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Two Novel Virulence Loci, *mxiA* and *mxiB*, in *Shigella flexneri* 2a Facilitate Excretion of Invasion Plasmid Antigens

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A bank of over 4,200 *lacZ* protein fusions in *Shigella flexneri* 2a was screened for fusions to temperature-regulated promoters. One mutant, BS260, was completely noninvasive on HeLa cells and mapped to a region on the 220-kb virulence plasmid in which we had previously localized several avirulent temperature-regulated operon fusions (A. E. Hromockyj and A. T. Maurelli, Infect. Immun. 57:2963–2970, 1989). The phenotype of BS260 was similar to that of the previously identified *mxi* (membrane expression of invasion plasmid antigens) mutants, since it made wild-type intracellular levels of the invasion plasmid antigens (Ipa) but was deficient in the surface expression of IpaB and IpaC. Six kilobases of DNA upstream of the BS260 fusion end joint were cloned, but no temperature-regulated promoter was found, whereas the fusion end joint clone of the noninvasive *mxi* operon fusion mutant BS226 contained a temperature-regulated promoter. The locus defined by BS260 was designated *mxiA*, and that defined by BS226 was designated *mxiB*. Closer analysis of the *mxiA* and *mxiB* phenotypes by a cell-free enzyme-linked immunosorbent assay revealed that the mutants failed to excrete IpaB and IpaC into the culture medium, whereas wild-type cells actively released these antigens. Excretion of the *ipa* polypeptides from wild-type bacteria was confirmed by Western blot analysis of culture supernatants. Protease protection experiments revealed that wild-type *S. flexneri* 2a actually had much lower levels of surface-exposed IpaB and IpaC relative to those in the total antigen pool. In addition, examination of cellular fractions showed that, although there was no IpaB or IpaC in the outer membrane of BS260 and BS226, the antigens did accumulate in the cytoplasmic membrane. A 76-kDa temperature-regulated polypeptide in wild-type *S. flexneri* was identified as the putative *mxiA* gene product. These results strongly suggest that IpaB and IpaC represent truly excreted proteins of *S. flexneri* and that the *mxiA* and *mxiB* loci on the plasmid code for accessory proteins required to facilitate their export through the bacterial outer membrane. These data also suggest that *mxiA* is part of an operon that specifies additional *mxi* genes. The products of this operon may constitute a unique multicomponent protein secretion apparatus involved in the transport of *Shigella* virulence determinants.

Shigella flexneri is an intestinal pathogen that produces dysentery as a result of invasion of the colonic epithelium. The invasive property of this microorganism is conferred by a 220-kb plasmid and is strongly temperature regulated; the organism is noninvasive (avirulent) at 30°C but fully invasive at 37°C (18). A 37-kb cloned region of the plasmid (pHS4108) codes for several temperature-regulated polypeptides that are associated with invasion, and transfer of this portion of the 220-kb plasmid into a plasmid-cured *Shigella* strain completely restores the invasive phenotype (17). Four of these proteins, designated Ipa (invasion plasmid antigen) A through D, have been identified by their high immunoreactivity with convalescent sera from both monkeys and humans infected with *Shigella* species (22). The *ipa* genes are organized as an operon (1a), and the polypeptide products of two of these genes (*ipaB* and *ipaC*) have been found to be exported to the surface of the bacterium (7, 21, 30). However, DNA sequence analysis indicates that *ipaB* and *ipaC* do not have the signal sequence that is typically found at the amino-terminal end of bacterial genes encoding exported products (1a, 27, 30).

Although IpaA through D have been identified as the major immunogenic antigens of *S. flexneri*, many other plasmid-associated, temperature-regulated proteins are involved in the initial steps in pathogenesis of this microorganism (7, 8). In a previous report from this laboratory, the random mutagenesis of *S. flexneri* 2a with λp*lac*Mu53 to create operon fusions to temperature-regulated genes was described. Three temperature-regulated operon fusion mutants were isolated that were avirulent and deficient in surface expression of IpaB and IpaC while still making wild-type intracellular levels of the antigens (12). All three of these mutations map outside the *ipa* coding region and represent novel virulence genes on the invasion plasmid. These findings suggest that the *ipa* products must utilize a system independent of the normal cellular export machinery for transport out of the cell. On the basis of this Ipa export-deficient phenotype, these loci were designated *mxi* for membrane expression of invasion plasmid antigens (12).

In the course of generating protein fusion mutants to identify new virulence gene loci, another *mxi* mutant was isolated that has permitted a more detailed analysis of the mechanism that *S. flexneri* employs for exporting essential virulence gene products to the exterior of the cell. We report here the characterization of two mutants defining the loci *mxiA* and *mxiB*. Additionally, we have identified a temperature-regulated polypeptide that is encoded by the *mxiA* locus. The results also demonstrate that wild-type *S. flexneri* excretes IpaB and IpaC extracellularly in vitro and that

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MxiA and MxiB are essential components of this export process.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The *Shigella* strains used in this study were derivatives of the wild-type *S. flexneri* 2a strain 2457T (6). All bacteria were grown in tryptic soy broth (Difco Laboratories, Detroit, Mich.) at 37°C unless otherwise stated.

Mutagenesis. Lysates of protein fusion phage λp*lac*Mu9 and helper phage λp*lac*Mu507 (3) were used to infect the wild-type strain of *S. flexneri* 2a, 2457T. Fusion mutants were selected for resistance to kanamycin (Km^r) and screened for the Lac^+ phenotype at 37°C on minimal medium containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) and kanamycin. The Km^r Lac^+ lysogens obtained were screened for the Lac^- phenotype at 30°C to determine temperature regulation. The fusion mutations were then transferred to a clean (nonmutagenized) *Shigella* background by P1 transduction (29) to segregate possible double insertions. The transductants were then retested for the temperature-regulated phenotype by a quantitative assay for β-galactosidase as previously described (20).

Virulence assays. The HeLa cell invasion assay and other in vitro tests for virulence were performed essentially as described previously (12). Bacterial invasion was enumerated as (number of HeLa cells invaded/number of total HeLa cells counted) × 100. The plaque assay (23), used to determine the degree of intracellular bacterial replication and spread, was quantitated by directly enumerating the plaques that formed from the destruction of a HeLa cell monolayer. The Sereny test was performed as an additional means to assay for intracellular bacterial multiplication and the ability of the microorganisms to cause an inflammatory response in a mammalian host (28). A positive test result was the development of a characteristic keratoconjunctivitis in guinea pigs after 48 h.

Cloning and mapping of protein fusion ends. The cloning and mapping of the protein fusion end joints was done by using the procedure we previously employed for cloning the operon fusion end of BS226 (12). Plasmid DNA from the BS260 protein fusion mutant was digested with *Eco*RI and ligated into the *Eco*RI-digested fusion vector pMLB524 (2), and the resulting recombinant DNA was transformed into *Escherichia coli* DH5-α. Clones were selected for vector-encoded ampicillin resistance and screened for the Lac^+ phenotype of the fusion. To determine whether the fusion end joints of the protein fusion mutants mapped within a 37-kb cosmid clone of the invasion plasmid (pHS4108) (12), the recombinant plasmids were purified, radiolabeled, and used as probes in a Southern hybridization analysis (16) of three *Sal*I subclones spanning pHS4108 (17).

Cell-bound Ipa suspension assay. Bacteria were grown to the log phase (optical density at 600 nm, 0.3 to 0.9), washed once in phosphate-buffered saline (PBS), and incubated with a 1:2,500 dilution of anti-IpaB or -IpaC monoclonal antibodies (MAbs; kindly provided by John Mills and Jerry Buysse) in PBS plus 3% casein at room temperature for 2 h. After three washes with PBS, the cells were labeled with a 1:500 dilution of alkaline phosphatase-conjugated rabbit anti-mouse immunoglobulin G (IgG; Boehringer Mannheim) for 1 h at room temperature. The PBS-washed, antibody-bound cells were transferred to clean plastic tubes. The substrate (Sigma 104; Sigma Chemical Co., St. Louis, Mo.) was added; after 30 min the reactions were stopped by the addition of 300 μl of 3 N NaOH, and the cells were pelleted by centrifugation. The A_{405} values of supernatants containing cleaved substrate were then read in an LKB model 4050 Ultraspec II spectrophotometer.

Cell-free Ipa ELISA. Exported IpaB and IpaC were measured by a modified enzyme-linked immunosorbent assay (ELISA). Wild-type *S. flexneri* and *mxi* mutants were grown to the log phase, centrifuged, and suspended in PBS to an optical density at 600 nm of 5.0. The suspended cells were then incubated at room temperature for 2 h and centrifuged, and the supernatant was passed through a 0.22-μm-pore-size low-protein-binding membrane filter (Millipore Corp., Bedford, Mass.). The cell-free PBS supernatants were then added to wells of a 96-well microtiter plate (Costar, Cambridge, Mass.) and incubated for 2 h at room temperature. After the wells were emptied of the supernatants, a 3% casein blocker was applied. After two washes with PBS–0.05% Tween 20 (PBST), anti-IpaB and IpaC MAbs were added to the wells at a 1:2,500 dilution and incubated for 2 h at 37°C. After three washes with PBST, a 1:500 dilution of alkaline phosphatase-conjugated anti-mouse IgG was added for 1 h at room temperature. After two washes with PBST, the wells were washed once with DEA buffer (8% diethanolamine, 500 mM $MgCl_2$, 3 mM NaN_3) and developed for 30 min with alkaline phosphatase substrate (1 mg/ml) in DEA buffer. Reactions in the wells were stopped with 3 N NaOH, and the A_{405} was recorded by using a Bio-Tek Microplate ELISA Reader.

Detection of IpaB and IpaC in culture supernatants. Supernatants from 20 ml of log-phase cultures were precipitated with ammonium sulfate to 70% saturation at 4°C. The protein pellets were suspended in 1 ml of distilled water containing 1 mM phenylmethylsulfonyl fluoride (Sigma). Samples were standardized to total protein by using the BCA-Lowry kit (Pierce, Rockford, Ill.) before application onto a 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gel. The gels were then electroblotted, and the blots were probed with a 1:1,000 dilution of anti-Ipa rabbit hyperimmune serum overnight, followed by a secondary label of alkaline phosphatase-conjugated anti-mouse IgG (1:1,000), and developed with fast red TR-salt and naphthol AS MX-phosphate (both from Sigma).

Cellular fractionation. Wild-type *S. flexneri* and selected *mxi* fusion mutants were fractionated into cytoplasmic, periplasmic, and total membrane components by a modification of reported methods (4, 5). Log-phase bacterial cultures (100 ml) were centrifuged and suspended in 0.1 volume of TEP (0.1 M Tris, 1.0 mM phenylmethylsulfonyl fluoride, 0.02% NaN_3), centrifuged, and then 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 were incubated at 37°C for 30 min with gentle agitation. Spheroplast formation was confirmed by using phase-contrast microscopy, and the periplasmic fraction was removed from the spheroplasts by centrifugation. Spheroplasts were then suspended in ice-cold TEP and subjected to sonication for four 15-s bursts. The total membrane fraction was separated from the cytoplasm by removing debris and unlysed cells from the sonicate by centrifuging first at 10,000 × *g* for 10 min and then at 50,000 × *g* for 2 h at 4°C. The membrane pellet was suspended in TEP with Triton X-100 added to 1.0% and incubated at 37°C for 10 min. The outer membrane (Triton X-100-insoluble fraction) was then separated from the inner membrane by pelleting at 50,000 × *g* for

2 h. Fractions were assayed for total protein by using the BCA-Lowry kit and stored at −20°C until needed.

Proteinase K digestion of IpaB and IpaC. The amount of surface-exposed, cell-associated IpaB and IpaC was assessed by proteolysis susceptibility. Proteinase K (Boehringer Mannheim) was added at a final concentration of 250 μg/ml to log-phase wild-type whole cells and to sonic lysates. After 1 h of incubation at 4°C, phenylmethylsulfonyl fluoride was added to 5 mM, and the samples were incubated on ice for an additional 10 min. The samples were then run on SDS-PAGE, electroblotted, and probed with anti-IpaB and -IpaC MAbs. Western blots were quantitated as described below.

Quantitative Western blot analysis. The intracellular distribution of IpaB and IpaC in the wild type and *mxi* mutants was determined by performing SDS-PAGE on cellular fractions standardized to total protein, electroblotting onto nitrocellulose, and immunolabeling blots overnight with a 1:10,000 dilution of anti-IpaB and -IpaC MAbs. Alkaline phophatase-conjugated anti-mouse IgG at a 1:1,000 dilution was added as the secondary label, and the blots were developed as described above. IpaB and IpaC signals on blots of cellular fractions were quantitated by 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 was determined with a CCS-1 integration program cassette and DR-2 data recorder. Alternatively, Western blots were scanned with a Datacopy model 230 digital scanner, and the mean density of each band was calculated by using Image v1.30 digital image analysis software on a Macintosh II computer.

Purification of BS260 fusion protein. The *mxi* hybrid protein (MxiHP) produced by BS260 was isolated by first growing approximately 7 liters of BS260 overnight and then lysing the cells in a French pressure cell at 1,000 lb/in^2 in the presence of 1% Nonidet P-40 (Sigma). The lysate was then concentrated in TBSN (50 mM Tris, 150 mM NaCl, 0.2% Nonidet P-40) and adjusted to 4 mg of total protein per ml. The lysate was then passed over a β-galactosidase affinity column (Promega Corp., Madison, Wis.) to bind the hybrid protein. After the column was washed with several volumes of TBSN, the bound MxiHP was eluted with a high-pH bicarbonate buffer and assayed for purity and yield by using the BCA-Lowry kit, SDS-PAGE, and Western blotting. The affinity-purified protein was used directly from the column to immunize rabbits for generation of monospecific polyclonal antisera.

Generation of hyperimmune rabbit serum. Monospecific rabbit antiserum to MxiA was generated by parenteral immunization with the affinity-purified MxiHP. A 6-week-old male New Zealand White rabbit was prebled and injected subcutaneously in each hind quarter with 1 ml of approximately 30 μg of MxiHP mixed 1:1 with Freund complete or incomplete adjuvant at 2- to 4-week intervals. The IgG fraction was purified from the rabbit hyperimmune serum by passage over a Staph-A Sepharose column (Pierce) and extensively adsorbed with acetone-dried wild-type *S. flexneri* grown at 30°C and plasmidless *S. flexneri* BS103 grown at 37°C. Anti-Ipa rabbit serum was generated in a fashion similar to that described above, except that the immunizing antigen consisted of a distilled water extract (7) of an overnight culture of *S. flexneri* 2457T. Whole serum was then adsorbed extensively with acetone-dried BS103 and used directly on electroblots at a 1:2,000 dilution.

TABLE 1. β-Galactosidase levels of the cloned BS260 and BS226 fusion end joints

Strain	U of β-galactosidase[a]		Ratio (37°C/30°C)
	37°C	30°C	
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)	55	69	<1
BS103(pAEH006)	63	67	<1
BS201(pAEH006)	456	51	9

[a] As defined by Miller (20).

RESULTS

Isolation and virulence phenotypes of temperature-regulated protein fusion mutants. Over 4,200 Km^r Lac$^+$ protein fusions were isolated, and 9 were found to be temperature regulated for β-galactosidase. Four of the mutants demonstrated high 37°C/30°C ratios characteristic of the pattern of temperature-regulated expression of *Shigella* virulence. 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. The remaining eight protein fusion mutants were fully invasive and behaved similarly to the wild-type parent in the other virulence assays. Consequently, these fusion mutants were not examined further.

Map location and expression of the gene fusion in BS260. To map the fusion phage insertion in BS260, the fusion end joint was cloned by *Eco*RI digestion and ligation into the fusion vector pMLB524, as was previously done for BS226 (12). The size of the *Eco*RI fragment in the resulting recombinant plasmid, pGPA001, was calculated to be 9 kb. This represented 6 kb of DNA upstream from the end joint and 3 kb of *lacZ* coding sequence. Southern hybridization and restriction mapping analyses (data not shown) placed the fusion phage insertion site in BS260 precisely between a *Xba*I site and a *Sal*I site, 11 kb downstream of the Ipa operon, and 4 kb upstream from the insertion in the avirulent operon fusion, BS226.

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 (DH5-α), however, exhibited low levels of expression of the fusion at both 30 and 37°C (Table 1). To test the possibility that these low enzyme levels were due to the absence of chromosomal regulatory genes present only in *S. flexneri*, 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 1). β-Galactosidase levels also remained low when plasmid-encoded activators were restored by transforming pGPA001 into an invasion plasmid-containing *Shigella* background (BS201). However, when the cloned end joint (pAEH006) (12) from the *mxi* operon fusion mutant BS226 was transformed into BS201, enzyme levels were elevated (Table 1). The above results suggested that the promoter for the gene

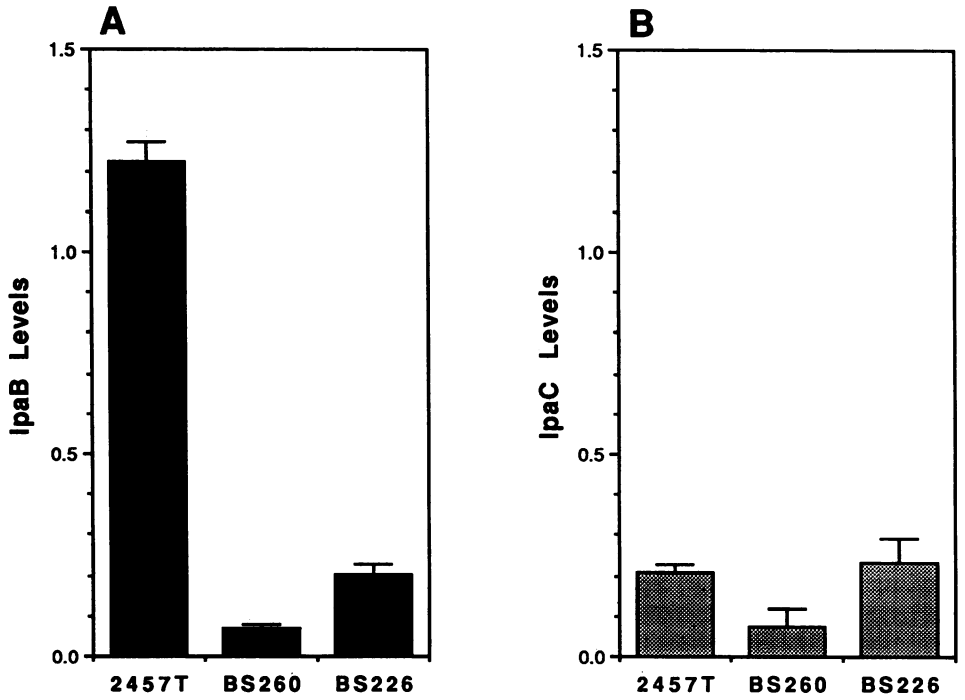

FIG. 1. Detection of cell surface-associated IpaB and IpaC on wild-type cells compared with that on fusion mutants BS260 and BS226. (A) IpaB levels, represented by reactivity of cells with anti-IpaB MAb; (B) IpaC levels, represented by reactivity of cells with anti-IpaC MAb. Samples were standardized by adjusting the volume against culture optical density. MAb reactivity is expressed as A_{405}, and values were corrected for background by subtracting the reactivity obtained for the plasmidless derivative BS103 (<5.0% of the wild-type reactivity). Error bars represent two standard deviations from the mean.

fusion in BS260 was not contained on the 6-kb end joint fragment cloned in pGPA001. The promoter for the *mxi* locus in BS226, however, did appear to be present on the *Eco*RI fragment containing the operon fusion end joint. Thus, BS260 and BS226 represent mutations in two independent transcription units.

Ipa export phenotype of BS260. To determine whether the noninvasive 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, and the previously identified *mxi* operon fusion mutant, BS226, was performed with anti-Ipa hyperimmune rabbit serum. IpaA through D were present at the same levels in all of the strains grown at 37°C; their expression was temperature regulated, since lysates from cultures of the same strains grown at 30°C possessed low levels of the antigens (data not shown).

An anti-Ipa MAb whole-cell suspension assay with log-phase cultures of BS260 was developed to test the ability of BS260 to express IpaB and IpaC on the cell surface. BS260 bound significantly lower levels of anti-IpaB MAb (Fig. 1A) but comparable levels of anti-IpaC relative to those of the wild type (Fig. 1B). A similar phenotype was seen with the *mxi* operon fusion mutant BS226. Two other *mxi* operon fusion mutants, BS230 and BS232 (12), behaved similarly to the protein fusion mutant (data not shown). These data suggest that BS260 was deficient in surface expression of IpaB. Since this at least partially defines 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 operons, the locus in BS226 was designated *mxiB*.

Detection of released and/or excreted IpaB and IpaC. We considered the possibility 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 with the anti-IpaB (and -IpaC) MAbs to detect any released antigen. As shown in Fig. 2, microtiter plate wells coated with cell-free PBS supernatant from the two *mxi* mutants (BS260 and BS226) were only weakly reactive to both MAbs. Wells exposed to supernatants from wild-type *S. flexneri*, however, reacted strongly with the MAbs, suggesting that excretion of the Ipa proteins was, in fact, a characteristic of the wild type. To confirm this finding, ammonium sulfate-precipitated culture supernatants from 2457T, BS260, and BS226 were reacted with rabbit anti-Ipa serum on a Western blot. Although all of the bacterial strains possessed comparable levels of total Ipa, the culture supernatants of the *mxi* mutants failed to yield significant levels of both antigens (Fig. 3). The wild-type (2457T) culture supernatant, however, was found to contain both IpaB and IpaC.

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, and inner and outer membrane fractions. The fractions were subjected to Western blotting, and the distribution of IpaB and IpaC was quantitated by laser densitometry. As shown in Fig. 4, analysis of the distribution of IpaB and IpaC in the *mxi* mutants revealed an accumulation of these antigens in the inner membrane and no IpaB or C in the outer membrane. There also appeared to be a slight accumulation of IpaB in the cytoplasm compared with that in the

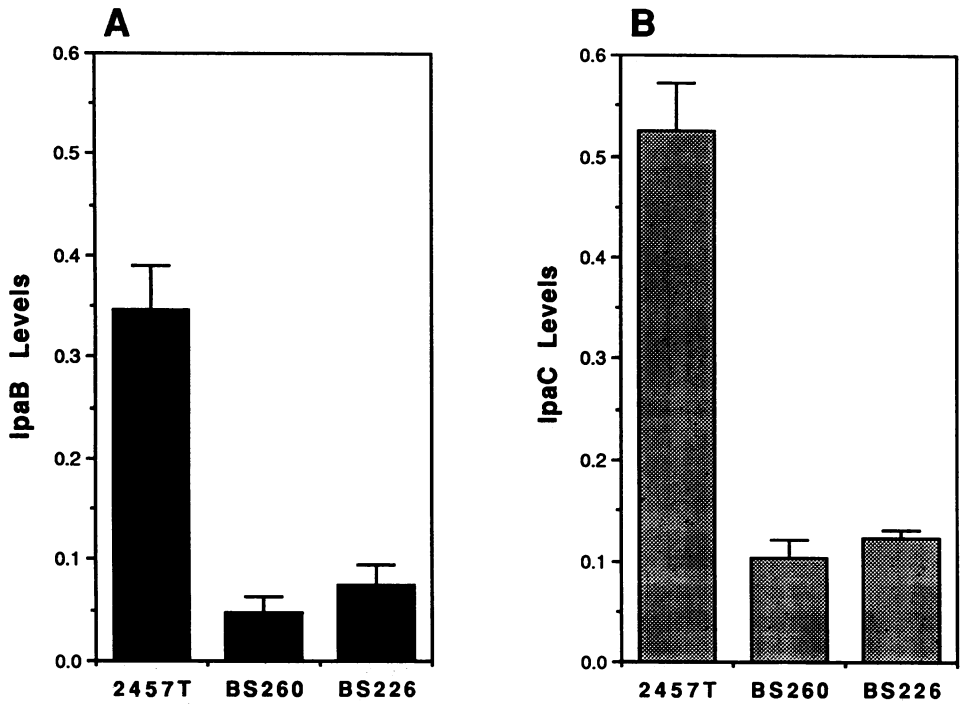

FIG. 2. Detection of released and/or excreted IpaB and IpaC by wild-type *S. flexneri* compared with that of the fusion mutants BS260 and BS226. (A) IpaB levels, represented by reactivity of PBS supernatants with anti-IpaB MAb; (B) IpaC levels, represented by reactivity of PBS supernatants with anti-IpaC MAb. Reactivities of MAbs are expressed as A_{405}. Values were corrected for background by subtracting reactivity of the plasmidless derivative, BS103. Error bars represent two standard deviations from the mean.

wild type. 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 strains, there was no difference in periplasmic levels between the mutants and the wild type (data not shown).

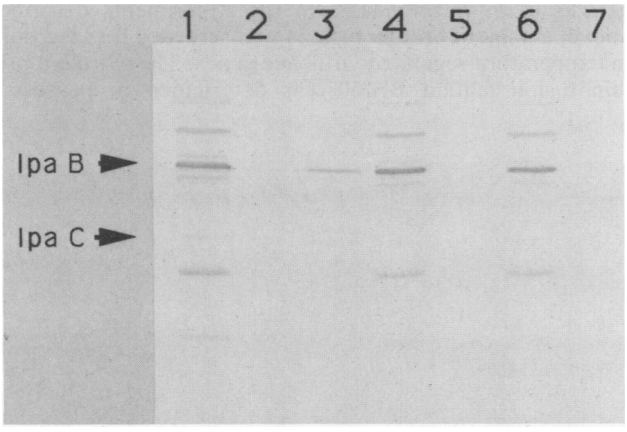

FIG. 3. Release and/or excretion of IpaB and IpaC from log-phase cultures. Supernatant proteins were separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with rabbit anti-Ipa serum. Lanes: 1, 4, and 6, whole-cell lysates of 2457T, BS260, and BS226, respectively; 3, 5, and 7, concentrated culture supernatants of 2457T, BS260, and BS226, respectively; 2, whole-cell lysate from 10^6 cells, representing the Ipa contribution of bacteria remaining in the culture supernatant after centrifugation. Lanes 1, 4, and 6 were standardized by optical density. Lanes 3, 5, and 7 were standardized by total protein.

Surface expression of IpaB and IpaC in wild-type *S. flexneri*. Based on the above data, it was concluded that IpaB was both cell surface associated and excreted, whereas IpaC appeared to be a predominantly excreted antigen. To confirm this conclusion, the amount of cell surface IpaB and IpaC on wild-type cells was assessed by determining the susceptibilities of the antigens to proteolytic digestion. Western blots of proteinase K-treated, wild-type whole cells probed with anti-IpaB MAb showed that the antigen was completely protected from degradation, whereas IpaB in sonic lysates showed exquisite sensitivity to the enzyme (Fig. 5). An identical blot probed with anti-IpaC MAb revealed similar results (data not shown). Quantitation of these blots confirmed that there was little difference in the total amount of IpaB and IpaC in the proteinase K-treated whole cells and the untreated controls. These results suggest that the bulk of IpaB and IpaC in wild-type *S. flexneri* is intracellular and/or cell free and not predominantly cell surface associated.

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 MAb to β-galactosidase (data not shown). After isolation and purification of this fusion protein, monospecific polyclonal rabbit antiserum was generated, and the IgG fraction was purified for use as an immunological reagent in the preliminary identification of the native *mxiA* gene product. The purified fusion protein was detected on a Western blot strip by using a 1:100 dilution of the anti-MxiHP IgG fraction (Fig. 6). Degradation products of the hybrid protein

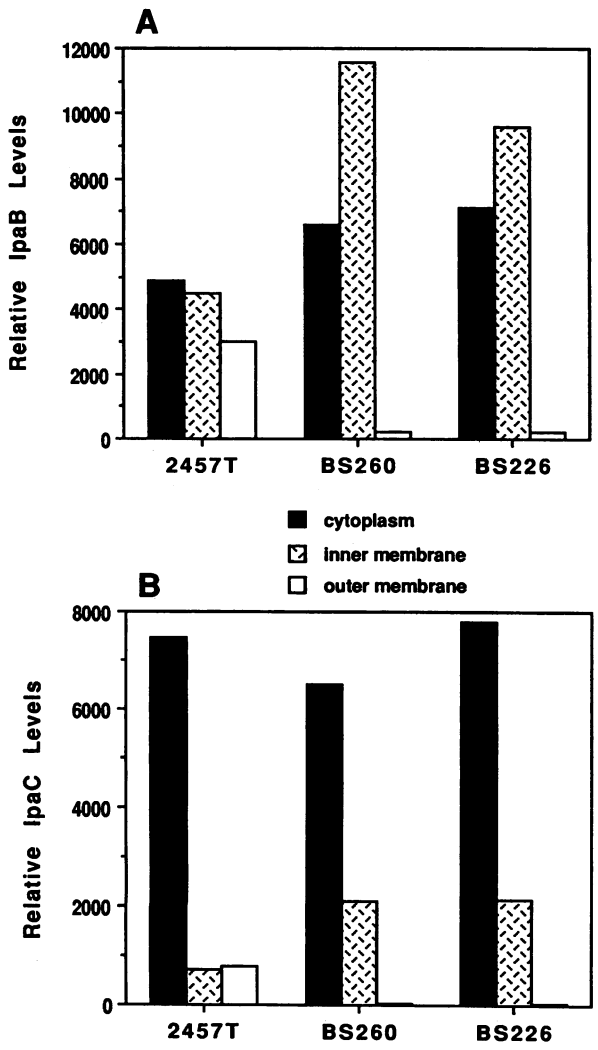

FIG. 4. Intracellular distribution of IpaB (A) and IpaC (B) in *S. flexneri* fusion mutants. Cells were fractionated, and immunoblots of the electrophoresed fractions were probed with MAbs to IpaB and IpaC. Blots were analyzed by laser densitometry, and the values were standardized to total protein. Symbols: ■, cytoplasmic fraction; ▨, inner membrane; □, outer membrane.

were also visible. Upon probing of whole-cell lysates of BS260, however, no fusion protein was detected. This result was unexpected and is addressed below. When lysates of wild-type *S. flexneri* were probed with the antiserum, a 76-kDa polypeptide was detected in lysates of cultures grown at 37°C but was absent in cultures grown at 30°C. Further, the invasion plasmid-cured strain BS103 and the protein fusion mutant BS260 produced no detectable reactivity at the position of 76 kDa. However, higher-molecular-weight cross-reactive antigens were weakly detected in all strains.

Absence of homology between the Ipa secretory system and the *E. coli hly* operon. One interesting characteristic of the *mxiA* mutant and the *mxi* operon fusion mutants was their low level of contact hemolytic activity. 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

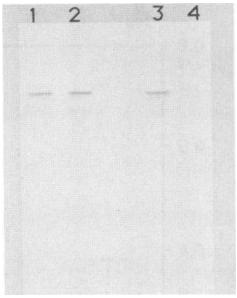

FIG. 5. Susceptibility of IpaB to digestion by proteinase K. Lanes: 1, whole cells (untreated); 2, whole cells plus 250 μg of proteinase K per ml; 3, sonic lysate (untreated); 4, sonic lysate plus 250 μg of proteinase K per ml. All samples were from the same culture of 2457T and were standardized by volume before SDS-PAGE and Western blotting with anti-IpaB MAb.

has been demonstrated to be exported by a unique multicomponent apparatus (11). Southern blot analysis was performed with the cloned *E. coli hly* operon, pSF4000 (32), 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 seemed reasonable, since it has been shown that secretion defects in some gram-negative organisms can be functionally complemented by the accessory gene products from different genera (10). However, although the transformed *mxi* mutants secreted functional hemolysin at wild-type *E. coli* levels (i.e., β-hemolysis on blood agar), ELISA analysis showed that the Ipa secretion defects failed to be complemented.

DISCUSSION

In our laboratory we have previously exploited gene fusion technology to identify new loci involved in the pathogenesis of *S. flexneri* (12). In the experiments described above, a bank of protein fusions was screened for insertions in temperature-regulated virulence genes. The avirulent protein fusion mutant BS260 was determined to possess a

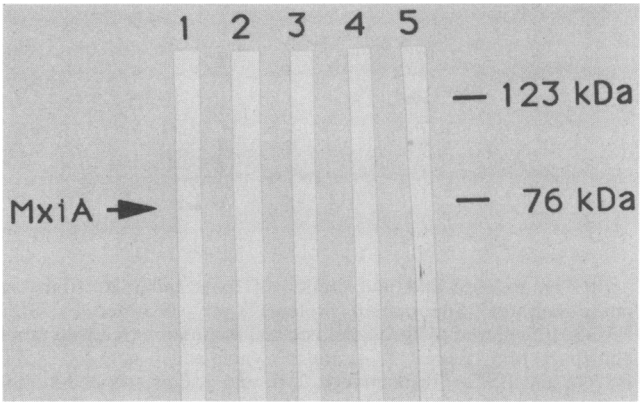

FIG. 6. Detection of the temperature-regulated *mxiA* gene product in whole-cell lysates of wild-type *S. flexneri*. Lanes: 1, 2457T (37°C); 2, 2457T (30°C); 3, BS103 (37°C); 4, BS260 (37°C); 5, purified MxiHP (5 μg).

phenotype similar to that of the previously described *mxi* operon fusion mutants, which are altered in extracellular expression of IpaB and IpaC (12). 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 11.5-kb *Eco*RI fragment (near the BS226 insert) of the cosmid clone pHS4108 produce a noninvasive phenotype (17). We initially chose to compare BS260 with the *mxi* operon fusion mutant BS226 because the map distance of the fusion inserts from one another (greater than 3 kb) suggested that the mutations were in different genes. This was confirmed by the failure to detect high levels of expression of the reporter gene, *lacZ*, from the cloned *mxiA* end joint fragment (pGPA001). However, expression of β-galactosidase from the BS226 end joint clone (pAEH006) was detected. Thus, the *mxiB* locus is driven by a separate promoter that is independent of *mxiA*. Additionally, these data strongly suggest that the promoter for *mxiA* lies at least 5 kb upstream from the translational start site of the gene. This implies that *mxiA* may be part of an operon that consists of additional Ipa accessory loci. This is further supported by the fact that no complementation of the secretion defect in BS260 was detected when a cloned wild-type *Eco*RI DNA fragment (pHS4011) (12) was transformed into the mutant (data not shown). In a previous report, a Tn*1* insertion in *Shigella sonnei* in a locus near the equivalent position of the BS226 insertion produced a noninvasive phenotype (31). Although the map positions of the insertion in this mutant (designated *invA* [13]) and BS226 (*mxiB*) differ slightly, they may be close enough to be in the same open reading frame. While this may suggest the existence of an *S. sonnei* homolog of *mxiB*, the mutation has not been characterized for the *mxi* phenotype.

An ELISA that employs whole bacteria as the coating antigen has previously been used to demonstrate that both IpaB and IpaC are expressed on the surface of the bacterium (7, 21, 22, 26). We had also found the whole cell ELISA to be useful in the initial characterization of the *mxi* operon fusion mutants (12). The experiments described here, however, indicate that only very low levels of IpaB and IpaC are associated with the surface of wild-type *S. flexneri*. Moreover, comparative studies in our laboratory suggest that the majority of the MAb reactivity detected in the whole-cell ELISA was due to excreted or released antigen rather than to expression of the antigens on the cell surface. PBS supernatants 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, we found that very low numbers of bacteria adhered to the microtiter plate wells under the conditions of this assay as measured by direct physical and immunological detection methods.

Earlier evidence suggesting that Ipa proteins are excreted came from the finding that IpaB and IpaC are present in distilled water extracts of the wild-type bacteria (22), although this phenomenon was attributed more to the release of the antigen from the surface of the organism. Our data, however, suggest that IpaB and IpaC are, in fact, excreted antigens and that the in vitro wild-type phenotype of *S. flexneri* is represented by the export of IpaB and IpaC into the culture medium. This characteristic was further demonstrated by testing culture supernatants on a Western blot for the presence of the two antigens. Wild-type culture supernatants showed detectable levels of the two antigens, whereas the *mxi* mutants failed to excrete them. The *mxi* mutants BS260 and BS226 did release low but detectable levels of both antigens in the cell-free ELISA. This observation was subsequently found to be an artifact of the assay procedure, since some cell lysis occurred 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 h (data not shown).

The interpretation of the results in Fig. 1 (suspension assay) should be taken in the context of the results of the cell-free ELISA (Fig. 2). Thus, although it appears that some surface-associated IpaC exists both on the wild type and the *mxi* mutants, the amount of antigen that is cell free is significantly different between the mutant and wild-type cells. Thus, the *mxi* mutations effect a significant decrease in the release or excretion of IpaC (and 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 our assay methods, we detected neither a higher level of surface-associated Ipa on these mutants nor an outer-membrane accumulation of these antigens. In fact, the *mxi* outer membranes were found to be deficient in both of these antigens. We have concluded, therefore, that the *mxiAB* functions are related to export of the Ipa proteins rather than to their release from the surface. The Ipa excretion phenomenon mediated by *mxi*, therefore, represents a specific mechanism which has not yet been reported for *Shigella*.

Despite all evidence that IpaB and IpaC are excreted proteins, an apparent discrepancy exists in the detection and quantitation of surface-associated IpaB between the whole-cell suspension assay and the proteinase K experiments. Although the 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 suspension assay represents a more sensitive means to detect low levels of surface-associated antigen relative to the protease protection method. In this regard, the suspension assay may be detecting only low levels of IpaB on wild-type cells and even lower levels of IpaC (on the *mxi* mutants as well as the wild type).

The intracellular distribution of IpaB and IpaC in the wild-type bacteria and the *mxi* mutants presents an interesting finding. Overall, a large intracellular pool of the antigens was detected in the cytoplasm and cell envelopes by quantitative Western blotting of cell fractions. These large pools of antigen may exist in *S. flexneri* because of a high turnover rate, not only from excretion of the proteins into the extracellular milieu but also as a compensatory effect in response to the extreme sensitivity of these proteins to degradation by endogenous bacterial proteases. Additionally, excreted (cell-free) antigen was found to represent only about 15% of the total Ipa synthesized (1), which indicates that the excretion is relatively inefficient or that extracellular Ipa is much more susceptible than the intracellular pool to proteolysis. Regardless, the bulk of the exported antigen appears to be in the cell-free form (versus cell surface associated). Although we also detected low levels of IpaB and IpaC in the periplasm after cell fractionation, this observation was probably

due to cytoplasmic cross-contamination, since significant levels of the cytoplasmic marker glucose-6-phosphate dehydrogenase were detected in the periplasmic fractions. The importance of the periplasmic space in the Ipa excretion process therefore still remains unclear. An accumulation of IpaB and IpaC was seen in the inner membrane of both *mxi* mutants, whereas very low levels of both antigens were detected in the outer membrane relative to those in wild-type bacteria (Fig. 4). This finding suggested a block in secretion between the inner and outer membrane. 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. Evidence from other gram-negative 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 nonionic detergents (19). Alternatively, outer membrane-associated Ipa may represent a transition-state pool as it passes from the inner membrane to the extracellular environment. One explanation for the reactivity of wild-type *S. flexneri* with anti-IpaB MAb in the whole-cell anti-Ipa binding assay is that the excreted antigens may readsorb to the cell surface at some point during cell growth. 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 (19). We are presently conducting experiments to address these possibilities.

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 parent strain (BS260), purified MxiHP was weakly reactive as a band of 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.

The mechanisms for secretion of virulence factors from gram-negative pathogens are quite complex and usually involve more than one accessory protein (11, 14, 15). Our findings indicate that *S. flexneri* is another gram-negative intestinal pathogen that employs a complex (multicomponent) secretion mechanism for its virulence factors. The finding that the Ipa secretion apparatus was dissimilar to the *E. coli* hemolysin A secretion mechanism lends support to the uniqueness of this system of protein export in shigellae.

The results presented here, along with published observations on the properties of the *ipa* gene products, lead to interesting speculation as to the role these antigens play in *Shigella* pathogenesis. Their primary function in facilitating invasion cannot be disputed (17). The way in which they produce this effect, however, still 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 that 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 *S. flexneri* is able to facilitate uptake by HeLa cells of a noninvasive strain (24). In contrast, pretreatment of whole bacteria with trypsin can entirely abolish the invasive property of the organism (25). This would argue against a specific role for the cell-free Ipa in invasion. Alternatively, if low levels of *ipa* proteins exist on the bacterial cell surface, they may function as ligands to a host cell receptor that, once bound, induce phagocytosis. This hypothesis implies a dual role for the *ipa* gene products in which the cell-free form of the antigens provides the pathogen with some other asset for establishing infection. Although Ipa may also have a role in virulence once inside the host cell, recent work by Headley and Payne (9) indicates that de novo synthesis of IpaB and IpaC does not occur after the bacteria have invaded. However, because our findings 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 that host-specified signals modulate the excretion of preexisting *ipa* proteins once the bacteria enter their epithelial host cells. We are currently undertaking experiments to address these hypotheses and to clarify the role that *mxi* gene products may play in the extracellular expression of the *ipa* virulence factors.

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