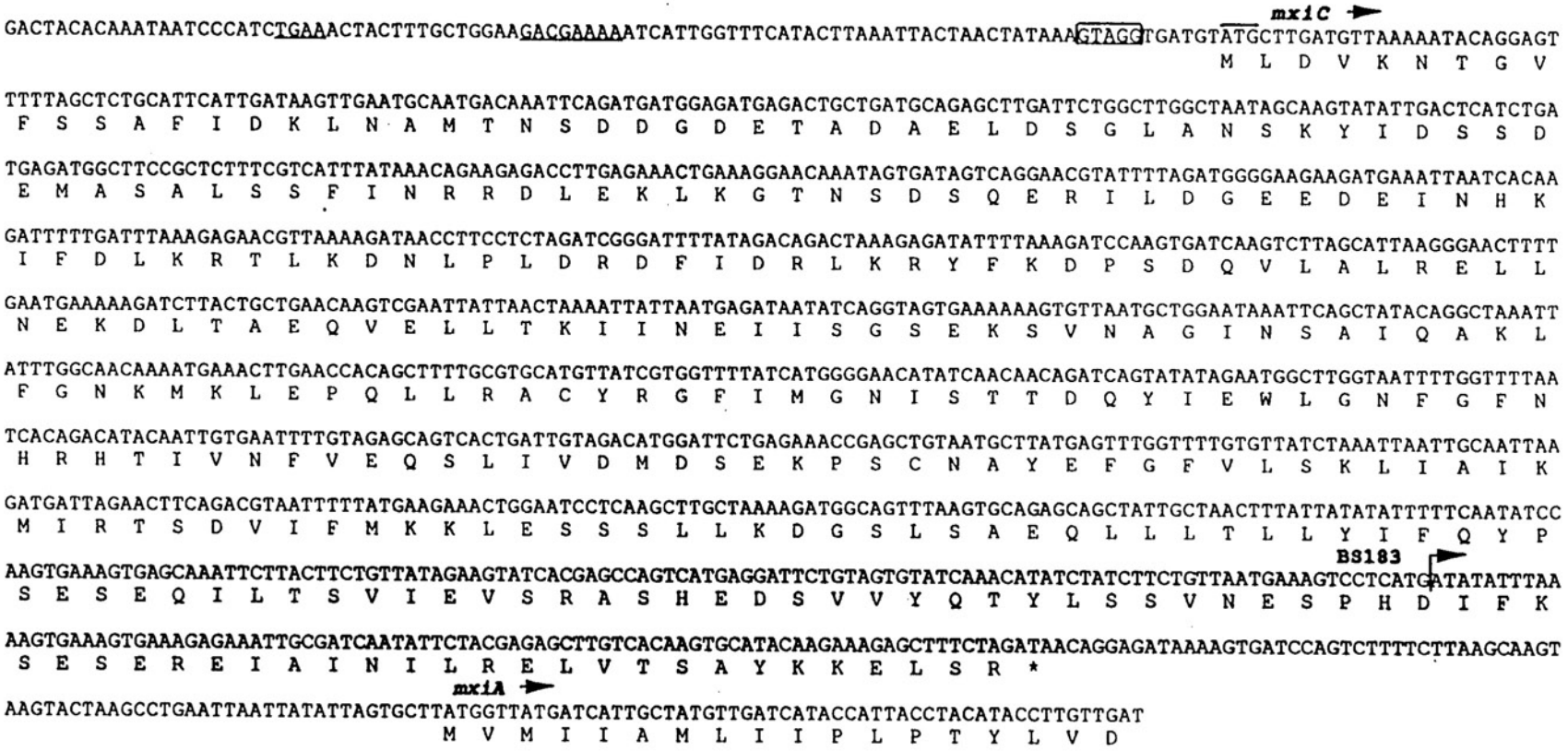
STRAINS	CONGO RED BINDING [a]	HeLa INVASION [b]	Ipa EXCRETION [c] IpaB	IpaC	SURFACE IpaB [c]
2457T	3.00	48	1.47	2.19	1.33
BS183 *mxiC*	0.36	1	0.03	0.05	0.19
BS183 (pGPA045)	3.22	22	0.37	0.61	0.54
2457T	4.32				
BS230 *mxi*	0.53	1	0.04	0.04	0.17
BS230 (pGPA045)	0.83	2	0.06	0.04	0.42

a Values for Congo red binding of BS183 and BS230 derived strains were determined from separate wild-type controls as indicated.

b HeLa cell invasion values are averaged from duplicate experiments.

c Ipa export values are averaged from triplicate experiments.

Figure 21. Nucleotide sequence of the *mxiC* coding region. The ATG start site (overline) and the putative Shine-Dalgarno sequence (boxed) are indicated. The -10 and -35 sites of a σ^{28}-like sequence upstream of the ribosomal binding site are underlined. The *mxiC* stop codon is indicated by an asterisk. The insert site of the transcriptional fusion phage in BS183 is shown by an arrow. Also shown is the 5' end of *mxiA*.

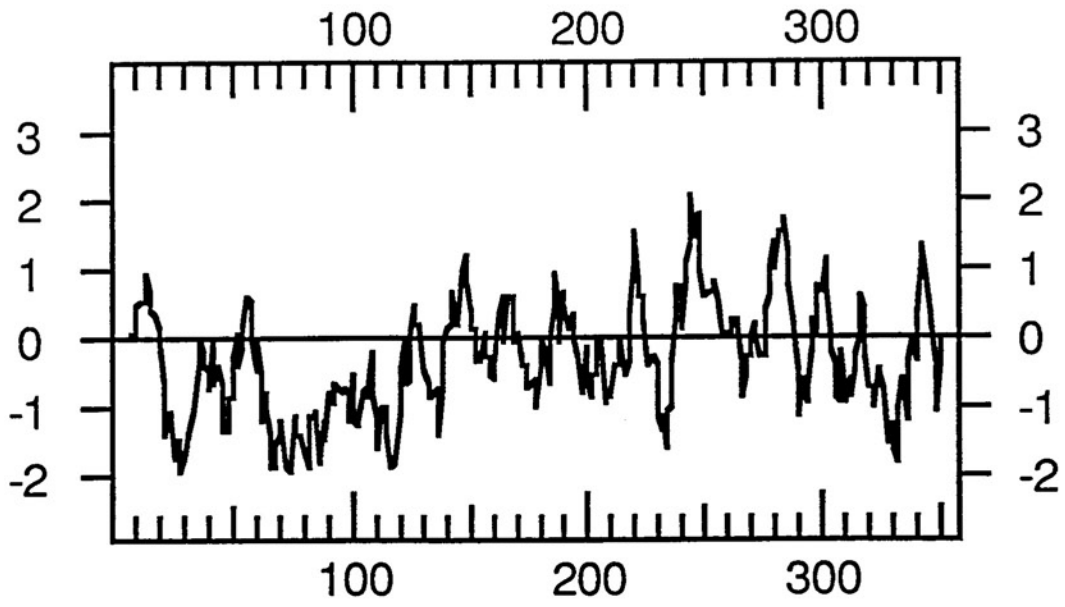

mxiC
GACTACACAAATAATCCCATCTGAAACTACTTTGCTGGAAGACGAAAAATCATTGGTTTCATACTTAAATTACTAACTATAAAGTAGGTGATGTATGCTTGATGTTAAAAATACAGGAGT
M L D V K N T G V
TTTTAGCTCTGCATTCATTGATAAGTTGAATGCAATGACAAATTCAGATGATGGAGATGAGACTGCTGATGCAGAGCTTGATTCTGGCTTGGCTAATAGCAAGTATATTGACTCATCTGA
F S S A F I D K L N A M T N S D D G D E T A D A E L D S G L A N S K Y I D S S D
TGAGATGGCTTCCGCTCTTTCGTCATTTATAAACAGAAGAGACCTTGAGAAACTGAAAGGAACAAATAGTGATAGTCAGGAACGTATTTTAGATGGGGAAGAAGATGAAATTAATCACAA
E M A S A L S S F I N R R D L E K L K G T N S D S Q E R I L D G E E D E I N H K
GATTTTTGATTTAAAGAGAACGTTAAAAGATAACCTTCCTCTAGATCGGGATTTTATAGACAGACTAAAGAGATATTTTAAAGATCCAAGTGATCAAGTCTTAGCATTAAGGGAACTTTT
I F D L K R T L K D N L P L D R D F I D R L K R Y F K D P S D Q V L A L R E L L
GAATGAAAAAGATCTTACTGCTGAACAAGTCGAATTATTAACTAAAATTATTAATGAGATAATATCAGGTAGTGAAAAAAGTGTTAATGCTGGAATAAATTCAGCTATACAGGCTAAATT
N E K D L T A E Q V E L L T K I I N E I I S G S E K S V N A G I N S A I Q A K L
ATTTGGCAACAAAATGAAACTTGAACCACAGCTTTTGCGTGCATGTTATCGTGGTTTTATCATGGGGAACATATCAACAACAGATCAGTATATAGAATGGCTTGGTAATTTTGGTTTTAA
F G N K M K L E P Q L L R A C Y R G F I M G N I S T T D Q Y I E W L G N F G F N
TCACAGACATACAATTGTGAATTTTGTAGAGCAGTCACTGATTGTAGACATGGATTCTGAGAAACCGAGCTGTAATGCTTATGAGTTTGGTTTTGTGTTATCTAAATTAATTGCAATTAA
H R H T I V N F V E Q S L I V D M D S E K P S C N A Y E F G F V L S K L I A I K
GATGATTAGAACTTCAGACGTAATTTTTATGAAGAAACTGGAATCCTCAAGCTTGCTAAAAGATGGCAGTTTAAGTGCAGAGCAGCTATTGCTAACTTTATTATATATTTTTCAATATCC
M I R T S D V I F M K K L E S S S L L K D G S L S A E Q L L L T L L Y I F Q Y P
BS183
AAGTGAAAGTGAGCAAATTCTTACTTCTGTTATAGAAGTATCACGAGCCAGTCATGAGGATTCTGTAGTGTATCAAACATATCTATCTTCTGTTAATGAAAGTCCTCATGATATATTTAA
S E S E Q I L T S V I E V S R A S H E D S V V Y Q T Y L S S V N E S P H D I F K
AAGTGAAAGTGAAAGAGAAATTGCGATCAATATTCTACGAGAGCTTGTCACAAGTGCATACAAGAAAGAGCTTTCTAGATAACAGGAGATAAAAGTGATCCAGTCTTTTCTTAAGCAAGT
S E S E R E I A I N I L R E L V T S A Y K K E L S R *
mxiA
AAGTACTAAGCCTGAATTAATTATATTAGTGCTTATGGTTATGATCATTGCTATGTTGATCATACCATTACCTACATACCTTGTTGAT
M V M I I A M L I I P L P T Y L V D

The -35 site was identified as a 3 of 4 base alignment and the -10 site as a 6 of 8 base alignment, 48 bases upstream of the translational start site. The spacer region of 15 bases matched the consensus. ORF2 specified a predicted polypeptide of 355 amino acids with a molecular weight of 40.17 kDa and a pI of 4.66. A hydropathy analysis of this putative gene product revealed an essentially hydrophilic molecule with two small hydrophobic domains in the C-terminus (Figure 22). A homology search at both DNA and amino acid levels yielded no significant similarity with any known prokaryotic genes or polypeptides.

Because of the extreme C-terminal position of the phage insert in ORF2 of BS183, it was thought that expression of *mxiA* may be affected and thus be responsible for the Mxi$^-$ phenotype. Therefore, to determine if the lesion in BS183 was able to be complemented with the cloned copy of *mxiA*, pGPA045 was transformed into BS183 and the transformants assayed for Congo red binding, HeLa cell invasion, and IpaB/C export. As shown in Table 13, the *mxiA* transformant of BS183 was able to bind Congo red to wild-type levels, but invaded HeLa cell monolayers at significantly reduced efficiency. Additionally, this transformant was unable to form plaques on HeLa cell monolayers (A. T. Maurelli, unpublished observations). Although the IpaB export profile was similar to the other *mxiA* transformants, levels of excreted IpaC were significantly reduced. Since this result suggested that the gene product of ORF2 may be associated with IpaC export, consequently affecting virulence, the locus defined by BS183 was designated as a third *mxi* gene, *mxiC*.

Identification of a fourth *mxi* locus. Another *mxi* transcriptional fusion mutant, BS230, which had been initially characterized by Hromockyj and Maurelli (1989), was previously mapped to a position upstream of the *mxiA* lesion in BS260. To determine if this mutation was within the *mxiC* ORF, the junction of the cloned fusion end joint, pAEH008 (Table 1) was sequenced and compared to the DNA sequence of the coding

Figure 22. Hydrophobicity/hydrophilicity plot of MxiC. Analysis was performed by DNA Stryder version 1.1 software for the Macintosh based on the algorithm of Kyte and Doolittle (1982). Positive values represent more hydrophobic residues while negative values indicate more hydrophilic residues.


100
200
300
3
2
1
0
-1
-2
100
200
300
3
2
1
0
-1
-2

regions specifying *mxiC* and *mxiA*. The mutation in BS230 was not localized within either of these two genes. This finding was consistent with previously reported Southern blot analysis and restriction mapping which placed the lesion within a locus further upstream of *mxiC* (Hromockyj and Maurelli, 1989). Moreover, when the cloned *mxiA* gene was placed into BS230, transformants were neither Congo red binding nor invasive (Table 13) which suggested that the insert in BS230 was not having a polar effect on *mxiA*.

Part IV. Induction of Ipa excretion in *Shigella* by extrinsic factors.

To address the possibility that excretion of the high intracellular pools of IpaB and IpaC may be modulated by extracellular cues, wild-type *Shigella* and *mxi*::*lacZ* reporter strains were grown under a variety of physical and nutritional conditions. Ipa excretion and reporter gene expression were assessed by SDS-PAGE, quantitative Western blotting, and enzymatic assay for β-galactosidase.

Stimulation of Ipa Excretion by Congo Red. In a previous report, the planar dye Congo red was shown to enhance expression of several membrane proteins in *Shigella*. (Sankaran, et al., 1989). To determine if a similar phenomenom could be demonstrated with the excreted Ipa pool, wild-type *S. flexneri* was grown at 37°C to late log phase in TSB in the presence and absence of 0.01% Congo red. Silver stained SDS-PAGE gels of supernatants from absorbance-standardized cultures grown in the presence of the dye showed elevated levels of four excreted proteins which corresponded to the molecular weights of IpaA through IpaD (see Table 7). Two other proteins also appeared under Congo red induction which were not detected in supernatants from untreated cultures. These species corresponded to molecular weights of 22 and 17 kDa. When an electroblot

of an identical gel was probed with anti-IpaB and anti-IpaC MAbs and the signals quantified, a significant increase in both excreted IpaB and IpaC was detected (38-fold and 7-fold respectively, Figure 23a). No affect of Congo red on excreted Ipa levels was seen in the *mxi* mutants BS260 and BS226 (Ipa levels were unmeasurable as tested). Stimulation by Congo red was apparently not due to lysis or leakage of the bacteria since the Congo red-binding *ipaB*::*lacZ* reporter strain, BS228, released little if any β-galactosidase into the medium even though the bacteria bound wild-type levels of the dye (Figure 24). Further, the effect was not the result of increased expression of Ipa since elevated levels of β-galactosidase (the reporter gene product for IpaB) were not detected in BS228 in the presence of the dye (Figure 24). Additionally, no detectable change in the total intracellular Ipa pool was seen on blots of wild-type whole cell lysates as well as the *mxi* mutants (Figure 25). To determine if the dye was inducing expression at the level of at least one of the *mxi* loci, the Congo red-binding *recA* derivative of wild-type *Shigella*, BS201, was transformed with the *mxiB* fusion end joint clone, pAEH006 (Table 3), and the resulting construct was grown in the presence of the dye. No effect of Congo red was seen on *mxiB* expression as measured by levels of β-galactosidase activity (no Congo red= 837 Miller units; + Congo red= 805 Miller units).

Stimulation of Ipa excretion by a host cell factor(s). One hypothesis for the above observations was that Congo red might be mimicking a host cell effect on the bacteria which stimulates excretion of the bacterial antigens. To determine if this hypothesis was true, wild-type *Shigella* were grown in the presence of HeLa cell sonic lysates. Confluent monolayers of HeLa cells were scraped, washed, resuspended in TSB, absorbance-standardized, and sonicated on ice. HeLa lysates were then inoculated 1:100 directly with an overnight culture of either 2457T or a *mxi* mutant. After 2 hours incubation at 37°C, the culture supernatants were analyzed for excreted Ipa proteins by quantitative Western blot. As shown in Figure 23b, an increase in excreted IpaB and IpaC

Figure 23. Stimulation of Ipa excretion by Congo red dye and HeLa cells. Culture supernatants from log phase 2457T grown in the presence of the planar dye Congo red or HeLa cell lysates were subjected to quantitative Western blot analysis to measure the levels of excreted IpaB and IpaC. (A) Effect of 0.01% Congo red on Ipa excretion after 2 hrs growth. (B) Effect of HeLa cell lysates on Ipa excretion after 2 hrs growth. Samples were standardized by culture absorbance prior to application on SDS-PAGE.

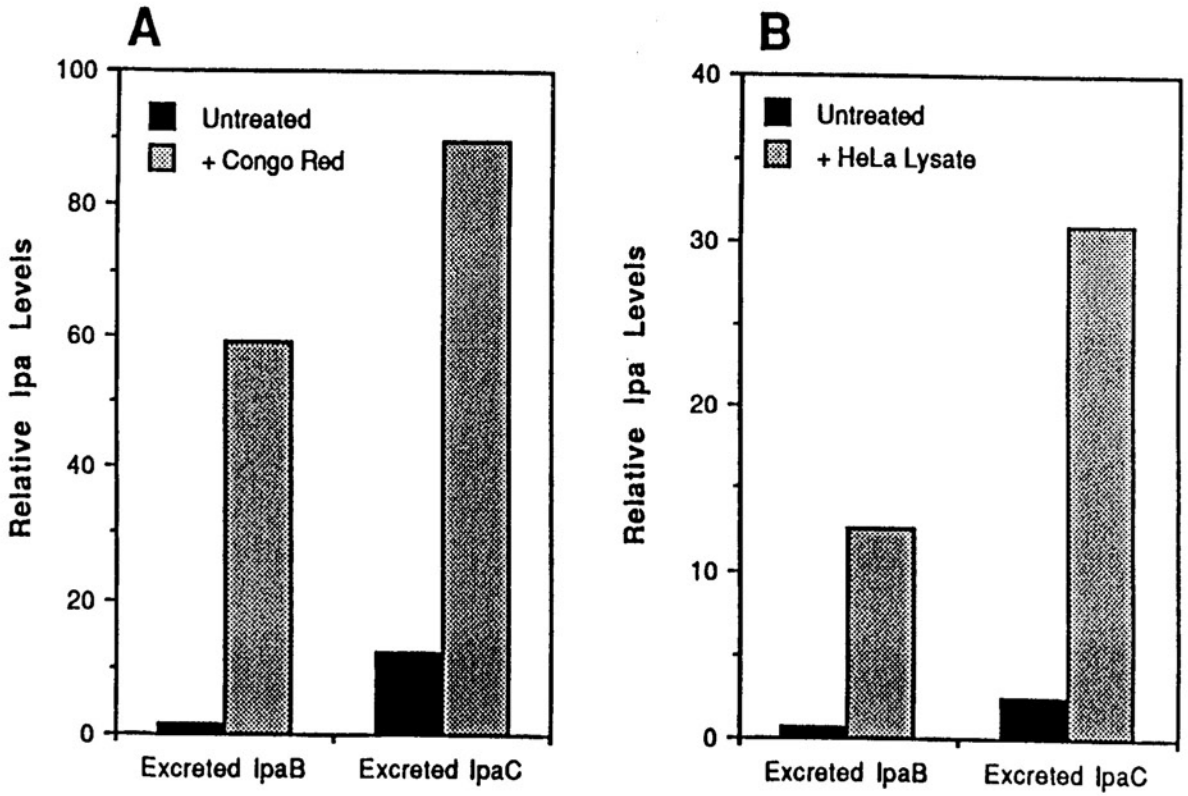

A
Relative Ipa Levels
100
80
60
40
20
0
Untreated
+ Congo Red
Excreted IpaB
Excreted IpaC
B
Relative Ipa Levels
40
30
20
10
0
Untreated
+ HeLa Lysate
Excreted IpaB
Excreted IpaC

Figure 24. Effect of Congo red on IpaB expression and non-specific release of β-galactosidase as a cytoplasmic marker. Quantitative Western blot analysis of intracellular and excreted β-galactosidase from the *ipaB::lacZ* reporter strain BS228 grown in the presence or absence of Congo red. Electroblots of whole cell lysates and culture supernatants were probed with MAb to β-galactosidase and the bands quantitated by digital analytic techniques (see materials and methods).

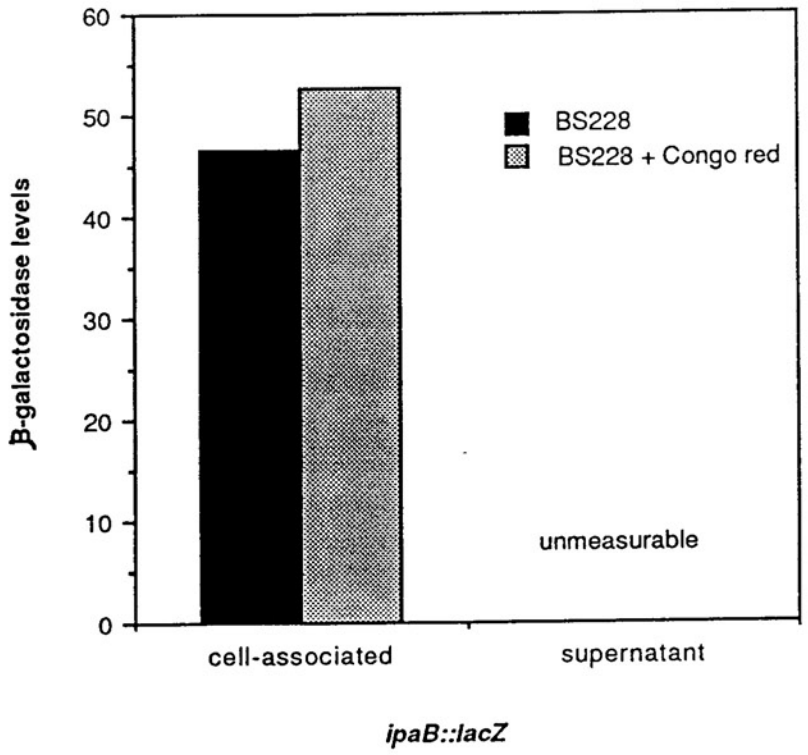
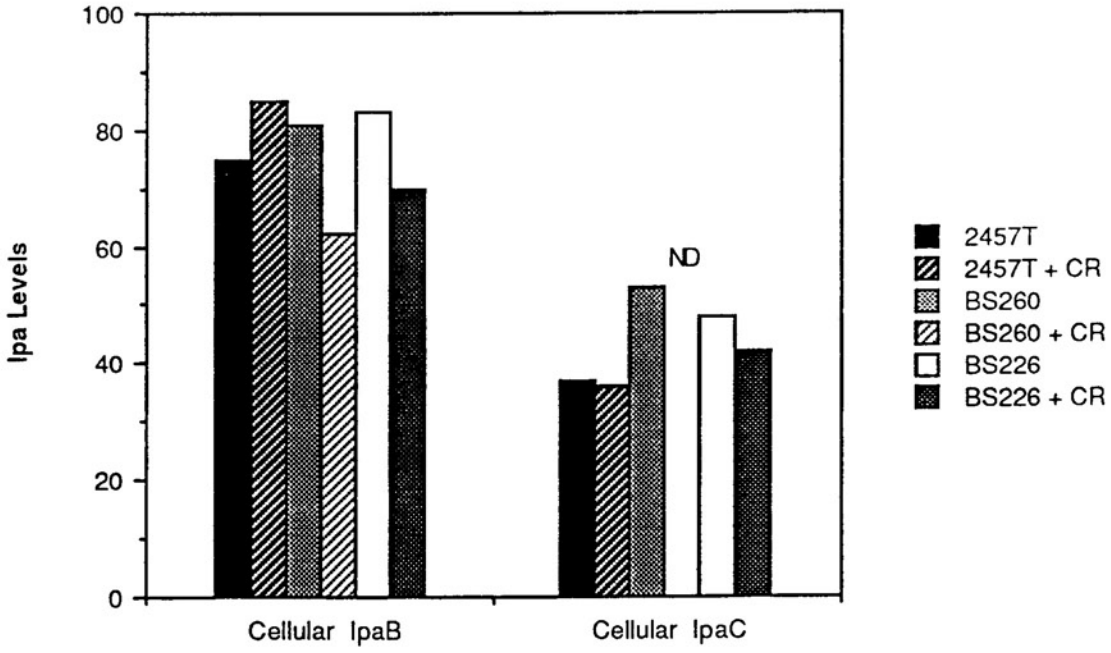

60
50
40
30
20
10
0
β-galactosidase levels
BS228
BS228 + Congo red
unmeasurable
cell-associated
supernatant
ipaB::lacZ

Figure 25. Effect of Congo red on cell-associated levels of IpaB and IpaC in wild-type *S. flexneri* and *mxi* mutants BS260 and BS226. Wild-type 2457T, BS260 and BS226 were grown in the presence or absence of Congo red and standardized to the A_{600} of the least dense culture. Whole cell lysates of each strain were electrophoresed, electroblotted, probed with MAb to IpaB and IpaC and quantitated densitometrically. CR = Congo red. ND = not determined.


100
80
60
40
20
0
Ipa Levels
ND
Cellular IpaB
Cellular IpaC
2457T
2457T + CR
BS260
BS260 + CR
BS226
BS226 + CR

was detected (39-fold and 10-fold, respectively) when wild-type bacteria were incubated with HeLa cell sonic lysates. The effect was not seen with the *mxi* mutant, BS230, when grown with the same HeLa preparation (not shown). HeLa cell lysates, like Congo red, did not appear to stimulate increased expression of Ipa as evidenced by quantitative assessment of Ipa B and IpaC signals on Western blots of bacterial lysates (Figure 26).

Effect of Ca^{++} concentration on *mxiAB* expression. Since it was found that MxiA is a homolog of LcrD, the low calcium response protein of *Y. pestis*, the effect of calcium cation concentration on *mxiAB* expression and Ipa export in *Shigella* was next tested. Strain 2457T and the *mxiA* mutant BS232 were grown at 37°C overnight and subcultured 1:100 in either TSB or calcium-chelated TSB (T-OX). The cultures were allowed to grow either to late log phase or stationary phase before precipitating the excreted bacterial proteins from the supernatants. No difference in levels of either excreted IpaB or IpaC were detected in the wild-type or the *mxi* mutant (data not shown). Additionally, when β-galactosidase levels were measured in the *mxiA* reporter, BS232, no difference in enzyme levels was seen between bacteria grown in the low calcium medium versus TSB (Table 14). Moreover calcium did not appear to modulate expression of *mxiB* since no difference in β-galactosidase levels was detected when the same experiment was performed on the *mxiB::lacZ* reporter, BS226.

Figure 26. Effect of HeLa lysates on cell-associated levels of IpaB and IpaC in wild-type *S. flexneri* and a *mxi* mutant (BS230). Wild-type 2457T and BS230 were grown in the presence and absence of HeLa cell extracts and standardized to the A_{600} of the least dense culture. Whole cell lysates of each strain were electrophoresed, electroblotted, probed with MAb to IpaB and IpaC and quantitated densitometrically.

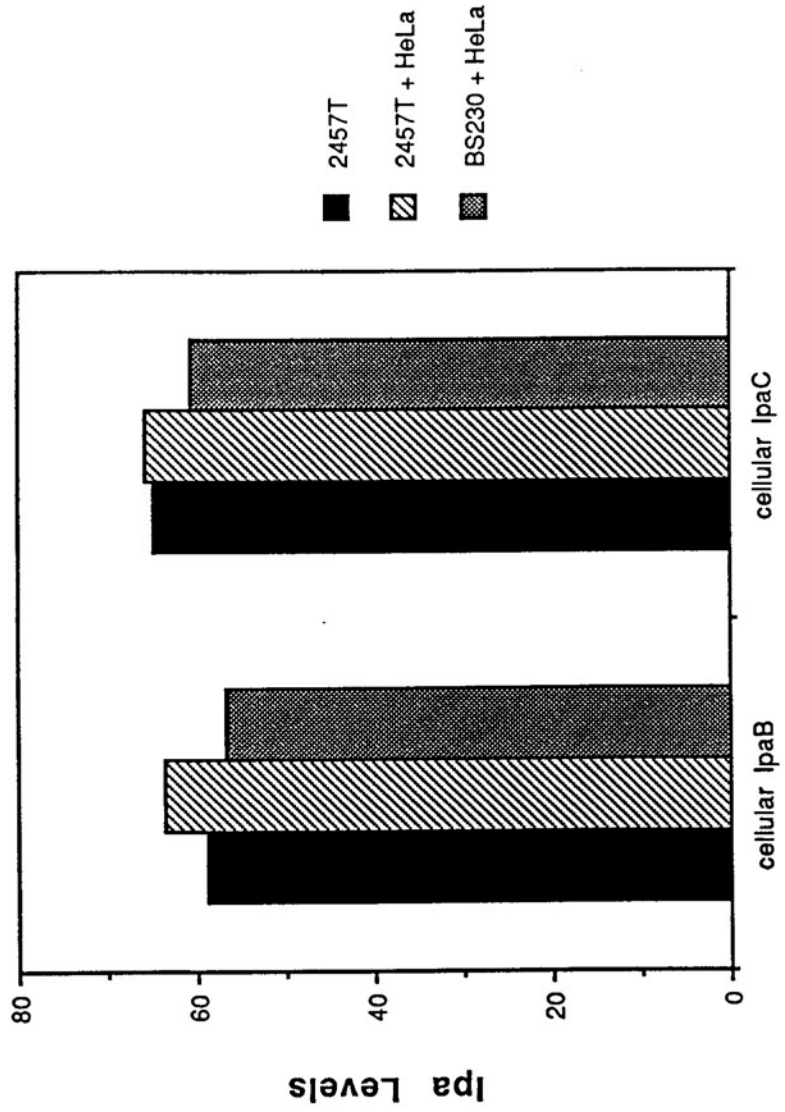

2457T
2457T + HeLa
BS230 + HeLa
Ipa Levels
0
20
40
60
80
cellular IpaB
cellular IpaC

Table 14. Effect of calcium concentration on expression of *mxiA* and *mxiB*.

Units, β-galactosidase[1]

Reporter Strains	High Ca++ (TSB)		Low Ca++ (T-OX)	
	Log	Stat.	Log	Stat.
BS232 (*mxiA*::*lacZ*)	660	893	693	672
BS226 (*mxiB*::*lacZ*)	668	868	654	843

1. As defined by Miller (1972).

DISCUSSION

Identification of the *mxiA* locus and characterization of IpaB and IpaC as excreted virulence factors.

Gene fusion technology has been previously exploited in this laboratory to identify new loci involved in the pathogenesis of *S. flexneri* (Hromockyj and Maurelli, 1989). In the experiments just described, a bank of protein fusions was screened for insertions in the chromosome and the invasion plasmid in an effort to further identify and characterize new virulence genes. A single avirulent protein fusion mutant BS260 was isolated and determined to possess a phenotype similar to the previously described *mxi* operon fusion mutants which are altered in extracellular expression of IpaB and IpaC (Hromockyj and Maurelli, 1989). Therefore, a more thorough characterization of this *mxi* locus (designated *mxiA*) and its role in facilitating export of the *Shigella ipa* gene products was conducted.

The region of the virulence plasmid near the site of the insertion in BS226 had previously been shown to contain essential virulence loci. Tn*5* insertions in the 11 kb *EcoR*I fragment (location of the BS226 insert) of the cosmid clone pHS4108 produce a non-invasive phenotype (Maurelli, et al., 1985). A comparison of BS260 with the *mxi* operon fusion mutant BS226 was initially made because the map distance of the fusion inserts from one another (greater than 3 kb) suggested that the mutations were in different *mxi* genes. This hypothesis was confirmed by the failure to detect high levels of expression of the reporter gene, *lacZ*, from the cloned *mxiA* end-join fragment (pGPA001), while expression of β-galactosidase from the BS226 end-join clone (pAEH006) was high. Thus, the *mxiB* locus, as defined by BS226, is driven by a separate promoter which is independent of *mxiA*. These data initially led to the hypothesis that the promoter for *mxiA* lay at least 5 kb upstream from the translational start site of the gene, which further implied that *mxiA* may be part of an operon which

consisted of additional Ipa accessory loci. Further, the *mxiA* locus was determined to be under the control of the chromosomal thermoregulatory gene, *virR*. This conclusion was based on the temperature deregulation of β-galactosidase expression when a *virR*::Tn*10* lesion was transduced into the parent mutant BS260. Deregulation of the reporter gene expression was unusual since it occured as a reduction in enzyme levels at 37°C and not an increase in levels at 30°C as was seen with other *mxi* mutants (Hromockyj and Maurelli, 1989). By further transductional analysis, it was concluded that this phenomenon was caused by a second site mutation on the invasion plasmid. Considering the size of the fusion phage insert (50 kb), the linkage distance of this secondary lesion from the insertion site (5' end) was determined to be approximately 17 kb. This value was derived from the Wu/Kemper mapping function curve for linkage distances between cotransduced markers (Wu, 1966; Kemper, 1974). If the secondary mutation is 17 kb upstream of the phage insert site, it would lie within a region downstream of the *ipa* gene cluster, possibly in the coding region of the trans-activator VirB (Adler, *et al.*, 1989). If *mxiA* is regulated by this gene, then the low level β-galactosidase phenotype seen with BS260 *virR*::Tn*10* may be accounted for in this way. Alternatively, if the second site lesion is 17 kb downstream of the phage insert, then it would lie in the uncharacterized 3' region of pHS4108. Thus, an unidentified downstream locus may exist which has a trans-activating effect on *mxiA* expression.

Earlier evidence suggesting that Ipa proteins are excreted came from the finding that IpaB and IpaC are found in distilled water extracts of the wild-type bacteria (Oaks, *et al.*, 1986) although this phenomenon was attributed to the "shedding" of the antigen from the surface of the organism. Data presented in this thesis, however, suggest that IpaB and C are, in fact, excreted antigens, and the *in vitro* wild-type phenotype of *S. flexneri* is represented by the export of IpaB and IpaC into the culture medium. These findings were further supported by the demonstration that wild-type culture

supernatants showed detectable levels of the two antigens on Western blots while the *mxi* mutants failed to excrete them (Figure 7).

The interpretation of the results in Figure 5 (SLIM) should be taken in the context of the results of the cell-free ELISA (Figure 6). Although it appears that some surface-associated IpaC exists both on wild-type as well as the *mxi* mutants, the amount of antigen that is cell-free is significantly different between the mutants and wild-type cells. Thus, the *mxi* mutations effect a significant decrease in the release or excretion of IpaC (as well as IpaB). *mxiA* and *mxiB* may, in fact, function in the release of "already" surface-associated IpaB and IpaC. If this were the case, however, one would expect to find an accumulation of Ipa proteins on the surface (or in the outer membrane) of the *mxi* mutants. By the assay methods used in this investigation, neither a higher level of surface-associated Ipa nor an outer membrane accumulation of these antigens in the mutants was detected. In fact, the outer membranes of the *mxi* mutants were found to be deficient in both of these antigens. Thus, it was concluded that the *mxiAB* functions are related to export of the Ipa proteins rather than their release from the surface. The Ipa excretion phenomena mediated by *mxi*, therefore, represents a specific virulence mechanism which has not yet been reported for *Shigella*.

The *mxi* mutants, BS260 and BS226, released low but detectable levels of both IpaB and IpaC in the cell-free ELISA. This observation was subsequently found to be an artifact of the assay procedure, since some cell lysis occured during the incubation step in PBS. β-galactosidase activity (a marker for cytoplasmic leakage) was detected in the *mxi* mutant PBS supernatants after the microorganisms were incubated in the buffer for 1 to 2 hrs (data not shown).

An ELISA which employs whole bacteria as the coating antigen has previously been used to demonstrate that both IpaB and IpaC are expressed on the surface of *Shigella* (Hale, et al., 1985; Mills, et al., 1988; Oaks, et al., 1986; Pal, et al., 1985). The whole cell ELISA was also found to be useful in the initial characterization of the *mxi*

operon fusion mutants (Hromockyj and Maurelli, 1989). The experiments described in this study, however, indicated that only very low levels of IpaB and almost no IpaC are associated with the surface of wild-type *Shigella*. Moreover, comparative studies have suggested that the majority of the MAb reactivity detected in the whole cell ELISA was due to excreted or released antigen rather than expression of the antigens on the cell surface. Cell-free PBS extracts were used to "coat" microtiter plate wells next to wells containing the equivalent number of bacteria from which the PBS extracts were derived. After the appropriate incubation period, only a 20% difference was found in Mab reactivity between the cell-free PBS supernatant-coated wells and the wells coated with whole cells (data not shown). Additionally, it was found that very low numbers of bacteria were found to be adhering to the microtiter plate wells under the conditions of this assay as measured by direct physical and immunological detection methods. Thus, these observations led to the development of two assays which separately measured cell-associated (SLIM assay) and released Ipa (cell-free ELISA).

Despite the evidence that IpaB and IpaC are excreted proteins, an apparent discrepancy exists in the detection/quantitation of surface-associated IpaB between the suspension labelling immunoassay and the proteinase K experiments. While results of the suspension assay showed a detectable level of surface-associated IpaB on wild-type cells, the proteinase K experiments indicated that very little was surface-exposed. This discrepancy can be reconciled by the fact that the SLIM assay represents a more sensitive means to detect low levels of surface-associated antigen relative to the protease protection method. In this regard, the assay may be detecting very low levels of IpaB on wild-type cells. One explanation for the reactivity of wild-type *Shigella* with anti-IpaB MAbs in the SLIM assay is that the excreted antigens may readsorb to the cell surface at some point in the cell's growth phase. Indeed, other investigators have suggested this reason for the apparent association of predominantly excreted proteins with the outer membrane of the bacteria that release them (Michiels, et al., 1990).

A third method was employed to detect cell surface-associated IpaB and IpaC. Immunoelectron microscopy using anti-IpaB and anti-IpaC MAbs as well as polyclonal monospecific antibody to these antigens failed to demonstrate a high level of either IpaB or IpaC on the surface of log phase wild-type cells even when surface-exposed proteins were cross-linked or fixed prior to labelling with the antibodies. In contrast, Ito and coworkers (Ito, et al., 1991) were able to label a significant amount of IpaB around the periphery of thin sections of an *E. coli* K-12 clone harboring the invasion plasmid from *S. sonnei*. It is not clear, however, from the electron microscopic data in these experiments, that the labelled antigen truly represented surface-exposed Ipa or merely membrane-associated antigen.

The intracellular distribution of IpaB and IpaC in the wild-type bacteria as well as the *mxi* mutants presents an interesting finding. Overall, large intracellular pools of both antigens were detected in the cytoplasm and cell envelopes by quantitative Western blotting of cell fractions. These large pools of antigen may exist in *Shigella* because of a high turnover rate, not only from excretion of the proteins into the extracellular milieu, but as a compensatory effect in response to the extreme sensitivity of these proteins to degradation by endogenous bacterial proteases (Mills, *et al.*, 1988). Additionally, excreted (cell-free) antigen was found to represent only a small portion of the total Ipa synthesized which indicates either that excretion is relatively inefficient or that extracellular Ipa is much more susceptible to proteolysis than the intracellular pool. A more intriguing possibility is that these antigens are stored by the bacteria for rapid dispersal at a later time (see below) and that the amount of excreted Ipa observed under the *in vitro* growth conditions may represent a leakage by the secretion machinery controlling export of these antigens. Regardless, the bulk of the exported antigen appears to exist as the cell-free form (versus cell surface-associated). Although low levels of IpaB and IpaC were also observed in the periplasm of the wild-type as well as the mxi mutants after cell fractionation, this observation was probably due to cytoplasmic

cross-contamination since significant levels of the cytoplasmic marker, G-6-P dehydrogenase, were detected in the periplasmic fractions (data not shown). Therefore, the importance of the periplasmic space in the Ipa excretion process still remains unclear. An accumulation (2 to 3-fold) of IpaB and IpaC was seen in the inner membrane of both *mxi* mutants, while very low levels of both antigens were detected in the outer membrane relative to wild-type bacteria (Figure 9a and b). This finding suggests a block in secretion between the inner and outer membrane may exist. Thus, at least two of the *mxi* gene products may function to facilitate movement of *ipa* proteins across the outer membrane.

Although a large amount of surface-exposed antigen (either IpaB or IpaC) was not detected by proteinase K digestion, an appreciable amount of IpaB (and some IpaC) was found in outer membrane fractions of wild-type cells. One explanation for the outer membrane-associated Ipa detected in the cellular fractions is that it may represent a "transition state" pool as it passes from inner membrane to the extracellular environment. Alternatively, evidence from other Gram negative bacteria excretion systems has suggested that the association of some exported proteins with the outer membrane may be due to their copurification with the membrane as insoluble aggregates in the presence of high concentrations of non-ionic detergents (Michiels, et al., 1990). The tendency of IpaB to form high molecular weight aggregates was, in fact, observed when clarified whole cell lysates of wild-type *Shigella* were passed over a Sephadex G-100 column. Void volume fractions (containing macromolecular species >100 kDa) contained the greatest amounts of both IpaB and IpaC. Since these proteins were previously characterized as being 62 kDa and 42 kDa respectively, they would be retained in the gel exclusion matrix if in monomeric form. Covalent cross-linking of IpaB in a complex of greater than 100 kDa confirmed that at least this antigen may be complexed with other proteins.

In addition to facilitating export of IpaB and IpaC, the *mxi* export apparatus directs excretion of other antigens, since two additional immunogenic proteins were detected in immunoblots of wild-type culture supernatants (Figure 7). These proteins, which corresponded approximately to the molecular weights of IpaA (78 kDa) and IpaD (37 kDa), were absent in culture supernatants of the *mxi* mutants. In contrast, the *virG* gene product appeared to be excreted independently of the two *mxi* loci, since both *mxiA* and *mxiB* mutants excreted this protein into culture supernatants at levels comparable to wild-type (Figure 7). This finding strongly suggests that more than one specific pathway exists for export of *Shigella* virulence factors.

Characterization of the *mxiA* locus and its product.

The putative native *mxiA* gene product has been identified as a temperature-regulated 76 kDa polypeptide in whole cell lysates of wild-type *S. flexneri* grown at 37°C. Although the anti-MxiHP polyclonal monospecific antiserum did not detect the native fusion protein in the fusion strain (BS260), affinity-purified MxiHP was weakly reactive as a band of molecular weight 123 kDa. The failure to detect the hybrid protein in BS260 was most likely due to low level production in the mutant combined with the weak reactivity of the antiserum. When whole cell lysates of other *mxi* mutants were probed with the anti-MxiHP IgG fraction, only the *mxiB* mutant, BS226, was found to make the putative MxiA protein. This coincided with previous findings, since the lesion in BS226 was localized downstream of *mxiA* and should not have affected expression of this gene unless *mxiB* had a positive regulatory effect on *mxiA* . The *mxi* mutant with a phage insert in a locus upstream of *mxiA*, BS230, may possess such a function, since no *mxiA* gene product was detected in this strain. Alternatively, since *mxiA* may be encoded on a polycistronic message, the lesion in BS230 may simply be a *mxiA* polar mutation. Based on *mxiA* complementation data however, the former may actually be true (see below). The 76 kDa putative *mxiA* gene product was also not detected in the *mxi* mutant,

BS232. This finding was not surprising since localization of the insert in BS232 had placed it within the *mxiA* coding region (see below).

An additional band corresponding to a protein of approximately 55 kDa was also seen in BS226 lysates probed with anti-MxiHP. Preliminary data indicated that a similar protein was also present in lysates of 2457T and BS260 as well as BS103 at both 37^{o} and 30^{o}C. The 55 kDa species in these lysates was detectable when lower dilutions of pre-adsorbed anti-MxiHP IgG were used as the primary label on Western blots Thus, the protein is a chromosomally-encoded and constitutively expressed. Interestingly, a species of similar molecular weight was detected as a co-elution product with the hybrid protein in some preparations of affinity-purified fusion protein. It is possible, therefore, that this protein is made at higher levels in the *mxiB* mutant, BS226 (based on the results presented in figure 15). One explanation for this observation is that the *mxiB* gene product may encode an outer membrane protein (Venkatesan, et al., 1991). The mutant may compensate for the absence of this protein in the cell envelope by upregulating synthesis of another outer membrane polypeptide which is required for cell membrane stability.

A DNA sequence analysis of the complete *mxiA* coding region and upstream DNA was performed on two contiguous *Hind*III fragments used as double-stranded templates. The fusion phage insertions in both *mxi* fusion mutants, BS232 and BS260, were subsequently found to map within the *mxiA* open reading frame. This finding confirmed the insertion in BS232 as being in *mxiA*.

A second open reading frame (ORF2) was found upstream and in frame to the *mxiA* coding sequence. This locus continued upstream of the 5' end of the *Hind*III restriction fragment in pGPA010 (Figures 13 and 14). A mutation in ORF2 (BS183, *mxiC*, see below) also conferred the non-invasive and Mxi^{-} phenotype. The first 10 codons of a third open reading frame immediately downstream of the *mxiA* coding region was found to exactly match an uncharacterized ORF, *spa15* (Venkatesan, et al., 1992). This ORF

predicts a hydrophilic polypeptide of 15 kDa. Examination of DNA sequence upstream from the ATG start of Spa15 revealed neither a σ^{28}-like promoter nor an *E. coli* consensus promoter. Thus, it is possible that *mxiA* and *spa15* are specified by a single transcript. Furthermore, the *spa15* locus lies immediately upstream of *spa47* (Venkatesan, et al., 1992) which is identical to the previously described *mxiB* locus (Andrews, et al., 1991). Two additional mxi loci have recently been identified upstream and adjacent to *mxiC*, designated *mxiD* and *mxiE* (P. J. Sansonetti, personel communication). *mxiDE* mutants are also defective for excretion of Ipa proteins and are Crb$^-$. Interestingly, MxiD has been identified as an outer membrane protein which shows significant homology to PulD (Allaoui, et al., 1992), the only outer membrane protein of the pullulanase export apparatus of *Klebsiella pneumoniae* (Pugsley, et al., 1990). Taken together, these findings suggest that the gene order of the *mxi* region is *mxiE mxiD mxiC mxiA spa15 mxiB*. Additional *mxi* loci may however extend beyond the 5' and 3' boundaries of this gene cluster.

DNA sequence homology to an alternate sigma factor (σ^{28}) binding sequence was identified upstream of the *mxiA* ORF. Transcriptional initiation sites of this type have been found to be involved in expression of chemotaxis and flagellar genes in *E. coli* as well as *Salmonella typhimurium* (Helmann and Chamberlin, 1987). Although the control of expression of the σ^{28} protein itself is not clearly understood, the genes specifying these alternate sigma factors possess CRP-cAMP complex binding sites. Hence, they may be susceptible to catabolite repression (Helmann, *et al.*, 1988; Bartlett, et al., 1988). Interestingly, in *B. subtilis*, expression of σ^{28} has been correlated with the transition from vegetative state to sporulation, since σ^{28}-directed transcripts are absent in certain sporulation deficient mutants (Gilman and Chamberlin, 1983). Taken together, these observations suggest that σ^{28} expression itself may be modulated by alterations in nutritional conditions such as the availability of certain carbon sources. Therefore, if *mxiA* transcription is controlled by σ^{28}, then it is

possible that optimal expression of *mxiA* occurs as a consequence of a nutrient-dependent transition from one growth state to another (*e.g.*, log phase to stationary phase) and coincides with optimal expression of the σ^{28} protein.

A sequence homology search of the complete *mxiA* ORF with other prokaryotic genes revealed an interesting finding. Although no homology at the DNA level was found, a strikingly high degree of homology (68%) was seen between the *lcrD* locus of *Y. pestis* and *mxiA* at the deduced amino acid level. This finding represents the first report of significant amino acid homology between a virulence protein of *Shigella* and that of *Yersinia* and strongly suggests the existence of a *Yersinia* homolog in *Shigella*.

The hydropathy plot of the predicted *mxiA* gene product also appeared very similar to that of LcrD (Plano, et al., 1991). In fact, the hydropathy profile of MxiA is consistent with the preliminary analysis in this study which suggested that MxiA was a membrane protein. Plano and coworkers (1991) reported that *lcrD* codes for an inner membrane protein with an N-terminal anchor consisting of several transmembrane spanning domains and a cytoplasmic C-terminus. Further, it appeared that MxiA possessed a non-cleavable signal sequence specified by the first 13 amino acids of the protein. This sequence may be all that is required to target MxiA to the inner membrane, since a significant amount of the β-gal/MxiA hybrid protein (possessing only these N-terminal 13 amino acids from MxiA) from the BS260 fusion mutant was driven into the membrane (Figure 3).

A more detailed analysis of the homology between MxiA and LcrD in relationship to the hydropathy profiles showed that the majority of the predicted membrane anchor sequence (amino acid 1 to 300) is conserved between the two organisms. The cytoplasmic domains of the two proteins, however, are more divergent with the exception of a region of high homology towards the carboxy-termini of the proteins. This pattern of homology (a conserved anchor sequence and a more divergent cytoplasmic region with a single domain of high homology) has been demonstrated between other

inner membrane proteins of different bacterial species which function as regulators (Miller, et al., 1989).

In addition to the homology reported with FlbF of *Caulobacter* (Ramankrishnan, et al., 1991), LcrD and the product of the *invA* gene of *S. typhimurium* are reported to be homologous (Plano, et al., 1991; Galan, et al., 1992). It is also interesting to note that *flbF* has recently been shown to possess a σ^{28}-like promoter sequence (Sanders, et al., 1992). These findings suggest that a family of proteins may exist among Gram negative bacteria which possess the same or a similar function (S. C. Straley, personal communication; Galan, et al., 1992). This function, as it pertains to MxiA, will be discussed below.

Complementation of the *mxiA* defect.

Despite the initial findings which suggested that *mxiA* transcription was driven by a distal promoter more than 6 kb upstream of the gene (Table 3), the results of the complementation experiments strongly suggest that *mxiA* is driven by a proximal promoter immediately adjacent to the coding region. The *mxiA* defect was complemented in two *mxiA* mutants (BS260 and BS232), using a cloned copy of the gene containing very little DNA upstream of the *mxiA* ORF. Complementation was also achieved with the *mxiA* ORF in reverse orientation to any vector-specified promoters (Figure 13, pGPA042 and pGPA045). Additional evidence supporting the hypothesis that *mxiA* is encoded on a much smaller transcription unit than was originally thought comes from the finding that the largest *mxiA*-specific message seen in a Northern blot analysis of wild-type *S. flexneri* is 2.6 kb (A. T. Maurelli, unpublished data), which would only be large enough to specify *mxiA* and possibly the 400 base ORF, *spa15* (Venkatesan, et al., 1992). If this is the case, than it is unlikely that the GTG codon identified in the *mxiA* ORF represents the true translational start site since there is not enough DNA upstream of the GTG codon in the smallest complementing *mxiA* clone (pGPA045) to contain a

promoter. One possible explanation for the discrepancy observed between the fusion end joint data of Table 5 and the complementation data is that transformation of the cloned BS260 fusion end joint into a wild-type *S. flexneri* background may have induced a second site mutation which represses *mxiA* promoter activity. This hypothesis would be similar to the explanation proposed for the phenotype of BS260 *virR*::Tn10 transductants, which may have created a condition in which overexpression of a membrane-driven hybrid protein was deleterious to the cell. If this hypothesis was true, then the cloned fusion end joint from BS232 should not have induced this type of mutational event, since it is a transcriptional gene fusion and does not make a hybrid protein. When the BS232 end joint was transformed into a *Shigella* background, however, the same results as with the BS260 end joint were seen, which suggested a distal *mxiA* promoter (Hromockyj and Maurelli, 1989). Despite these findings, the position of the insert in BS232 predicts that a 9 kDa *mxiA* truncate could still be made which may have the same deleterious effect when overproduced as the MxiA/β-gal hybrid protein.

When the smaller *XbaI-EcoRI* fragment of pGPA045 was placed in the p*lac*-driven orientation in the vector pBS (similar to pKS but with polylinker in reverse orientation), the resultant transformants of *E. coli* were extremely slow growing and appeared to readily lyse in liquid culture (data not shown). Consequently, transformants of *S. flexneri mxi* mutants with this DNA were never able to be isolated. Difficulty with this recombinant plasmid was not surprising in light of the findings suggesting that MxiA is an inner membrane protein. Overexpression of this polypeptide may lead to blockage or interference of essential export function in the bacteria. Additionally, in support of this hypothesis, the *mxiA* mutants transformed with *mxiA* cloned into either pUC19 or pKS appeared to secrete reduced levels of IpaB (both cell-associated and excreted) even though they were all invasive. It is possible, therefore, that because *mxiA* is in a high copy vector in these strains, any increased level of expression of this

gene product may interfere with efficient export of IpaB. One phenotypic effect which may be correlated with decreased export of IpaB is the reduced capacity of *mxiA* transformants to produce plaques on HeLa cell monolayers. This finding suggests that decreased excretion of IpaB may lead to a decrease in the efficiency of the organisms to replicate intracellularly and/or to spread from cell to cell. Alternatively, the export of other excreted proteins may be affected in the *mxiA* transformants which may account for the reduced plaquing efficiency. This hypothesis may, in fact, explain the reason for differences seen in the plaque assay between BS232/pGPA043 (*mxiA*, p*lac*-driven) and BS232/pGPA043 (*mxiA*, non p*lac*-driven), both of which export the same intermediate levels of IpaB. Greater expression of *mxiA* from the former may have a greater effect on export of additional proteins, such as VirG, associated with post-invasion events. In contrast to IpaB, wild-type levels of excreted IpaC were seen in all the *mxiA* transformants. Thus, export of this antigen may be less effected by overexpression of MxiA.

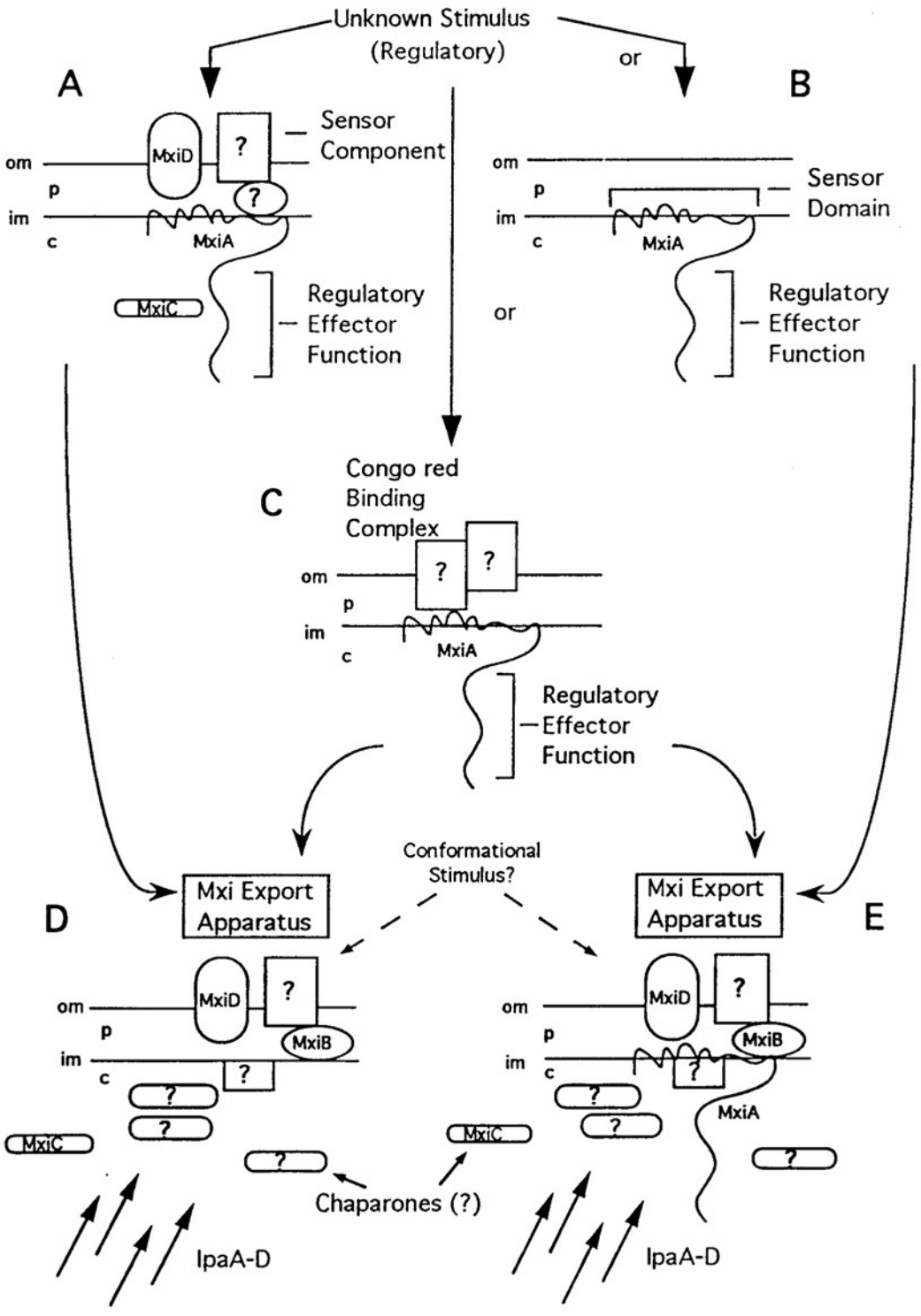
Role of the *mxiABC* gene products.

In this study, it was demonstrated that MxiA functions in facilitating export of IpaB and IpaC. Similarly, one of the functions proposed for LcrD in *Y. pestis* is to export virulence factors, since a mutation in this locus results in loss of *Yersinia* outer membrane proteins (Yops) H and M in the outer membrane and their accumulation within the bacteria (Plano, et al., 1991). The *lcrD* locus also plays a role in the regulation of gene expression during the low calcium reponse (Goguen, et al., 1984). In addition to displaying a secretion-defective phenotype, *lcrD* mutants are also down-regulated for expression of the V antigen when grown under conditions for optimal expression of this protein (37^{o}C, no Ca^{++}). Thus, it appears that LcrD can function as a trans-activating factor as well as facilitating secretion of Yops. It is highly possible that LcrD actually regulates expression of those genes directly involved in export of these

proteins (Plano, et al., 1991). Since *mxiA* may function as the *lcrD* homolog in *Shigella*, it is not unrealistic to envision *mxiA* as having a role in the regulation of *Shigella* genes similar to the proposed role for *lcrD* in *Y. pestis.* Although *Shigella* proteins have not yet been specifically identified which are down-regulated as a result of mutating the *mxiA* locus, several regulatory loci, both chromosomal- and plasmid-encoded, have been identified and characterized in *Shigella* (Adler, et al., 1989; Buysse, et al., 1990; Kato, et al., 1989; Maurelli and Sansonetti, 1988). Up to now, response to temperature and osmolarity have been implicated as environmental factors modulating gene expression in this organism (Maurelli, et al., 1992). It is not clear, however, if cationic (or anionic) effects are important. In this regard, an initial attempt was made to determine if calcium plays a role in modulating excretion of IpaB and IpaC in wild-type *S. flexneri.* No difference was seen in the Ipa excretion phenotype of bacteria grown under high or low calcium conditions. Additionally, no effect on either *mxiA* or *mxiB* expression was seen in *mxi*::*lacZ* reporter strains by reducing calcium alone. It is possible, therefore, that calcium does not function to modulate these genes in *Shigella* as it does in *Yersinia.* Alternatively, a combination of several physical conditions may be necessary to effect modulation of virulence factor export. A more thorough investigation of the effects of additional conditions, such as osmolarity, pH, etc., should prove interesting.

If MxiA does have a regulatory role in modulating export, it is possible that it may function as a sensor/effector molecule in response to a specific environmental stimulus (Figure 27a). In this case, the N-terminal hydrophilic loops of the protein may perform a sensory function while the C-terminus acts as the effector or regulator with the conserved region representing a common functional domain. Similar structure/function relationships have been proposed for sensor/effector molecules in other bacteria (Miller, et al., 1989). Since, however, a functional comparison between MxiA and other sensor/effector molecules has not yet been made, other roles for this accessory protein are equally as probable. In fact, it is quite possible that MxiA

Figure 27. Models for the role of the mxi gene products in the export of IpaA-D. (A) An outer membrane sensor protein, possibly MxiD (or in conjunction with MxiD), responds to an external environmental stimulus. The signal is then transduced across the outer membrane to MxiA with the potential involvement of a periplasmic component. The hydrophilic regulatory domain of MxiA (together with MxiC?) ultimately responds to the extracellular signal to regulate expression of genes encoding structural Mxi proteins more directly involved in export of Ipa polypeptides. (B) Same as (A) but here the N-terminus of MxiA functions directly to receive the modulatory extracellular signal. (C) Same as (A) but MxiA is in a regulatory complex with one or more unidentified components which bind Congo red. (D) Scenario which portrays Mxi products as structural components of the Ipa export apparatus. The expression of these proteins may respond to an effector signal from MxiA (A, B or C). Alternatively, an extrinsic signal may conformationally alter the export complex which then makes it excretion-competent. (E) Same as (D) but MxiA has a structural role as part of the Ipa export apparatus. In these last two examples, MxiB could couple ATP hydrolysis to the translocation of IpaA-D across the cell envelope. Additionally, MxiC and three other unidentified proteins may function as product-specific cytoplasmic chaparones for each of the Ipa proteins which then target each of the Ipa species to the membrane-bound export complex. **om** = outer membrane, **p** = periplasm, **im** = inner membrane, **c** = cytoplasm.


Unknown Stimulus
(Regulatory)
or
A
B
om
p
im
c
MxiD
?
Sensor
Component
MxiA
MxiC
Regulatory
Effector
Function
Sensor
Domain
or
C
Congo red
Binding
Complex
Conformational
Stimulus?
Mxi Export
Apparatus
D
E
MxiB
Chaparones (?)
IpaA-D

functions as part of a membrane-bound regulatory complex which has multiple components, each with a different specific function (Figure 27b and c). In this regard, the family of inner membrane proteins, to which MxiA belongs, may function as a core component of the regulatory complex. Functional diversity, such as the ability of the regulatory complex to respond to different environmemtal stimuli, may be dictated by other components of the complex which may vary greatly from organism to organism depending on its particular lifestyle. Thus, the MxiA/LcrD family may regulate genes involved in export of virulence factors, but the signals which modulate the regulators themselves may be quite different between organisms.

mxiB has been found to be identical to the recently reported *spa47* locus which encodes a product that has high homology with the product of a flagellar gene of *Salmonella typhimurium, fliI* (Venkatesan, et al., 1992). Several genes in this organism, *fliAHIN*, have been deduced to play a role in the export of flagellar structural components (Vogler, et al., 1991). Specifically, FliI has been identified as having high homology to an ATPase β subunit (Albertini, et al., 1991), and it has been suggested that it may be a part of an ATP-driven protein translocase (Vogler, et al., 1991). Thus, MxiB (Spa47) may function in a manner similar to the FliI protein as the ATP-dependent translocator component of the Mxi export complex (Figure 27d and e). A similar component of the hemolysin A export complex of *E. coli*, HlyB, has been characterized (Holland, et al., 1990). HlyB also functions in an ATP-dependent manner in this system, although no homology was found between this protein and MxiB at either the DNA or the amino acid levels (Venkatesan, et al., 1992). Additionally, in this study, an attempt to functionally complement *mxi* defects with the *hly* accessory loci failed, lending support to the uniqueness of the Mxi export system in *Shigella*.

Since the transcriptional fusion in BS183 was characterized as another *mxi* (*mxiC*) mutant in terms of its Ipa excretion phenotype, further characterization of this strain was carried out to yield additional information about the Ipa export mechanism. Although

the mutation in BS183 mapped upstream of the *mxiA* locus, a high copy clone of *mxiA* was able to partially restore the virulence phenotype of the mutant. The lesion in BS183 appeared to have a polar effect on *mxiA* since the cloned *mxiC* locus alone failed to restore the invasive phenotype to BS183 (S. C. Tucker and A. T. Maurelli, unpublished observations). Since the mutation in *mxiC* was 121 bases upstream of the putative σ^{28} promoter of *mxiA*, it was hypothesized that the effect on *mxiA* was due to an inactivation of a regulatory region of this gene. A model for one such regulatory region controlling expression from a σ^{28} promoter has been recently proposed for the flagellin gene of *Pseudomonas aeruginosa* (Starnbach and Lory, 1992). An interesting finding with BS183 transformed with *mxiA* alone was that only intermediate levels of excreted IpaC were detected. This was in contrast to the wild-type levels of IpaC detected in the *mxiA* mutants, BS260 and BS232, transformed with *mxiA*. Despite the polar effect on *mxiA*, it was found that both loci (*mxiA* and *mxiC*) were required to confer the wild-type phenotype in BS183, since only a *mxiA*/*mxiC* double transformant completely restored invasion and plaque formation in this mutant (S. C. Tucker and A. T. Maurelli, unpublished data). Additionally, excreted IpaC was significantly increased in BS183 containing cloned copies of both *mxiC* and *mxiA* compared with BS183 transformed with *mxiA* alone (S. C. Tucker and A. T. Maurelli, unpublished data). Thus, the gene product of *mxiC* may be more directly involved in the export of IpaC. Although these data suggest that excreted IpaC is one factor necessary for efficient invasion by the bacteria, it is possible that a mutation in *mxiC* effects excretion of other virulence factors as well which may also be required for invasion (e.g., IpaD). The experiments performed in this study were designed only to detect IpaB and IpaC. Moreover, the findings presented here may be complicated by the fact that the mutation in BS183 also effects *mxiA* expression. The generation of a mutation in *mxiC* which does not have a polar effect on *mxiA* should provide further clarification of the role of the *mxiC* gene product in excretion of IpaC as well as export of other virulence-associated products.

The complete coding sequence of *mxiC* was identified and found to encode a predicted polypeptide of approximately 40 kDa. Both the DNA sequence and the putative gene product showed no homology to any known prokaryotic genes or their products. Because no signal sequence was detected in the N-terminus of the predicted protein and the hydropathy plot revealed an essentially hydrophilic species with a slightly hydrophobic C-terminus, it is possible that MxiC is a cytoplasmic protein or a peripheral inner membrane protein which functions as a structural component of the *mxi* export apparatus. In fact, it is tempting to speculate that MxiC may function as a cytoplasmic molecular chaperone (possibly IpaC-specific) which "holds" a protein destined to be exported in a conformation which allows it to be recognized and/or translocated by the Mxi export machinery (Figure 27d and e). This hypothetical function for MxiC would, therefore, be analagous to the function of the *secB* gene product for proteins secreted through the general (*sec*-dependent) export pathway (Schatz and Beckwith, 1990).

An insert within a previously isolated *mxi* operon fusion mutant, BS230 (Table 3) was not found to map within either of the two sequenced *mxi* loci (*mxiA* and *mxiC*). Based on this finding and previous mapping data, the insertion in this mutant would be localized upstream of *mxiC*. A Western blot analysis of BS230 with anti-MxiHP, however, revealed that MxiA was not made. The possibility that this insert has a polar effect on the *mxiA* locus was eliminated when a cloned copy of *mxiA* transformed into BS230 failed to restore any of the virulence phenotypes. Thus, the locus defined by BS230 may represent a new *mxi* locus whose gene product has a trans-activating function on *mxiA* expression. Alternatively, the mutation in BS230 may have a polar effect on other downstream loci which then effect *mxiA* expression.

Functions for Excreted IpaB and IpaC.

The results presented here, along with published observations on the properties of the *ipa* gene products, lead to interesting speculations as to the roles these antigens play

in the pathogenesis of *Shigella*. The primary function of these molecules in facilitating invasion can not be disputed (Maurelli, et al., 1985). The way in which they produce this effect however, remains unclear. If the finding that IpaB and IpaC are excreted *in vitro* can be extended to the *in vivo* situation, then one may envision cell-free *ipa* proteins as possible inducers of phagocytosis of the bacteria which are in close proximity to the host epithelial cells without the organisms actually having to contact these cells. This hypothesis is supported by the observation that an unknown soluble factor in culture supernatants from wild-type *Shigella* is able to facilitate uptake by HeLa cells of a non-invasive strain (Osada and Ogawa, 1977). In this study however, Ipa-enriched culture supernatants failed to restore the invasive phenotype to an *S. flexneri mxi* mutant. Thus, it is possible that Ipa proteins in cell-free form are not the only factors required for efficient uptake of the bacterium into their host cells. Moreover, the levels of Ipa that were added may have been insufficient to induce uptake of the mutant. Despite the difficulty involved in interpreting these results, findings with the *mxiA* transformant of BS183 (*mxiA*$^-$ *mxiC*$^-$) support a role for cell-free IpaC in invasion (see above).

Alternatively, if low levels of *ipa* proteins exist on the bacterial cell surface, they may function as ligands to a host cell receptor which, once bound, induce phagocytosis. This hypothesis implies that a dual role for the *ipa* gene products may exist in which the cell-free form of the antigens provides the pathogen with some other asset for establishing infection. One intriguing hypothesis is that IpaB and IpaC may be excreted in the host extracellular environment as a means to adsorb neutralizing secretory immunoglobulin directed against the bacteria. This type of "immune avoidance" strategy has, in fact, not only been reported for Gram positive pathogens (Mims, 1988) but also may be utilized by Gram negative microorganisms as well. *Neisseria gonorrhoeae* has been shown to release membrane blebs which contain several outer membrane proteins in addition to LPS (Pettit and Judd, 1992a). These blebs were demonstrated to bind

serum immunoglobulin, and it was suggested that this phemonenon may contribute to the serum resistance of some strains (Pettit and Judd, 1992b). Although no evidence exists that *Shigella* produces similar blebs, it is conceivable that low level excreted Ipa may act as an immunological "decoy" which would allow the bacteria to establish infection without interference from a host secretory immunoglobulin response. An unusual finding in *S. typhimurium* is that a normally structural flagellar component (hook protein) continues to be excreted even though flagella are completely assembled on the surface of the bacteria (Homma and Iino, 1985). Although a dual structural/immunological role of this protein has yet to be found, it is interesting to speculate that immune avoidance mechanisms of this type may exist in other Gram negative enteric pathogens.

A role for excreted Ipa in the host intracellular environment also can not be discounted. In a recent report, high immunological cross-reactivity was demonstrated between epitopes of IpaB and myosin, a host protein which is a component of actin filaments (Oaks and Turbyfill, 1992). In this regard, it is entirely possible that a host intracellular role of IpaB may be related to the VirG-independent movement of the bacteria along myosin-containing actin cables within the host cell (Vasselon, et al., 1991). Additionally, IpaB has been demonstrated to be necessary for the rapid lysis of the host vacuole with the subsequent escape of the bacteria into the host cytoplasm (High, et al., 1992). An immediate requirement, therefore, may exist for higher levels of excreted cytolytic IpaB once the bacteria have entered their host cells.

Regulation of Ipa excretion.

Although Ipa proteins may also have a role in virulence once inside the host cell, recent work by Headley and Payne (1990) indicated that *de novo* synthesis of IpaB and IpaC does not occur after the bacteria have invaded. However, because findings in this investigation indicate that high intracellular pools of the antigens can already exist in

the bacteria (in the absence of host-specified induction signals), the need for continued synthesis of these antigens may not be necessary once the bacteria have invaded. It is conceivable, therefore, that host-specified signals modulate the excretion of preexisting intracellular *ipa* proteins - the "bee venom" hypothesis. This hypothesis presents two possibilities for the induction of Ipa excretion. The first scenario is that pre-existing antigenic pools are exported in response to a signal upon contact of the bacteria with the host cell. The second is that a host-specified intracellular signal stimulates excretion of the bacterial antigens after the bacteria have invaded. Experiments to address the general concept of the "bee venom" hypothesis were conducted and yielded some interesting findings. The ability of Congo red to bind virulent strains of *Shigella* was extended in a report which describes the dye-induced elevation of expression of three proteins associated with the inner membrane, two of which correspond to the molecular weights of IpaB and IpaC (Sankaran, et al., 1989). The third species, an uncharacterized 53 kDa protein, was also significantly elevated by Congo red. Experiments conducted in this laboratory to look specifically at the effects of the dye on Ipa export showed that while overall intracellular levels of the antigens were not significantly effected, levels of excreted IpaB and IpaC from the dye-treated bacteria were significantly higher than the untreated control. This effect appeared to be specific for IpaB, IpaC, excreted proteins corresponding to molecular weights of IpaA and IpaD, and two lower molecular weight species of 22 kDa and 17 kDa (see below). It is possible, therefore that the previously reported increase in synthesis of certain inner membrane proteins in *S. flexneri* in the presence of the dye may represent a shift in localization of pre-existing protein and/or more efficient translocation of newly synthesized protein from cytoplasm to inner membrane, ultimately destined for export. IpaB and IpaC have, in fact, been shown to be associated with both the cytoplasm and inner membrane during normal *in vitro* growth conditions (Andrews, et al., 1991).

The mechanism of Congo red binding to various bacterial pathogens is unclear, although it has been suggested that the dye may bind at the inner membrane and may even be found in the cytoplasm (K. Sankaran, personal communication). If this is the case, then it could be that Congo red binds directly to an inner membrane protein or perturbs the membrane around the protein which may alter its activity in a fashion similar to certain phenolic compounds or hydrophobic amino acids binding to and modulating the activity of chemotactic receptors (Tsang, et al., 1973; Tso and Adler, 1974). In this regard, it is possible that MxiA or a gene product associated with MxiA is functionally modulated by the dye either directly or indirectly. Congo red binding to the *Shigella* outer membrane has also been reported (Daskalaros and Payne, 1989). If this is the case, than the "signal" induced by the binding of the dye may be transmitted across the bacterial envelope through a complex of membrane proteins. If one assumes a regulatory role for MxiA, then it may function directly as a part of this regulatory complex or may trans-activate expression of genes specifying components of this complex (Figure 27c).

As implied above, the ability of Congo red dye to modulate the Ipa excretion phenotype of *S. flexneri* may be mimicking events that immediately precede (contact) or follow (intracellular spread) invasion *in vivo*. A study by Headley and Payne (1990) indicated that a 58 kDa protein, which is not synthesized under in vitro growth conditions (absence of host cells), appears to be induced during the attachment and invasion steps of *S. flexneri* on HeLa cell monolayers. This species may, in fact, be identical to the 53 kDa Congo red-induced inner membrane protein described by Sankaren et al. (1989), which was not found in culture supernatants (this study). Therefore, it is possible that this species represents a cell-associated protein whose expression and/or localization is effected by Congo red as well as an unidentified component of HeLa cells. Headley and Payne (1990) also found that two new proteins of 25 kDa and 18 kDa appear only during intracellular multiplication of the bacteria in HeLa cells. These species correspond closely to the molecular weights of two new proteins which were detected in

culture supernatants of Congo red-induced wild-type *Shigella* in this study. It is interesting to note that two uncharacterized ORFs (24 kDa, Sasakawa, et al., 1989, and *ippI*, Baudry, et al., 1988) immediately upstream of *ipaBCDA* specify polypeptides resembling the molecular weights of these two induced proteins. In addition to the appearance of new proteins, polypeptides of 97 kDa, 80 kDa (IpaA), 62 kDa, and 50 kDa made *in vitro* are upregulated during infection of HeLa cells (Headley and Payne, 1990). In this study, only one of these species (IpaA) appeared to be excreted upon induction of the wild-type bacteria by Congo red. Thus, the 97 kDa, 62 kDa, and 50 kDa proteins may be cell-associated, in addition to the 53 kDa species. An examination of membrane fractions was not conducted in this study, so alteration of these proteins by Congo red would not have been observed. Alternatively, these proteins may represent species which are simply uneffected by Congo red, regardless of their location in the bacteria.

Considering the hypothesis that Congo red may be mimicking a host cell component, induction of Ipa excretion by HeLa cells was examined. Wild-type bacteria incubated with the HeLa cell extracts for two hours stimulated excretion of both IpaB and IpaC to levels significantly above the control (no extract added). These results suggested that a host cell factor or factors may exist which triggers excretion of at least two of the Ipa proteins. It is entirely possible that some or all of the pre-existing bacterial proteins described by Headley and Payne (1990) which are upregulated within HeLa cells may be involved in the export of IpaB and IpaC (*i.e.*, the *mxi* machinery).

Interestingly, Sankaren and coworkers (1989) concluded that IpaC synthesis is upregulated in response to a HeLa cell factor since intracellular bacteria are able to be surface-immunolabelled with anti-Ipa antibody. As mentioned above, Headley and Payne (1990) demonstrated by [^{35}S]-methionine incorporation that *de novo* synthesis of IpaC (as well as IpaB and IpaD) is shut down once the bacteria penetrate their host cells. It is possible, therefore, that the observations in the former experiments were the result of preexisting intracellular Ipa pools being excreted. Thus, these findings lend support to a

HeLa cell factor-induced synthesis of proteins involved in export of Ipa rather than significant upregulation of synthesis of the Ipa polypeptides themselves. Furthermore, taken together, the results of the Congo red and HeLa induction experiments suggest that the host signal(s) which upregulates expression of certain bacterial proteins (*e.g.*, Mxi) may not be the same signal(s) which downregulates other proteins (*e.g.*, Ipa).

Finally, the question arises as to exactly what Congo red is mimicking within the host cell. Evidence that the dye may be resembling an iron-binding porphyrin comes from the work of Daskalaros and Payne (1987) who observed that strains of *Shigella* which do not bind Congo red (Crb^-) also do not bind hemin. Furthermore, Congo red competes with hemin for the same site on cells of *S. flexneri*, which strongly suggests that these compounds bind to the same bacterial receptor. This finding is interesting in light of the fact that the availability of host intracellular iron is severely limited and that it exists mostly complexed with iron-binding compounds (Finkelstein, et al., 1983).

Curiously, the ability of the bacteria to bind hemin is not related to its ability to utilize the compound as a sole iron source, even in the absence of the production of the siderophore, aerobactin (Daskalaros and Payne, 1987). Also, the addition of a HeLa cell lysate to iron-depleted media is able to restore growth of both Crb^+ and Crb^- aerobactin mutants. Therefore, the ability of the bacteria to bind certain porphyrins *in vivo* does not correlate with their ability to aquire iron from them (Daskaleros and Payne, 1987). Another question then arises as to the exact function of hemin binding on the bacterial surface. Interestingly, bacteria prebound with either hemin or Congo red are able to invade HeLa cell monolayers at significantly higher efficiency than untreated bacteria (Daskaleros and Payne, 1987). Conclusions drawn from these observations suggested that surface-to-surface interactions between *Shigella* and the host HeLa cells are enhanced by porphyrin-binding, hence invasion efficiency is increased. Thus, a similar situation may occur *in vivo* in which bacteria in the gut lumen bind hemin or hemin-like molecules, present in partially digested food, which then enhances their

interaction with host target tissue by the binding of the bacteria-hemin complex to a host hemin uptake receptor (Daskalaeros and Payne, 1987). Based on the data obtained in this study, however, an alternate hypothesis can be proposed. Porphryin-binding to the bacteria may represent a signaling mechanism by which virulence factor export accessory proteins are upregulated, which consequently results in the increased excretion of critical antigens. Thus, if invasion is enhanced by hemin in the gut lumen, then it may be the result of increased levels of excreted Ipa proteins rather than direct interaction of porphyrin receptors on host tissue with hemin-bound bacteria. Additionally, the "porphryin-signaling" hypothesis does not constrain the hemin-binding phenomenon to just the invasion step of *Shigella* pathogenesis. The signaling cue may occur not only in the gut lumen but in the host intracellular environment where several iron-binding compounds are known to be present. In fact, stimulation of virulence factor excretion by iron-binding compounds may occur predominantly in the host intracellular environment where these compounds might be expected to be more concentrated.

Finally, the possibility that Congo red may be mimicking something other than a porphryin can not be ruled out. Prebinding *Shigella* with hemin does not completely inhibit Congo red binding, which suggests that Congo red may also bind an alternate site on the bacteria (Daskaleros and Payne, 1987). This alternate site, whether it is on the outer membrane or inner membrane, may represent a signal transducer which responds to an entirely different environmental stimulus.

Summary.

The mechanisms for secretion of virulence factors from Gram negative pathogens are quite complex and usually involve more than one accessory protein (Holland, et al., 1990; Lazdunski, et al., 1990; Lindberg, et al., 1989; Pugsley et al., 1990). The conclusions drawn from this study indicate that *Shigella* is another Gram negative

intestinal pathogen which employs a complex, multi-component secretion mechanism for at least some of its virulence factors. The *mxiA* locus is the first of these accessory loci of *S. flexneri* to be identified as playing a pivotal role in conferring the virulent phenotype to the organism by controlling export of Ipa polypeptides. Further clarification of this role, in addition to the characterization of the *mxiC* gene product and the locus defined by BS230, will contribute significantly to the formulation of a model for export of *Shigella* virulence factors.

Evidence obtained from the experiments in this study strongly suggests that at least two of the invasion plasmid antigens, IpaB and IpaC, are truly excreted antigens, and not predominantly cell surface-associated as was previously suggested. These findings have initiated a reevaluation of the role these antigens play in the pathogenesis of *S. flexneri* and the specific mechanisms by which they facilitate the establishment of this pathogen within its host.

LITERATURE CITED

Adler, B., C. Sasakawa, T. Tobe, S. Makino, K. Komatsu, and M. Yoshikawa. 1989. A dual transcriptional activation system for the 230 kb plasmid genes coding for virulence-associated antigens of *Shigella flexneri*. Mol. Microbiol. **3**:627-635.

Akita, M., S. Sasaki, S. Matsuyama, and S. Mizushima. 1990. SecA interacts with secretory proteins by recognizing the positive charge at the amino terminus of the signal peptide in *Escherichia coli*. J. Biol. Chem. **265**:8164-8169.

Albertini, A. M., T. Caramori, W. D. Crabb, F. Scoffone, and A. Galizzi. 1991. The *fla* locus of *Bacillus subtilis* is part of a large operon coding for flagellar structures, motility functions, and an ATPase-like polypeptide. J. Bacteriol. **173**:3573-3579.

Allaoui, A., P. J. Sansonetti, and C. Parsot. 1992. MxiD, an outer membrane protein necessary for the secretion of the *Shigella flexneri* Ipa invasins, belongs to the PulD family of secretory proteins. Submitted.

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

Andrews, G. P., and A. T. Maurelli. 1992. *mxiA* of *Shigella flexneri* 2a, which facilitates export of invasion plasmid antigens, encodes a homolog of the low calcium response protein, LcrD, of *Yersinia pestis*. Infect. Immun. **60**:3287-3295.

Bally, M., A. Filloux, M. Akrim, G. Ball, A. Lazduski, and J. Tommassen. 1992. Protein secretion in *Pseudomonas aeruginosa*: characterization of seven *xcp* genes and processing of secretory apparatus components by prepilin peptidase. Mol. Microbiol. **6**:1121-1131.

Bartlett, D. H., B. B. Frantz, and P. Matsumura. 1988. Flagellar transcriptional activators FlbB and FlaI: gene sequences and 5' consensus sequences of operons under FlbB and FlaI control. J. Bacteriol. **170**:1575-1581.

Baudry, B., M. Kaczorek, and P. J. Sansonetti. 1988. Nucleotide sequence of the invasion plasmid antigen B and C genes (*ipaB* and *ipaC*) of *Shigella flexneri*. Microbial Pathogen. **4**:345-357.

Baudry, B., A. T. Maurelli, P. Clerc, J. C. Sadoff, and P. J. Sansonetti. 1987. Localization of plasmid loci necessary for the entry of *Shigella flexneri* into HeLa cells and characterization of one locus encoding four immunogenic polypeptides. J. Gen. Microbiol. **133**:3403-3413.

Bayer, M. E. 1968. Areas of adhesion between wall and membrane of *Escherichia coli*. J. Gen. Microbiol. **53**:395-404.

Bennish, M. L., J. R. Harris, B. J. Wajtyniok, and M. Strueleos. 1990. Death in shigellosis: incidence and risk factors in hospitalized patients. J. Infect. Dis. **161**:500-506.

Berman, M. L., D. E. Jackson, A. Fowler, I. Zabin, L. Christensen, N. P. Fiil, and M. N. Hall. 1984. Gene fusion techniques: cloning vectors for manipulating *lacZ* gene fusions. Gene. Anal. Tech. **1**:43-51.

Bernardini, M. L., J. Mounier, H. d'Hauteville, M. Coquis-Rondon, and P. J. Sansonetti. 1989. Identification of *icsA*, a plasmid locus of *Shigella flexneri* that governs bacterial intra- and intercellular spread through interaction with F-actin. Proc. Natl. Acad. Sci. USA. **86**:3867-3871.

Birnboim, H. C., and J. Doly. 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7**:1513-1523.

Blight, M. A., and I. B. Holland. 1990. Structure and function of haemolysin B, P-glycoprotein and other members of a novel family of membrane translocators. Mol. Microbiol. **4**:873-880.

Bremer, E., T. J. Silhavy, J. M. Weisemann, and G. M. Weinstock. 1984. λp*lac*Mu: a transposable derivative of bacteriophage lambda for creating *lacZ* protein fusions in a single step. J. Bacteriol. **158**:1084-1093.

Buysse, J. M., C. K. Stover, E. V. Oaks, M. Venkatesan, and D. Kopecko. 1987. Molecular cloning of invasion plasmid antigen (*ipa*) genes from *Shigella flexneri*: analysis of *ipa* gene products and genetic mapping. J. Bacteriol. **169**:2561-2569.

Buysse, J. M., M. Venkatesan, J. A. Mills, and E. V. Oaks. 1990. Molecular characterization of a trans-acting, positive effector (*ipaR*) of invasion plasmid antigen synthesis in *Shigella flexneri* serotype 5. Microbial Pathogen. **8**:197-211.

Clerc, P., and P. J. Sansonetti. 1987. Entry of *Shigella flexneri* into HeLa cells: evidence for directed phagocytosis involving actin polymerization and myosin accumulation. Infect. Immun. **55**:2681-2688.

Csonka, L. N. 1989. Physiological and genetic responses of bacteria to osmotic stress. Microbiol. Rev. **53**:124-147.

Dagert, M., and S. D. Erlich. 1979. Prolonged incubation in calcium chloride improves the competence of *Escherichia coli* cells. Gene **6**:23-28.

Daskalaros, P. A., and S. M. Payne. 1985. Cloning the gene for Congo red binding in *Shigella flexneri*. Infect Immun. **48**:165-168.

Daskalaros, P. A., and S. M. Payne. 1986. Characterization of *Shigella flexneri* sequences encoding Congo red binding (*crb*): conservation of multiple *crb* sequences and role of IS*1* in loss of the Crb^+ phenotype. Infect. Immun. **54**:435-443.

Daskalaros, P. A., and S. M. Payne. 1987. Congo red binding phenotype is associated with hemin binding and increased infectivity of *Shigella flexneri* in the HeLa cell model. Infect. Immun. **55**:1393-1398.

Davis, R. W., D. Botstein, and J. R. Roth. 1980. A manual for genetic engineering: advanced bacterial genetics. Cold Spring Harbor Laboratories, Cold Spring Harbor, N.Y.

Deidrich, D. K., A. O. Summers, and C. A. Schnaitman. 1977. Outer membrane proteins of *Escherichia coli*. V. Evidence that Protein 1 and bacteriophage-directed protein 2 are different polypeptides. J. Bacteriol. **131**:598-607.

d'Enfert, C., A. Ryter, and A. P. Pugsley. 1987. Cloning and expression in *Escherichia coli* of the *Klebsiella pneumoniae* genes for production, surface localization and secretion of the lipoprotein pullulanase. EMBO J. **6**:3531-3538.

Devereux, J., P. Haeberli, and O. Smithies. 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. **12**:387-407.

Donohue-Rolfe, A., and G. T. Keusch. 1983. *Shigella dysenteriae* 1 cytotoxin: periplasmic protein releasable by polymyxin B and osmotic shock. Infect Immun. **39**:270-274.

Dupont, H. L., M. M. Levine, R. B. Hornick, and S. B. Formal. 1989. Inoculum size in shigellosis and implications for expected mode of transmission. J. Infect. Dis. **159**:1126-1128.

Finkelstein, R. A., C. V. Sciortino, and M. A. McIntosh. 1983. Role of iron in microbe-host interactions. Rev. Inf. Dis. **5**:S759-S777.

Flanagan, J. L., L. Ventra, and A. S. Weiss. 1989. Rapid method for preparation and cleavage of bacterial DNA for pulsed-field gel electrophoresis. Nucleic Acids Res. **17**:814.

Fontaine, A., J. Arondel, and P. J. Sansonetti. 1988. Role of *Shiga* toxin in the pathogenesis of bacillary dysentery studied by using a Tox$^-$ mutant of *Shigella dysenteriae* 1. Infect. Immun. **56**:3099-3109.

Formal, S. B., G. J. Dammin, E. H. LaBrec, and H. Schneider. 1958. Experimental *Shigella* infections: characteristics of a fatal infection produced in guinea pigs. J. Bacteriol. **75**:604-610.

Forsberg, A., A.-M. Viitanen, M. Skurnik, and H. Wolf-Watz. 1991. The surface-located YopN protein is involved in calcium signal transduction in *Yersinia pseudotuberculosis*. Mol. Microbiol. **5**:977-986.

Galan, J. E., C. Ginocchio, and P. Costeas. 1992. Molecular and functional characterization of the *Salmonella* invasion gene *invA*: homology of InvA to members of a new protein family. J. Bacteriol. **174**:4338-4349.

Gilman, M. Z., and M. J. Chamberlin. 1983. Developmental and genetic regulation of *Bacillus subtilis* genes transcribed by σ^{28} RNA polymerase. Cell **35**:285-293.

Goguen, J. D., J. Yother, and S. C. Straley. 1984. Genetic analysis of the low calcium response in *Yersinia pestis* Mu d1 (Ap^r *lac*) insertion mutants. J. Bacteriol. **160**:842-848.

Griffen, D. E., and P. Gemski. 1983. Release of Shiga toxin from *Shigella dysenteriae* 1 by polymyxin B. Infect. Immun. **40**:425-428.

Hale, T. L., and S. B. Formal. 1981. Protein synthesis in HeLa or Henle 407 cells infected with *Shigella dysenteriae* 1, *Shigella flexneri* 2a, or *Salmonella typhimurium* W118. Infect. Immun. **32**:137-144.

Hale, T. L., E. V. Oaks, and S. B. Formal. 1985. Identification and antigenic characterization of virulence-associated, plasmid-coded proteins of *Shigella* spp. and enteroinvasive *Escherichia coli*. Infect. Immun. **50**:620-629.

Hale, T. L., P. J. Sansonetti, P. A. Schad, S. Austin, and S. B. Formal. 1983. Characterization of virulence plasmids and plasmid-associated outer membrane proteins in *Shigella flexneri*, *Shigella sonnei*, and *Escherichia coli*. Infect. Immun. **40**:340-350.

Hanahan, D. 1985. Techniques for transformation of *E. coli*, p. 109-135. *In* D. M. Glover (ed.), *DNA cloning, vol. 1*. IRL Press, Oxford.

Hartman, A. B., M. Venkatesan, E. V. Oaks, and J. M. Buysse. 1990. Sequence and molecular characterization of a multicopy invasion plasmid antigen gene, *ipaH*, of *Shigella flexneri*. J. Bacteriol. **172**:1905-1915.

Hawley, D. K., and W. R. McClure. 1983. Compilation and analysis of *Escherichia coli* promoter DNA sequences. Nucleic Acids Res. **11**:2237-2255.

Headley, V. L. and S. M. Payne. 1990. Differential protein expression by *Shigella flexneri* in intracellular and extracellular environments. Proc. Natl. Acad. Sci. USA **87**:4179-4183.

Helmann, J. D., and M. J. Chamberlin. 1987. DNA sequence analysis suggests that expression of flagellar and chemotaxis genes in *Escherichia coli* and *Salmonella typhimurium* is controlled by an alternate σ factor. Proc. Natl. Acad. Sci. USA. **84**:6422-6424.

Helmann, J. D., L. M. Marquez, and M. J. Chamberlin. 1988. Cloning, sequencing, and disruption of the *Bacillus subtilis* σ^{28} gene. J. Bacteriol. **170**:1568-1574.

High, N., J. Mounier, M. C. Prevost, and P. J. Sansonetti. 1992. IpaB of *Shigella flexneri* causes entry into epithelial cells and escape from the phagocytic vacuole. EMBO J. **11**:1991-1999.

Highlander, S. K., M. J. Engler, and G. M. Weinstock. 1990. Secretion and expression of the *Pasteurella haemolytica* leukotoxin. J. Bacteriol. **172**:2343-2350.

Higuchi, K., I. L. Kupferburg, and J. L. Smith. 1959. Studies on the nutrition and physiology of *Pasteurella pestis.* III. Effects of calcium ions on the growth of virulent and avirulent strains of *Pasteurella pestis*. J. Bacteriol. **77**:317-321.

Hirst, T. R., J. Sanchez, J. B. Kaper, S. J. S. Hardy, J. Holmgren. 1984. Mechanism of toxin secretion by *Vibrio cholerae* investigated in strains harboring plasmids that encode heat-labile enterotoxins of *Escherichia coli.* Proc. Nat. Acad. Sci. USA **81**:7752-7756.

Hirst, T. R., and R. A. Welch. 1988. Mechanisms for secretion of extracellular proteins by Gram-negative bacteria. Trends Biochem. Sci. **13**:265-269.

Holland, I. B., B. Kenny, and M. Blight. 1990. Haemolysin secretion from *E. coli*. Biochimie. **72**:131-141.

Homma, M., and T. Iino. 1985. Excretion of unassembled hook-associated proteins by *Salmonella typhimurium*. J. Bacteriol. **164**:1370-1372.

Hromockyj, A. E. 1991. Temperature regulation of *Shigella* virulence: identification of temperature-regulated *Shigella* virulence genes by the isolation of *inv::lacZ* operon fusions and characterization of the virulence gene regulator *virR*. Ph.D. Dissertation. Uniformed Services University of the Health Sciences, Bethesda, MD.

Hromockyj, A. E. and A. T. Maurelli. 1989. Identification of *Shigella* invasion genes by isolation of temperature-regulated *inv::lacZ* operon fusions. Infect. Immun. **57**:2963-2970.

Hromockyj, A.E., S.C. Tucker, and A.T. Maurelli. 1992. Temperature-regulation of *Shigella* virulence: Identification of the repressor *virR*, an analog of *hns*, and partial complementation by tyrosyl transfer RNA ($tRNA_1^{Tyr}$). Mol. Microbiol. **6**:2113-2124

Ito, K., T. Nakajima, T. Sasaki, and H. Watanabe. 1991. Localization of IpaB protein in *Escherichia coli* K-12 MC1061 strain carrying *Shigella sonnei* Form I plasmid pSS120. Microbiol. Immunol. **35**:335-341.

Kato, J., K. Ito, A. Nakamura, and H. Watanabe. 1989. Cloning of regions required for contact hemolysis and entry into LLC-MK2 cells from *Shigella sonnei* Form

I plasmid: *virF* is a positive regulator gene for these phenotypes. Infect. Immun. **57**:1391-1398.

Kellenberger, E. 1990. The "Bayer bridges" confronted with results from improved electron microscopy methods. Mol. Microbiol. **4**:697-705.

Kozak, M. 1983. Comparison of initiation of protein synthesis in procaryotes, eukaryotes, and organelles. Microbiol. Rev. **47**:1-45.

Kumamoto, C. A. 1991. Molecular chaperones and protein translocation across the *Escherichia coli* inner membrane. Mol. Microbiol. **5**:19-22.

LaBrec, E. H., H. Schneider, T. K. Magnani, and S. B. Formal. 1964. Epithelial cell penetration as an essential step in the pathogenesis of bacillary dysentery. J. Bacteriol. **145**:1365-1373.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head bacteriophage T4. Nature. **227**:680-685.

Lawlor, K., P. A. Daskaleros, R. E. Robinson, and S. M. Payne. 1987. Virulence of iron transport mutants of *Shigella flexneri* and utilization of host iron compounds. Infect. Immun. **55**:594-599.

Lazdunski, A., J. Guzzo, A. Filloux, M. Bally, and M. Murgier. 1990. Secretion of extracellular proteins by *Pseudomonas aeruginosa*. Biochimie **72**:147-156.

Lett, M.-C., C. Sasakawa, N. Okada, T. Sakai, S. Makino, M. Yamada, K. Komatsu, and M. Yoshikawa. 1989. *virG*, a plasmid-encoded virulence gene of *Shigella flexneri*: identification of the *virG* protein and determination of the complete coding sequence. J. Bacteriol. **171**:353-359.

Leung, K. Y., B. S. Reisner, and S. Straley. 1990. YopM inhibits platelet aggregation and is necessary for virulence of *Yersinia pestis* in mice. Infect. Immun. **58**:3262-3271.

Lindburg, F., J. M. Tennent, S. J. Hultgren, B. Lund, and S. Normark. 1989. PapD, a periplasmic transport protein in P-pilus biogenesis. J. Bacteriol. **171**:6052-6058.

Macnab, R. M., 1987. Motility and Chemotaxis. p. 742. *In*, F.C. Neidhardt (ed.), *Escherichia coli* and *Salmonella typhimurium*: Cellular and Molecular Biology. American Society for Microbiology, Washington, DC.

Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

Maurelli, A. T., B. Baudry, H. d'Hauteville, T. L. Hale, and P. J. Sansonetti. 1985. Cloning of virulence plasmid DNA sequences involved in invasion of HeLa cells by *Shigella flexneri*. Infect. Immun. **49**:164-171.

Maurelli, A. T., Б. Blackmon, and R. Curtiss III. 1984a. Temperature-dependent expression of virulence genes in *Shigella* species. Infect. Immun. **43**:195-201.

Maurelli, A. T., B. Blackmon, and R. Curtiss III. 1984b. Loss of pigmentation in *Shigella flexneri* 2a is correlated with loss of virulence and virulence-associated plasmid. Infect. Immun. **43**:397-401.

Maurelli, A. T., and R. Curtiss III. 1984. Bacteriophage Mu*d*1 (Ap^r *lac*) generates *vir-lac* operon fusions in *Shigella flexneri* 2a. Infect. Immun. **45**:642-648.

Maurelli, A. T., A. E. Hromockyj, and M. L. Bernardini. 1992. Environmental regulation of *Shigella* virulence. Curr. Top. Microbiol. Immunol. **180**:95-116.

Maurelli, A. T., and P. J. Sansonetti. 1988. Identification of a chromosomal gene controlling temperature-regulated expression of *Shigella* virulence. Proc. Natl. Acad. Sci. USA **85**: 2820-2824.

McIver, J., G. F. Grady, and G. T. Keusch. 1975. Production and characterization of exotoxin(s) of *Shigella dysenteriae* type 1. J. Infect. Dis. **131**:559-566.

Michiels, T., P. Watliau, R. Brasseur, J. Ruysschaert, and G. Cornelis. 1990. Secretion of Yop proteins by *Yersiniae*. Infect. Immun. **58**:2840-2849.

Miller, J. H. 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

Miller, J. F., J. J. Mekalanos, and S. Falkow. 1989. Coordinate regulation and sensory transduction in the control of bacterial virulence. Science **243**:916-922.

Mills, J. A., J. M. Buysee, and E. V. Oaks. 1988. *Shigella flexneri* invasion plasmid antigens B and C: Epitope location and characterization with monoclonal antibodies. Infect. Immun. **56**:2933-2941.

Mims, C. A., 1988. p. 167-168. *In* The Pathogenesis of Infectious Disease. Academic Press, London.

Miyazaki, H., N. Yanagida, S. Horinouchi, and T. Beppu. 1989. Characterization of the precursor of *Serratia marcescens* serine protease and COOH-terminal processing of the precursor during its excretion through the outer membrane of *Escherichia coli*. *J. Bacteriol.* **171**:6566-6572.

Neill, R. J., B. E. Ivins, and R. K. Holmes. 1983. Synthesis and secretion of the plasmid-coded heat-labile enterotoxin of *Escherichia coli* in *Vibrio cholerae*. Science **221**:289-291.

Nikaido, H., and M. Vaara. 1985. Molecular basis of bacterial outer membrane permeability. Microbiol. Rev. **49**:1-32.

Oaks, E. V., T. L. Hale, and S. B. Formal. 1986. Serum immune response to *Shigella* protein antigens in Rhesus monkeys and humans infected with *Shigella* spp. Infect. Immun. **53**:57-63.

Oaks, E. V., and K. R. Turbyfill. 1992. Myosin-cross-reactive epitope of *Shigella flexneri* invasion plasmid antigen B. Infect. Immun. **60**:557-564.

Oaks, E. V., M. E. Wingfield, and S. B. Formal. 1985. Plaque formation by virulent *Shigella flexneri*. Infect. Immun. **48**:124-129.

O'Brien, A. D., and R. K. Holmes. 1987. Shiga and Shiga-like toxins. Microbiol. Rev. 1987. **51**:206-220.

Obrig, T. G., P. J. Del Vecchio, J. E. Brown, T. P. Moran, B. M. Rowland, T. K. Judge, and S. W. Rothman. 1988. Direct cytotoxic action of Shiga toxin on human vascular endothelial cells. Infect. Immun. **56**:2373-2378.

Osada, Y. and H. Ogawa. 1977. Phagocytosis stimulation by an extracellular product of virulent *Shigella flexneri* 2a. Microbiol. Immunol. **21**:49-55.

Pages, J. M., J. M. Bolla, A. Bernadac, and D. Fourel. 1990. Immunological approach to assembly and topology of OmpF, an outer membrane protein of *Escherichia coli*. Biochimie **72**:169-176.

Pal, T. and T. L. Hale. 1989. Plasmid-associated adherence of *Shigella flexneri* in a HeLa cell model. Infect. Immun. **57**:2580-2582.

Pal, T., S. Pasca, L. Emody, S. Voros, and E. Selley. 1985. Modified enzyme-linked immunoabsorbant assay for detecting enteroinvasive *Escherichia coli* and virulent *Shigella* strains. J. Clin. Micobiol. **21**:415-418.

Payne, S. M., and R. A. Finkelstein. 1977. Detection and differentiation of iron-responsive avirulent mutants on Congo red agar. Infect. Immun. **18**:94-98.

Pettit, R. K., and R. C. Judd. 1992a. Characterization of naturally elaborated blebs from serum-susceptible and serum-resistant strains of *Neisseria gonorrhoeae*. Mol. Microbiol. **6**:723-728.

Pettit, R. K., and R. C. Judd. 1992b. The interaction of naturally elaborated blebs from serum-susceptible and serum-resistant strains of *Neisseria gonorrhoeae* with normal human serum. Mol. Microbiol. **6**:729-734.

Plano, G. V., S. S. Barve, and S. C. Straley. 1991. LcrD, a membrane-bound regulator of the *Yersinia pestis* low-calcium response. J. Bacteriol. **173**:7293-7303.

Pohlner, J., R. Halter, K. Beyreuther, and T. F. Meyer. 1987. Gene structure and extracellular secretion of *Neisseria gonorrhoeae* IgA protease. Nature. **325**:458-462.

Pugsley, A. P., C. d'Enfert, I. Reyss, and M. G. Kornacker. 1990. Genetics of extracellular protein secretion by Gram-negative bacteria. Annu. Rev. Genet. **24**:67-90.

Pugsley, A. P., and M. Schwartz. 1984. Colicin E2 release: lysis, leakage or secretion? Possible role of a phospholipase. EMBO J. **3**:2393-2397.

Ramakrishnan, G., J.-L. Zhao, and A. Newton. 1991. The cell cycle-regulated flagellar gene *flbF* of *Caulobacter crescentus* is homologous to a virulence locus (*lcrD*) of *Yersinia pestis*. J. Bacteriol. **173**:7283-7292.

Saier, M. H., P. K. Werner, and M. Muller. 1989. Insertion of proteins into bacterial membranes: mechanism, characteristics, and comparisons with the eukaryotic process. Microbiol. Rev. **53**:333-366.

Sakai, T., C. Sasakawa, C. Makino, K. Kamata, and M. Yoshikawa. 1986a. Molecular cloning of a genetic determinant for Congo red binding ability which is essential for the virulence of *Shigella flexneri*. Infect. Immun. **51**:476-482

Sakai, T., C. Sasakawa, S. Makino, and M. Yoshikawa. 1986b. DNA sequence and product analysis of the *virF* locus responsible for Congo red binding and cell invasion in *Shigella flexneri* 2a. Infect. Immun. **54**:395-402.

Sanders, L. A., S. V. Way, and D. A. Mullin. 1992. Characterization of the *Caulobacter crescentus* FlbF product as a homolog of the LcrD protein from a *Yersinia enterocolitica* virulence plasmid. J. Bacteriol. **174**:857-866.

Sankaran, K., V. Ramachandran, Y. V. B. K. Subrahmanyam, S. Rajarathnam, S. Elango, and R.K. Roy. 1989. Congo red-mediated regulation of levels of *Shigella flexneri* 2a membrane proteins. Infect. Immun. **57**:2364-2371.

Sansonetti, P. J., D. J. Kopecko, and S. B. Formal. 1981. *Shigella sonnei* plasmids: evidence that a large plasmid is necessary for virulence. Infect. Immun. **34**:75-83.

Sansonetti, P. J., D. J. Kopecko, and S. B. Formal. 1982. Involvement of a plasmid in the invasive ability of *Shigella flexneri*. Infect. Immun. **35**:852-860.

Sansonetti, P. J., A. Ryter, P. Clerc, A. T. Maurelli, and J. Mounier. 1986. Multiplication of *Shigella flexneri* within HeLa cells: lysis of the phagocytic vacuole and plasmid-mediated contact hemolysis. Infect. Immun. **51**:461-469.

Sasakawa, C., B. Adler, T. Tobe, N. Okada, S. Nagai, K. Komatsu, and M. Yoshikawa. 1989. Functional organization and nucleotide sequence of virulence region-2 on the large virulence plasmid in *Shigella flexneri* 2a. Mol. Microbiol. **3**:1191-1201.

Schatz, P. J., and J. Beckwith. 1990. Genetic analysis of protein export in *Escherichia coli*. Annu. Rev. Genet. **24**:215-248.

Schiebel, E., H. Schwarz, and V. Braun. 1989. Subcellular location and unique secretion of the hemolysin of *Serratia marcescens*. J. Biol. Chem. **264**:16311-16320.

Scott, W. A. 1975. Glucose-6-phophate dehydrogenase from *Neurospora crassa*. p. 177-182. *In* W. A. Wood (ed.), *Methods in Enzymology, Vol. XLII*. Academic Press, New York.

Sekizaki, T., S. Harayama, G. M. Brazil, and K. N. Timmis. 1987. Localization of *stx*, a determinant essential for high-level production of Shiga toxin by *Shigella dysenteriae* 1, near *pyrF* and generation of *stx* transposon mutants. Infect. Immun. **55**:2208-2214.

Sereny, B. 1955. Experimental *Shigella* conjunctivitis. Acta Microbiol. Acad. Sci. Hung. **2**:293-296.

Silhavy, T. J., and J. R. Beckwith. 1985. Uses of *lac* fusions in the study of biological problems. Microbiol. Rev. **49**:398-418.

Silhavy, T. J., M. L. Berman, and L. W. Enquist. 1984. Experiments with gene fusions. Cold Spring Harbor Laboratory, Cold Spring Harbor, N. Y.

Southern, E. M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:242-253.

Starnbach, M. N., and S. Lory. 1992. The *fliA* (*rpoF*) gene of *Pseudomonas aeruginosa* encodes an alternative sigma factor required for flagellin synthesis. Mol. Microbiol. **6**:459-469.

Strockbine, N. A., M. P. Jackson, L. M. Sung, R. K. Holmes, and A. D. O'Brien. 1988. Cloning and sequencing of the genes for Shiga toxin from *Shigella dysenteriae* type 1. J. Bacteriol. **170**:1116-1122.

Stugard, C. E., P. A. Daskaleros, and S. M. Payne. 1989. A 101-kilodalton heme-binding protein associated with Congo red binding and virulence of *Shigella flexneri* and enteroinvasive *Escherichia coli* strains. Infect. Immun. **57**:3534-3539.

Takeuchi, A., H. Spring, E. H. Labrec, and S. B. Formal. 1965. Experimental bacillary dysentery: an electron microscopic study of the response of intestinal mucosa to bacterial invasion. Am. J. Pathol. **4**:1011-1044.

Tsang, N., R. Macnab, and D. E. Koshland, Jr. 1973. Common mechanisms for repellents and attractants in bacterial chemotaxis. Science **181**:60-63.

Tso, W.-W., and J. Adler. 1974. Negative chemotaxis in *Escherichia coli.* J. Bacteriol. **118**:560-576.

Venkatesan, M. M., and J. M. Buysse. 1989. Nucleotide sequence of invasion plasmid antigen gene *ipaA* from *Shigella flexneri* 5. Nucleic Acids Res. **18**:1648.

Venkatesan, M. M., J. M. Buysse, and A. B. Hartman. 1991. Sequence variation in two *ipaH* genes of *Shigella flexneri* 5 and homology to the LRG-like family of proteins. Mol. Microbiol. **5**:2435-2445.

Venkatesan, M. M., J. M. Buysse, and D. J. Kopecko. 1988. Characterization of invasion plasmid antigen genes (*ipaBCD*) from *Shigella flexneri.* Proc. Natl. Acad. Sci. USA. **85**:9317-9321.

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

Viitanen, A. M., P. Toivanen, and M. Skurnik. 1990. The *lcrE* gene is part of an operon in the *lcr* region of Yersinia enterocolitica O:3. J. Bacteriol. **172**:3152-3162.

Vogler, A. P., M. Homma, V. M. Irikura, and R. M. Macnab. 1991. *Salmonella typhimurium* mutants defective in flagellar filament regrowth and sequence similarity of FliI to F_0F_1, vacuolar, and archaebacterial ATPase subunits. J. Bacteriol. **173**:3564-3572.

Vasselon, T., J. Mounier, M. C. Prevost, R. Hellio, and P. J. Sansonetti. 1991. Stress fiber-based movement of *Shigella flexneri* within cells. Infect. Immun. **59**:1723-1732.

Wandersman, C., and P. Delepelaire. 1990. TolC, an *Escherichia coli* outer membrane protein required for hemolysin secretion. Proc. Natl. Acad. Sci. USA. **87**:4776-4780.

Watanabe, H. and A. Nakamura. 1986. Identification of *Shigella sonnei* Form I plasmid genes necessary for cell invasion and their conservation among *Shigella* species and enteroinvasive *Escherichia coli.* Infect. Immun. **53**:352-358.

Weiss, J. B., P. H. Ray, and P. J. Bassford. 1988. Purified SecB protein of *Escherichia coli* retards folding and promotes membrane translocation of the maltose-binding protein in vitro. Proc. Natl. Acad. Sci. USA **85**:8978-8982.

Welch, R. A., R. Hull, and S. Falkow. 1983. Molecular cloning and physical characterization of a chromosomal hemolysin from *Escherichia coli.* Infect. Immun. **42**:178-186.

Wu, H. C., and M. Tokunaga. 1986. Biogenesis of lipoproteins in bacteria. Curr. Top. Microbiol. Immun. **125**:127-157.

Young, D. B., and D. A. Broadbent. 1982. Biochemical characterization of extracellular proteases from *Vibrio cholerae.* Infect. Immun. **37**:875-883.