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# Nonpolar Mutagenesis of the *ipa* Genes Defines IpaB, IpaC, and IpaD as Effectors of *Shigella flexneri* Entry into Epithelial Cells

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**A 31-kb fragment of the large virulence plasmid of *Shigella flexneri* is necessary for bacterial entry into epithelial cells in vitro. One locus of this fragment encodes the IpaA, -B, -C, and -D proteins, which are the dominant antigens of the humoral immune response during shigellosis. To address the role of the *ipa* genes, which are clustered in an operon, we constructed a selectable cassette that does not affect transcription of downstream genes and used this cassette to inactivate the *ipaB*, *ipaC*, and *ipaD* genes. Each of these nonpolar mutants was defective in entry and lysis of the phagocytic vacuole but was not impaired in adhesion to the cells. We showed that, like IpaB and IpaC, IpaD is secreted into the culture supernatant and that none of these proteins is necessary for secretion of the other two. This result differentiates the Ipa proteins, which direct the entry process, from the Mxi and Spa proteins, which direct secretion of the Ipa proteins. Moreover, lack of either IpaB or IpaD resulted in the release of larger amounts of the other Ipa polypeptides into the culture medium, which indicates that, in addition to their role in invasion, IpaB and IpaD are each involved in the maintenance of the association of the Ipa proteins with the bacterium.**

*Shigella* species cause bacillary dysentery in humans through a complex series of events which include entry into colonic epithelial cells, intracellular multiplication, and spread of bacteria to adjacent cells and to the lamina propria of intestinal villi (19). The molecular and cellular basis of the entry process have been studied in vitro by using various epithelial cell lines (for a review, see reference 14). *Shigella* cells enter semiconfluent HeLa cells through a mechanism similar to phagocytosis, since actin polymerization and myosin accumulation occur beneath the plasma membrane at the entry site of virulent bacteria (12). Following entry, *Shigella* cells rapidly lyse the membrane of the phagocytic vacuole with a contact hemolysin (15, 33) and subsequently spread within the infected cell (33). With the human colonic epithelial cell line Caco-2, which differentiates into a polarized epithelium expressing a well-established brush border, the invasion process occurs through the basolateral surfaces (27).

The invasive phenotype of *Shigella flexneri* is conferred by a 220-kb virulence plasmid (32). By cosmid cloning (21) and Tn*5* mutagenesis (34, 35), entry-associated sequences have been identified in six distinct loci of the large plasmid. Two of these loci, *virB* and *virF*, encode transcriptional activators of invasion gene expression (1), while the remaining four loci map close to one another and cover a 31-kb fragment of DNA (35). This segment contains numerous closely linked genes, including the *ipa* (invasion plasmid antigen), *mxi* (membrane expression of invasion plasmid antigen), and *spa* (surface presentation of invasion plasmid antigen) genes. The *ipa* genes encode the IpaA, IpaB, IpaC, and IpaD polypeptides, which are the dominant antigens eliciting a humoral immune response during shigellosis (29). Recent reports have indicated that these antigens are presented at the bacterial surface and released into the culture supernatant by a secretion apparatus encoded by the *mxi* and *spa* genes (4–6, 39).

The Ipa-encoding region is a cluster of eight genes transcribed in the following order: *icsB*, *ipgA*, *ipgB*, *ipgC*, *ipaB*, *ipaC*, *ipaD*, and *ipaA*. Transcriptional analysis of this region indicated that the three *ipg* and the four *ipa* genes belong to a single transcriptional unit (3, 9, 34). Transposon insertions in *ipgA*, *-B*, and *-C* and in *ipaB*, *-C*, and *-D*, but not in *ipaA*, abolished invasion of epithelial cells and the ability to provoke keratoconjunctivitis in mice (35). Testing of some transposon insertion mutants complemented with various recombinant plasmids in the focus plaque assay confirmed the importance of *ipaB*, *ipaC*, and *ipaD* in the virulence phenotype of *Shigella* species, although their role in the entry process was not specifically addressed (34). Recently, the *ipaB* gene has been mutated with an interposon that directs downstream transcription. IpaB was found to be necessary for entry into cells and lysis of the phagocytic vacuole (15).

We report here the construction of a mutagenic cassette that can be inserted into a gene of an operon without affecting transcription of downstream genes. This cassette was used to inactivate each of the *ipaB*, *ipaC*, and *ipaD* genes on the large virulence plasmid of *S. flexneri*. While still adhering to HeLa cells, the *ipaB*, *ipaC*, and *ipaD* nonpolar mutants displayed similar phenotypes in that they failed to invade HeLa cells, trigger actin polymerization at sites of bacterial interaction with the cell membrane, or lyse erythrocytes. For each of these mutants, these defects, as well as the ability to induce keratoconjunctivitis in guinea pigs, were restored by complementation with a plasmid expressing a wild-type copy of the mutated gene. In addition, we showed that IpaD, like IpaB and IpaC, is secreted into the culture medium of the wild-type strain. Analysis of the culture supernatant of each of the *ipaB*, *ipaC*, and *ipaD* mutants indicated that none of the Ipa proteins was necessary for secretion of the others, which, together with the similar

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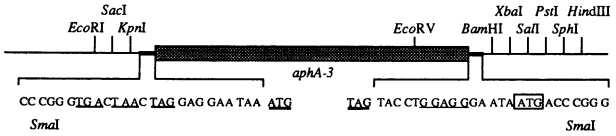


FIG. 1. Schematic representation of the *aphA-3* cassette carried by plasmid pUC18K. Plasmid pUC18K contains a 850-bp cassette inserted into the *Sma*I site of plasmid pUC18. The kanamycin resistance gene (*aphA-3*), whose start and stop codons are underlined by boldface lines, is indicated by a stippled box. The resistance gene is preceded by translation stop codons in all three reading frames (underlined by thin lines) and is immediately followed by a consensus ribosome-binding site, GGAGG (underlined by a thin line), and a start codon (boxed). Restriction enzyme sites of the pUC18 polylinker are shown.

defective phenotype of the mutants, suggests that IpaB, IpaC, and IpaD are effectors of the entry process. Moreover, lack of IpaB or IpaD resulted in the release of larger amounts of the other Ipa polypeptides into the culture medium, which indicates that, in addition to their essential role in invasion, IpaB and IpaD are each involved in the maintenance of the association of the Ipa proteins with the bacterium.

## MATERIALS AND METHODS

**Bacterial strains and growth media.** *Escherichia coli* MC1061 (10) was used as a host for constructs involving usual plasmid vectors, and *E. coli* DH5αλpir (*endA1 hsdR17* [$r_k^-m_k^+$] *supE44 thi-1 recA1 gyrA* [Nal$^r$] *relA1* Δ[*lacZYA-argF*]*U169*, F'[*Φ80dlac*Δ(*lacZ*)*M15*] [λ*pir*]) was used as a host for constructs involving the suicide plasmid pGP704 (*oriR6K mob* Ap$^r$) (25). *E. coli* SM10λpir (*thi thr leu tonA lacY supE recA*::RP4-2Tc::Mu [Km$^r$] [λ*pir*]) (36) was used to transfer suicide plasmids to *S. flexneri* M90T-Sm. *S. flexneri* wild-type M90T (32); M90T-Sm, a spontaneous streptomycin-resistant derivative of M90T (3); and BS176, a plasmidless derivative of the wild-type strain (21), have been previously described. *E. coli* strains were grown on Luria-Bertani agar or in Luria-Bertani broth, and *S. flexneri* strains were grown on Trypticase soy agar or in Trypticase soy broth at 37°C. Antibiotics were used at the following concentrations: ampicillin, 100 μg/ml; kanamycin, 50 μg/ml; and streptomycin, 100 μg/ml.

**Construction of a nonpolar cassette.** A cassette containing a kanamycin resistance gene was constructed by polymerase chain reaction (PCR) with plasmid pAT21 (38), which carries the *aphA-3* gene from *Enterococcus faecalis*. One primer was 44 nucleotides in length (5'-CCCCCCGGGTGACTAAC TAGGAGGAATAAATGGCTAAAATGAGA-3') and contained 20 bases complementary to the 5' region of the *aphA-3* gene (underlined). The other primer was 43 nucleotides in length (5'-CCCCCCGGGTCATTATTCCCTCCAGGTACT AAAACAATTCATC-3') and contained 20 nucleotides complementary to the opposite strand of the 3' region of the *aphA-3* gene (underlined). A *Sma*I site (5'-CCCGGG-3') was included in each oligonucleotide to allow cloning of the PCR product. DNA amplification was performed by using 2 ng of circular template DNA and 1 μM each primer in a 100-μl reaction mixture containing 50 mM Tris-HCl (pH 8.8), 15 mM ammonium sulfate, 3 mM $MgCl_2$, 10 mM β-mercaptoethanol, and 200 μM each deoxynucleoside triphosphate. Thirty cycles of 1 min of denaturation at 94°C, 2 min of primer annealing at 42°C, and 3 min of extension by *Taq* polymerase at 72°C were carried out.

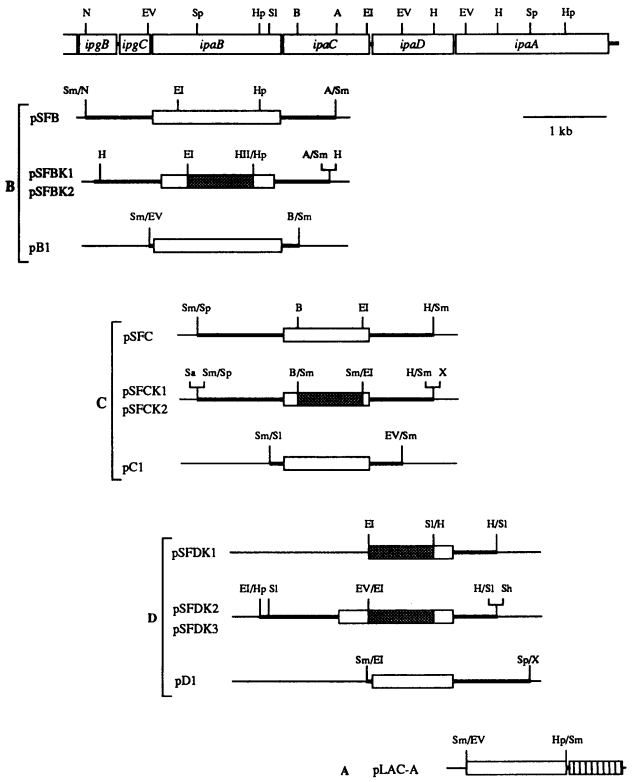


FIG. 2. Genetic organization of part of the *ipa* operon (top) and plasmids used in this study. Construction of plasmids used to generate and complement the *ipaB2* mutant (B), the *ipaC2* mutant (C), and the *ipaD2* mutant (D) and construction of plasmid pLAC-A (A) are detailed in Materials and Methods. The *ipaB*, *ipaC*, and *ipaD* genes of the wild-type strain M90T were inactivated by allelic exchange between the large virulence plasmid pWR100 and the suicide plasmids pSFBK2 (*ipaB*), pSFCK2 (*ipaC*), or pSFDK3 (*ipaD*). The *ipaA* gene was inactivated by integration of pLAC-A, which carries a DNA fragment internal to *ipaA*, into pWR100. The *aphA-3* resistance gene is indicated by a stippled box, and the *lacZ* gene (not shown to scale) is indicated by a striped box. Restriction enzyme sites: A, *Afl*II; B, *Bcl*I; EI, *Eco*RI; EV, *Eco*RV; H, *Hin*dIII; HII, *Hin*cII; Hp, *Hpa*I; N, *Nru*I; Sa, *Sac*I; Sh, *Sph*I; Sl, *Sal*I; Sm, *Sma*I; Sp, *Spe*I; X, *Xba*I.

The PCR products were resolved on 0.8% agarose gels, and a DNA fragment of the expected size (850 bp) was purified, cleaved by *Sma*I, and cloned into the *Sma*I site of pUC18 (23) to generate plasmid pUC18K. In this construct (Fig. 1), the *aphA-3* gene is transcribed from the *lac* promoter of the vector and confers resistance to kanamycin. Nucleotide sequencing of the *aphA-3* flanking regions confirmed that no error had occurred at the extremities of the cloned fragment.

**Construction of strains SF620 (*ipaB2*), SF621 (*ipaC2*), and SF622 (*ipaD2*) and plasmids pB1, pC1, and pD1.** The different steps in the construction of mutant strains SF620 (*ipaB2*), SF621 (*ipaC2*), and SF622 (*ipaD2*) are shown in Fig. 2. All plasmids are derivatives of pHS4108 (21).

To construct the *ipaB2* mutant, plasmid pSFB was first obtained by cloning a *Nru*I-*Afl*II fragment that contains the *ipaB* gene and flanking regions into a derivative of vector pUC18 that lacks the *Eco*RI site. The *Eco*RI-*Hpa*I segment of pSFB, internal to *ipaB*, was then replaced by an *Eco*RI-*Hin*cII fragment of pUC18K that carries the *aphA-3* cas-

sette. Recombinant plasmid pSFBK1 contained the *aphA-3* gene inserted in the correct orientation, thereby placing the ATG codon downstream from the resistance gene and in frame with the TGA codon of *ipaB*. A filled-in *Hin*dIII fragment of pSFBK1 that encompasses the mutated *ipaB* gene was then cloned into pGP704, a derivative of the Ap$^r$ suicide vector pJM703.1 (25), to yield plasmid pSFBK2. This plasmid was transferred to the *S. flexneri* strain M90T-Sm by conjugal mating, and, among transconjugants, clones in which a double recombinational event had exchanged the wild-type *ipaB* gene for the mutated copy were screened for their sensitivity to ampicillin, i.e., loss of the suicide plasmid. Southern analysis of one such strain, called SF620, confirmed the expected structure of the large plasmid carrying the *ipaB2* mutation.

To generate the *ipaC2* mutant, a filled-in *Spe*I-*Hin*dIII fragment that encompasses the *ipaC* gene was first cloned into a derivative of pUC18 that lacks the *Eco*RI site to yield plasmid pSFC. Plasmid pSFCK1 was obtained by replacing the *Bcl*I-*Eco*RI fragment of plasmid pSFC, internal to *ipaC*, by the *Sma*I insert of plasmid pUC18K that carries the *aphA-3* gene. The *Sac*I-*Xba*I segment of plasmid pSFCK1 was then inserted into vector pGP704 to give rise to pSFCK2. The latter plasmid was transferred to M90T-Sm by conjugal mating, and transconjugant SF621, screened as described for SF620, was shown to have undergone the expected double recombinational event by Southern analysis.

To construct the *ipaD2* mutant, plasmid pSFDK1 was obtained by cloning a filled-in *Hin*dIII fragment containing the 3′ region of *ipaD* into the filled-in *Sal*I site of plasmid pUC18K, thereby placing the start codon of the cassette in the same reading frame as the stop codon of *ipaD*. Plasmid pSFDK2 was constructed by inserting the *Hpa*I-*Eco*RV fragment that carries the 5′ region of *ipaD* into the filled-in *Eco*RI site of pSFDK1. The *Sal*I-*Sph*I fragment of plasmid pSFDK2 that contains the *ipaD2* mutation was then cloned into vector pGP704 to yield plasmid pSFDK3. The latter plasmid was transferred to M90T-Sm by conjugal mating, and transconjugant strain SF622, identified as described for SF620, was shown by Southern blotting to harbor the *ipaD2* mutation on its large plasmid.

Plasmids pB1, pC1, and pD1 were used for complementation assays. Plasmid pB1 was obtained by cloning the *Eco*RV-*Bcl*I fragment of plasmid pSFB that encompasses *ipaB* into pUC18. Plasmid pC1 was constructed by cloning the *Sal*I-*Eco*RV fragment of plasmid pSFC that contains *ipaC* into pUC18. Plasmid pD1 was constructed by cloning the *Eco*RI-*Spe*I fragment that encompasses *ipaD* into pUC18. The *ipaB*, *ipaC*, and *ipaD* genes in plasmids pB1, pC1, and pD1, respectively, are in the same orientation as the *lac* promoter of pUC18.

**Construction of strains SF623 (*ipaA*::*lacZ*) and SF624 (*ipaA*::*lacZ ipaD2*) and β-galactosidase assays.** The *ipaA* mutant was obtained by integration of the suicide plasmid pLAC-A (Fig. 2). Plasmid pLAC-A was constructed by cloning the *Eco*RV-*Hpa*I fragment, internal to *ipaA*, into the *Sma*I site located upstream from the *lacZ* reporter gene in vector pLAC-1 (3). In pLAC-A, the *ipaA* fragment is in the same orientation as *lacZ*. This plasmid was transferred to M90T-Sm, and, among Ap$^r$ clones that arose through integration of the suicide plasmid, strain SF623 harbored the expected *ipaA1* mutation, which consists of two truncated copies of *ipaA*, the first of which is part of an *ipaA*::*lacZ* transcriptional fusion.

Plasmid pLAC-A was also transferred to strain SF622 (*ipaD2*). Integration of pLAC-A into the large plasmid of SF622 gave rise to strain SF624, which thus carries the *ipaA*::*lacZ* fusion and the *ipaD2* mutation. Southern analysis confirmed the correct structure of the large plasmid derivatives of strains SF623 and SF624.

β-Galactosidase activity was assayed as described by Miller (24) with the substrate *o*-nitrophenol-β-D-galactoside.

**Preparation of secreted proteins, SDS-PAGE, and immunoblotting.** Bacteria in the exponential phase of growth were harvested by centrifugation (13,000 × *g*, 20 min). Crude extracts of bacteria were obtained from the bacterial pellet, and proteins of the culture supernatant were precipitated by the addition of 1/10 (vol/vol) trichloracetic acid. Electrophoresis in 10% polyacrylamide gels in the presence of sodium dodecyl sulfate (SDS-PAGE) was performed as described by Laemmli (20). After electrophoresis, proteins were transferred to a nitrocellulose membrane. Immunoblotting procedures were carried out with mouse monoclonal antibodies (MAbs) H4 and J22, which recognize IpaB and IpaC, respectively (8, 31), and rabbit polyclonal antisera directed against IpaD (see below) or alkaline phosphatase. Horseradish peroxidase-labelled goat anti-mouse or goat anti-rabbit antibodies were used as secondary antibodies and visualized by enhanced chemiluminescence.

**Virulence assays.** Bacterial invasion of HeLa cells was performed as previously described (27). Briefly, bacteria at a multiplicity of infection of 100 were centrifuged onto semiconfluent monolayers (700 × *g*, 10 min), and infection was carried out for 3 h without antibiotics, followed by a further 3 h with gentamicin (50 μg/ml) to eliminate extracellular bacteria. Giemsa-stained preparations were examined for intracellular bacteria. Contact hemolytic activity was detected as previously described (33). Zones of F-actin accumulation within HeLa cells were visualized by fluorescence microscopy by staining cells with 7-nitrobenz-2-oxy-1,3-diazole (NBD)-Phalloidin (Molecular Probes, Junction City, Oreg.) for 20 min, essentially as described elsewhere (12).

Adhesion tests were performed as follows. Bacteria grown in Trypticase soy broth ($A_{600}$ = 0.3) were harvested by low-speed centrifugation (2,000 × *g*, 10 min), resuspended in minimum essential medium, and centrifuged onto HeLa cells (700 × *g*, 10 min) at a multiplicity of infection of 10 or 100. After various infection periods at 37°C, infected cells were washed five times in phosphate-buffered saline (PBS), fixed, and Giemsa stained. The numbers of adherent bacteria per cell, bound at the periphery or over the surface of the cells, were evaluated by examining 100 HeLa cells in each of three separate experiments.

**Preparation of anti-IpaD polyclonal antiserum.** The *Eco*RV fragment that contains the 3′ portion of *ipaD* (Fig. 2) was cloned into the filled-in *Eco*RI site of plasmid pMAL-p2 (New England Biolabs, Beverly, Mass.) to generate a gene fusion between the *E. coli malE* gene, which encodes the periplasmic maltose-binding protein, and the 3′ portion of the *ipaD* gene. The fusion protein expressed in *E. coli* was purified from periplasmic extracts (28) by affinity chromatography over an amylose column (11). The purified MalE-IpaD protein was used to immunize male New Zealand White rabbits (Charles River, Saint Aubin les Elbeuf, France) according to the following protocol. Initially, 100 μg of the protein mixed with complete Freund adjuvant was injected intradermally. Subsequently, two booster injections of 100 μg of antigen, each without adjuvant, were given: the first subcutaneously after 3 weeks, and the second intramuscularly 2 weeks later. Three weeks after the last booster injection, the rabbits were bled. The serum samples were

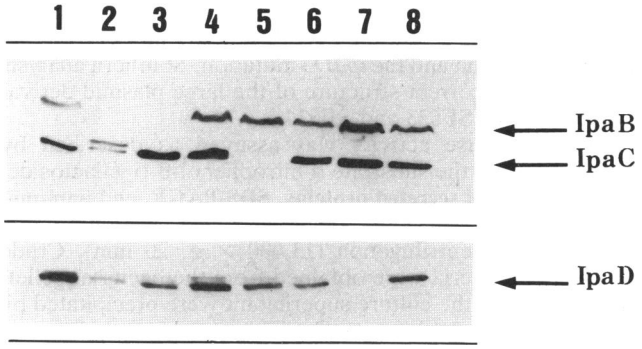


FIG. 3. Immunoblot analysis of the IpaB, IpaC, and IpaD proteins produced in the *ipa* mutants. Samples of crude extracts of bacteria corresponding to 50 μl of bacterial culture ($A_{600}$ = 1) were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and reacted with a mixture of MAbs directed against IpaB and IpaC (upper panel) or an anti-IpaD polyclonal antiserum (lower panel). Lanes: 1, M90T (wild type); 2, SC403 (*ipaB1*); 3, SF620 (*ipaB2*); 4, SF620(pB1); 5, SF621 (*ipaC2*); 6, SF621(pC1); 7, SF622 (*ipaD2*); 8, SF622(pD1).

tested on crude extracts of the wild-type M90T, the plasmidless derivative BS176, the *ipaD2* mutant, and *E. coli* MC1061 carrying plasmid pD1. The serum samples strongly recognized the 36-kDa IpaD protein.

## RESULTS

**Construction of a nonpolar cassette.** In a previous work, the *ipaB1* noninvasive mutant SC403 was constructed by allelic exchange of the wild-type gene with an interposon-mutagenized *ipaB* gene (15). Despite the presence of a constitutive promoter inserted at the 3′ end of the interposon, the mutant strain produced only small amounts of the downstream gene products IpaC and IpaD (Fig. 3, lane 2). To generate nonpolar mutations in the *ipa* operon, we constructed a selectable cassette that does not contain a promoter or transcription terminator by using PCR on the cloned *aphA-3* resistance gene (see Materials and Methods and Fig. 1). The *aphA-3* gene carried by this cassette is preceded by translation stop codons in all three reading frames and is immediately followed by a consensus ribosome-binding site and a start codon. Use of appropriate restriction sites allows the placement of the start codon at the 3′ end of the cassette in the reading frame of the stop codon of the mutated gene, so that translation of the remaining 3′ portion of the mutated gene could allow translational coupling, if any, with the downstream gene.

**Construction of *ipaB*, *ipaC*, *ipaD*, and *ipaA* mutants.** The *ipa* genes on the large plasmid of *S. flexneri* M90T were mutated by either allelic exchange or a plasmid integration technique. Constructions are detailed in Materials and Methods and are summarized in Fig. 2. Each of the mutations in the *ipaB*, *ipaC*, and *ipaD* genes consists of the replacement of an internal fragment of the gene by the *aphA-3* cassette. Since the gene located downstream from *ipaA*, the *virB* gene, is transcribed from its own promoter (37), the *ipaA* gene was mutated by integration of a suicide plasmid. The latter, pLAC-A, contains an internal fragment of *ipaA* inserted upstream from a promoterless *lacZ* gene. The integration event thus generated two truncated copies of *ipaA*, the first of which creates an *ipaA*::*lacZ* transcriptional fusion. The mutant alleles of the genes were designated *ipaB2*, *ipaC2*, *ipaD2*, and *ipaA1*, and *S. flexneri* strains carrying these mutations were named SF620, SF621, SF622, and SF623, respectively.

**The *ipaB2*, *ipaC2*, and *ipaD2* mutations are nonpolar.** To confirm the nonpolar nature of the *ipaB2* and *ipaC2* mutations, immunoblot analysis (Fig. 3) was performed on whole-cell extracts of strains M90T (wild type), SC403 (*ipaB1*), SF620 (*ipaB2*), and SF621 (*ipaC2*), with a mixture of MAbs directed against the 62-kDa IpaB and 41-kDa IpaC polypeptides and a polyclonal antiserum directed against the 36-kDa IpaD protein. Strain SC403 (lane 2) produced a 42-kDa protein, corresponding to the truncated IpaB protein encoded by the *ipaB1* allele (15), and amounts of IpaC and IpaD that were less than those produced by the wild type (lane 1), thus confirming the polar effect of the *ipaB1* mutation. In contrast, IpaC and IpaD were produced in similar amounts by the wild-type (lane 1) and *ipaB2* (lane 3) strains, and IpaD was produced in similar amounts by the wild-type and *ipaC2* (lane 5) strains.

We confirmed the nonpolar nature of the *ipaD2* mutation by comparing the β-galactosidase activities produced by the *ipaA*::*lacZ* transcriptional fusion in the presence or absence of the upstream *ipaD2* mutation. Totals of 530 and 550 U of β-galactosidase activity were produced by strains SF623 (*ipaA*::*lacZ*) and SF624 (*ipaA*::*lacZ ipaD2*), respectively, indicating that the *ipaD2* mutation did not affect *ipaA* transcription. To ensure that downstream transcription was not initiated from a promoter located in the *aphA-3* coding sequence, we made use of the transcriptional control of the *ipa* operon by growth temperature (22) and compared thermoregulation of the *ipaA*::*lacZ* fusion in strains SF623 (*ipaA*::*lacZ*) and SF624 (*ipaA*::*lacZ ipaD2*). The low levels of β-galactosidase activity obtained at 30°C were similar in the two strains, indicating that insertion of the *aphA-3* cassette preserves wild-type transcription of downstream genes.

**The *ipaB2*, *ipaC2*, and *ipaD2* mutants are defective in invasion-related tests.** The Sereny test and the HeLa cell infection assay were first used to assess the virulence properties of the *ipa* mutants. The *ipaB2*, *ipaC2*, and *ipaD2* mutants were each unable to provoke keratoconjunctivitis in guinea pigs. In addition, none of these three mutants could be detected inside HeLa cells, even after long incubation periods (4 h). They were each restored to virulence in both tests when complemented with a plasmid expressing a wild-type copy of the mutated gene, pB1 (*ipaB*), pC1 (*ipaC*), and pD1 (*ipaD*) (Fig. 2), respectively. In contrast, the *ipaA1* mutant could still invade HeLa cells and induce keratoconjunctivitis in guinea pigs. The phenotype of the *ipaA1* mutant was not further characterized.

Penetration of HeLa cells by invasive *Shigella* cells has been shown to coincide with localized and transient actin polymerization at the site of bacterial entry (12). We tested whether the *ipaB2*, *ipaC2*, and *ipaD2* mutants could still trigger actin polymerization despite their noninvasive phenotype. None of the *ipaB*, *ipaC*, and *ipaD* mutants was able to induce the actin polymerization at the site of bacterial attachment which is characteristic of the first step of entry of wild-type *Shigella* strains (12). To allow strong binding to HeLa cells, the ability to induce actin polymerization was also studied with strains carrying the pIL22 plasmid, which encodes the *E. coli* afimbrial adhesin AFA-I and confers a highly adhesive phenotype on cells of human origin (12, 18). The three noninvasive mutants remained uniformly bound to unaltered cells, and upon labelling with NBD-Phalloidin, no evidence of aggregates of filamentous actin could be ob-

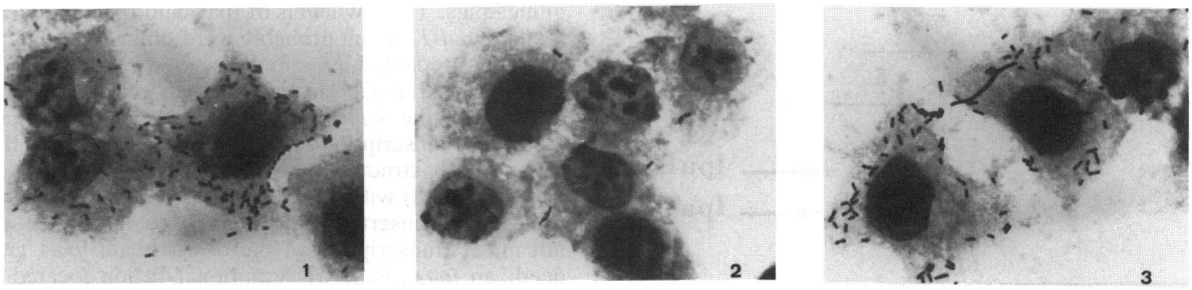


FIG. 4. Adhesion assay. HeLa cells were infected with the *ipaB2* (1), *ipaC2* (2), and *ipaD2* (3) mutants. Bacteria were centrifuged onto HeLa cells at a multiplicity of infection of 10. After 2 h at 37°C, infected cells were washed five times in PBS, fixed, and Giemsa stained.

served, whereas large foci of F-actin were labelled at sites of interaction of the wild-type strain M90T (harboring pIL22) with cytoplasmic membranes (data not shown).

Intracellular multiplication, ability to lyse the phagocytic vacuole, and contact hemolysis are three correlated virulence phenotypes having thus far indistinguishable genetic bases (33). The contact hemolytic activities of the *ipaB2*, *ipaC2*, and *ipaD2* mutants, M90T, and BS176 (a plasmidless derivative of M90T) were compared. Whereas M90T was strongly hemolytic, the levels of hemolysis provoked by each of the mutants were negligible (less than 10% of that of M90T) and similar to that of the plasmidless strain. These results indicate that IpaB, IpaC, and IpaD are each necessary for both inducing entry and lysing of the phagosomal membrane.

**The *ipaB2*, *ipaC2*, and *ipaD2* mutants are not defective in adhesion to HeLa cells.** Although *S. flexneri* is only poorly adherent to HeLa cells in vitro (30), we investigated whether the noninvasive phenotype of the *ipaB2*, *ipaC2*, and *ipaD2* mutants could be due to an impairment in adhesion to the cells. To evaluate the adhesion phenotypes of the *ipa* mutants, their adhesion capabilities were compared with those of M90T and BS176 after a short infection period (15 min) to avoid entry and intracellular multiplication of M90T. Results of four independent experiments indicated that the mutants and M90T displayed similar levels of adhesion to HeLa cells, which were fivefold higher than that of BS176.

The noninvasive phenotype of the mutants allowed examination of their attachment to the cells during prolonged infection periods. After 2 h, the *ipaB2* and *ipaD2* strains appeared to be highly adherent to HeLa cells and were frequently seen as clusters of bacteria on the surface of the monolayer (Fig. 4). In contrast, the *ipaC2* mutant displayed a weaker adhesive phenotype, consisting of isolated bacteria bound predominantly at the periphery of the cells in regions of focal contacts. On average, after a 2-h infection period, 10- to 50-fold more bacteria were adherent for each of the *ipaB2* and *ipaD2* mutants than were adherent for the *ipaC2* mutant.

We also tested whether the *ipaC2* mutant could be made invasive by enabling it to strongly adhere to the cells, i.e., when carrying plasmid pIL22. The *ipaC2* mutant harboring pIL22 could not be found within cells after treatment of the monolayers with gentamicin, whereas M90T(pIL22) was invasive. These results suggest that loss of invasiveness of the *ipaC2* mutant, like that of the *ipaB2* and *ipaD2* mutants, is not due to impairment of adhesion.

**The *ipaB2*, *ipaC2*, and *ipaD2* mutants still secrete the other Ipa proteins.** Recent work has shown that IpaB and IpaC are released into the culture supernatant of wild-type bacteria grown in vitro (4–6, 39). Using a polyclonal antiserum raised against IpaD, we found that this protein, like IpaB and IpaC, was secreted into the culture supernatant of the wild-type strain (Fig. 5, lane 1). In light of the similar phenotypes of the *ipaB2*, *ipaC2*, and *ipaD2* mutants, we tested whether one of the IpaB, IpaC, and IpaD proteins could be involved in export of the other two by analyzing the secreted Ipa proteins in these three mutants. Immunoblot analysis showed that lack of IpaB, IpaC, or IpaD did not impede secretion of each of the other two (Fig. 5, lanes 2, 5, and 7, respectively), which indicates that the loss of invasion of the three *ipa* mutants is not due merely to a defect in secretion of the other Ipa proteins.

**The *ipaB2* and *ipaD2* mutants oversecrete the other Ipa proteins.** Comparison of the amounts of antigens secreted by the wild type and the *ipa* mutants indicated that increased amounts of IpaC and IpaD were present in the culture supernatant of the *ipaB2* mutant (Fig. 5, lane 2) compared with that of the wild type and that, similarly, increased amounts of IpaB and IpaC were secreted into the supernatant of the *ipaD2* mutant (lane 7). In contrast, the *ipaC2* mutant (lane 5) secreted similar or only slightly increased levels of IpaB and IpaD compared with those of the wild-type strain. These alterations could be reverted by complementation of each of the mutants with a plasmid carrying the wild-type copy of the mutated gene, thus confirming the role of the respective mutations in oversecretion.

To determine whether the *ipaB2* and *ipaD2* mutations had

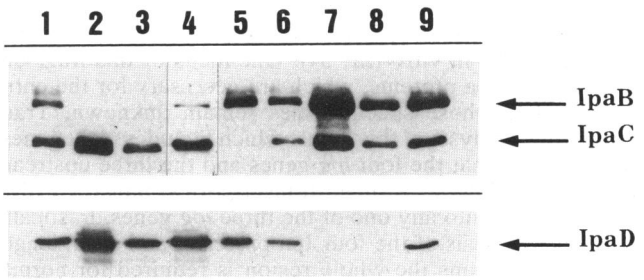


FIG. 5. Immunoblot analysis of the IpaB, IpaC, and IpaD proteins secreted into the supernatant by the *ipa* mutants. Samples of secreted proteins corresponding to 1 ml of bacterial culture ($A_{600}$ = 1) were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and reacted with a mixture of MAbs directed against IpaB and IpaC (upper panel) or an anti-IpaD polyclonal antiserum (lower panel). Lanes: 1, M90T (wild type); 2, SF620 (*ipaB2*); 3, SF620, diluted 1:10; 4, SF620(pB1); 5, SF621 (*ipaC2*); 6, SF621 (pC1); 7, SF622 (*ipaD2*); 8, SF622, diluted 1:20; 9, SF622(pD1).

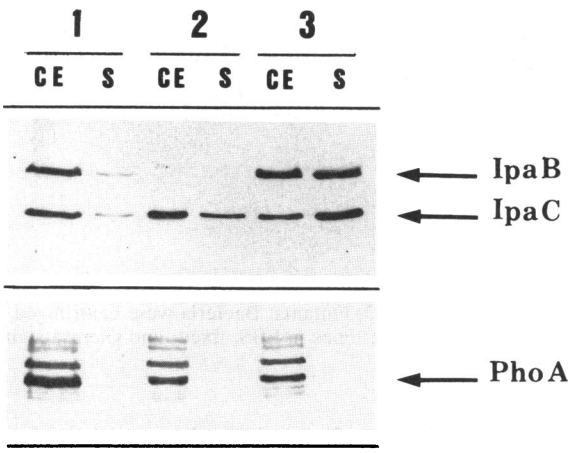


FIG. 6. Immunoblot analysis of the IpaB, IpaC, and PhoA proteins produced and secreted into the supernatant by the *ipaB2* and *ipaD2* mutants. Analysis was performed on samples of crude extracts (CE) and secreted proteins (S) corresponding to 15 μl of bacterial culture ($A_{600}$ = 1), with a mixture of MAbs directed against IpaB and IpaC (upper panel) or an anti-PhoA polyclonal antiserum (lower panel). Lanes: 1, M90T(pAB44); 2, SF620(pAB44); 3, SF622 (pAB44).

any effect on bacterial lysis or on the release of periplasmic proteins, the amount of periplasmic alkaline phosphatase in the supernatant of the mutants was compared with that of the wild-type strain. Plasmid pAB44, which contains the *phoA* gene fused to the first 64 codons of the *ipgF* gene (2) and produces an exported alkaline phosphatase, was transformed into the wild-type, the *ipaB2*, and the *ipaD2* strains. Immunoblot analysis with a polyclonal antiserum directed against *E. coli* alkaline phosphatase and the mixture of anti-IpaB and anti-IpaC MAbs was performed on crude extracts and on secreted proteins of the transformed strains (Fig. 6). No alkaline phosphatase was detected in the supernatants of the strains, implying that the Ipa antigens found in the supernatants of the *ipaB2* and *ipaD2* mutants were not released through bacterial lysis or nonspecific leakage of periplasmic proteins.

## DISCUSSION

A 31-kb fragment of pWR100, the 220-kb virulence plasmid of *S. flexneri* (32), is necessary for bacterial entry into epithelial cells in vitro (21, 34). One locus of this fragment encodes the Ipa proteins, which are necessary for the entry process but whose specific roles remain unknown. Transcriptional analysis of this locus, which includes eight genes, has revealed that the four *ipa* genes and the three upstream *ipg* genes belong to a single transcriptional unit (3, 9, 34). Tn*5* insertion into any one of the three *ipg* genes drastically reduces synthesis of the four Ipa proteins, and a full-length mRNA that spans the whole region is required for normal expression of the *ipa* genes (34). In a recent work, the *ipaB1* mutant SC403 was constructed by allelic exchange of the wild-type gene with an interposon-mutagenized *ipaB* gene (15). However, although a constitutive promoter was inserted at the 3′ end of the interposon, the *ipaB1* mutant produced only small amounts of the downstream gene products IpaC and IpaD. While in *trans* expression of *ipaB* in the *ipaB1* mutant could restore invasion into HeLa cells, it could not restore the capacity to induce keratoconjunctivitis in guinea pigs. The low levels of IpaC and IpaD in the complemented *ipaB1* mutant probably were sufficient for invasion in vitro but precluded infection in vivo.

The partial polar effect observed in the *ipaB1* mutant highlighted the need for a cassette that could preserve wild-type transcription of downstream genes. For this purpose, we constructed a cassette carrying the *aphA-3* resistance gene (38) without its promoter or transcription terminator. When inserted into the *ipaD* gene, this cassette did not affect transcription of the downstream *ipaA* gene. Indeed, an *ipaA*::*lacZ* transcriptional fusion located on the large plasmid downstream from the cassette-induced *ipaD2* mutation produced levels of β-galactosidase activity similar to those for the same fusion located downstream from the wild-type *ipaD* gene. Furthermore, transcription of the *ipaA*::*lacZ* fusion located downstream from the *ipaD2* mutation was under the control of the natural promoter of the *ipaA* gene, since expression of this fusion was still under wild-type temperature regulation. This suggests that insertion of the *aphA-3* cassette into any gene within an operon of gram-negative bacteria should respect transcription of downstream genes.

It is likely that the involvement of the Ipa proteins in the entry process requires stoichiometric amounts of each of these proteins. In an attempt to ensure synthesis of wild-type amounts of downstream gene products, a translation start signal was placed at the 3′ end of the cassette. This signal includes the Shine-Dalgarno sequence most often encountered in genes of the 31-kb invasion fragment (5′-GGAGG-3′) and a start codon which initiates translation of the 3′ remaining portion of the mutated gene. Translation of this fragment should allow translational coupling with a downstream gene(s), which might occur between some of the overlapping or closely linked genes of the *Shigella* virulence operons. We have confirmed by immunoblot analysis that similar amounts of IpaC were synthesized by the wild-type strain and the *ipaB2* mutant and that similar amounts of IpaD were produced by the wild-type strain and the *ipaB2* and *ipaC2* mutants. Moreover, each of the three noninvasive mutants was reverted to a fully virulent phenotype, both in cell invasion in vitro and in the Sereny test in vivo, by in *trans* expression of the corresponding wild-type gene alone. Similarly, an *aphA-3* cassette-generated mutant of the *mxiD* gene, which belongs to the *mxi* operon of the 31-kb invasion fragment, could be restored to invasion when carrying the *mxiD* gene in *trans* (5). Thus, when inserted into a gene of the virulence-associated operons of *Shigella* strains, the *aphA-3* cassette also preserves synthesis of wild-type amounts of downstream gene products.

Analysis of the phenotypes of the *ipaB2*, *ipaC2*, and *ipaD2* nonpolar mutants and of an *ipaA* mutant obtained by integration of a suicide plasmid has confirmed that IpaB, IpaC, and IpaD, but not IpaA, are involved in cell invasion in vitro (15, 35). In addition, the three nonpolar mutants each failed to enter into HeLa cells or to trigger even low levels of actin polymerization, which demonstrates that IpaB, IpaC, and IpaD are each necessary for entry into epithelial cells. We have shown that the noninvasive phenotype of the *ipaB2*, *ipaC2*, and *ipaD2* mutants could not be ascribed to a loss of adhesion to the cells. However, whereas the *ipaC2* mutant displayed a wild-type adhesive phenotype, the *ipaB2* and *ipaD2* mutants bound strongly to HeLa cells. After long infection times, the *ipaB2* and *ipaD2* mutants appeared in clusters of bacteria covering HeLa cells, a phenotype that is similar to that of the *ipaB1* mutant SC403 (15). The molecular basis of this phenotype remains unclear. Since the *ipaC2*

mutant did not display such a phenotype, the mere multiplication of noninvasive bacteria on HeLa cell membranes cannot explain this overadhesive phenotype. Rather, since the *ipaB2* and *ipaD2* mutants also secrete higher amounts of the Ipa proteins, these or other oversecreted proteins could be involved in the enhanced adhesive properties and in the clustering of bacteria. In any case, a defect in cell adhesion of the *ipaB2*, *ipaC2*, and *ipaD2* mutants cannot account for loss of their invasiveness. In addition, these three *ipa* mutants did not produce any hemolytic activity, which suggests that IpaB, IpaC, and IpaD are also involved in escape from the phagosome.

Although IpaB and IpaC have been detected at the surface of bacteria with MAbs in a whole-cell enzyme-linked immunosorbent assay (26), protection experiments have shown that the bulk of IpaB and IpaC in wild-type cells is intracellular rather than surface associated (6). Approximately 10% of the total IpaB and IpaC is released into the supernatant of wild-type bacteria (5, 6, 39). However, the biological function of the secreted Ipa antigens is unclear. Secreted proteins could be active proteins involved in the entry process or molecules detached from the surface or released from an inner compartment of bacteria. Both surface presentation and secretion of IpaB and IpaC are dependent on numerous *mxi* and *spa* genes, which span a 20-kb region of the invasion fragment (4–6, 39). All *mxi* and *spa* mutants characterized so far are noninvasive and do not present at the surface or secrete IpaB and IpaC. We have shown that IpaD is also secreted and that the lack of IpaB, IpaC, or IpaD does not impede secretion of the other two Ipa proteins. This indicates that none of these Ipa proteins is necessary for the secretion of the others and thus strongly suggests that each of these Ipa proteins is an effector of the entry process. Since IpaB, IpaC, and IpaD are each secreted and required for the achievement of entry into epithelial cells and escape from the phagosome, two major hypotheses of interactions of these three Ipa can be considered: (i) IpaB, IpaC, and IpaD and possibly other proteins form a complex endowed with invasive and membranolytic capacities; and (ii) one or more of these Ipa proteins is involved in the correct presentation or activation of another Ipa protein that can act alone as an invasin or a membrane-lysing toxin.

Moreover, in contrast to the *ipaC2* mutant, which secreted normal amounts of IpaB and IpaD, the *ipaB2* and *ipaD2* mutants secreted much larger amounts of the remaining Ipa. In the *ipaB2* and *ipaD2* mutants, 50% of the total Ipa antigens were found in the culture supernatant. This phenotype is not due to bacterial lysis or to a nonspecific release of periplasmic proteins, since alkaline phosphatase produced in the *ipaB2* and *ipaD2* mutants was not recovered from the supernatants of these cells. This oversecretion phenotype is reminiscent of that of the *papH* mutant of Pap pilus-producing uropathogenic *E. coli*, which releases large amounts of the major pilin subunit PapA into the culture medium (7). PapH anchors the pilus to the outer membrane of the bacterial cell (17). However, macromolecular structures, such as pili, have not been detected at the bacterial surfaces of invasive *Shigella* strains. IpaB and IpaD and to a lesser extent IpaC might be necessary for stable maintenance of the Ipa antigens as a complex. This putative Ipa complex could be located either at the bacterial surface prior to invasion or in an inner compartment from which it could be efficiently secreted after binding to the cell. In any case, the association of the putative complex with bacteria requires the presence of both the IpaB and the IpaD proteins.

The Ipa proteins appear to be functionally distinct from other bacterial invasins described so far, such as invasin of *Yersinia pseudotuberculosis* (16) and internalin of *Listeria monocytogenes* (13), which are able to induce entry into epithelial cells by themselves. The invasion process of *S. flexneri*, including induced phagocytosis and lysis of the phagosomal membrane, emerges as a complex process directed by the IpaB, IpaC, and IpaD proteins whose functions include stable maintenance of one another within or at the surface of bacteria. The existence of an invasion complex and, if it exists, its localization, will be important topics in the understanding of the molecular basis of the invasive process of *S. flexneri* into epithelial cells.

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