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Characterization of the *Shigella flexneri ipgD* and *ipgF* Genes, Which Are Located in the Proximal Part of the *mxi* Locus

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The *Shigella flexneri* invasion process requires the synthesis of the Ipa proteins and their secretion by specific factors encoded by the *mxi* and *spa* genes, which are clustered upstream from the *ipa* operon. We report here the characterization of the *ipgD*, *ipgE*, and *ipgF* genes, which are located in the 5′ end of the *mxi* locus. Analysis of IpgF-PhoA fusions endowed with high levels of alkaline phosphatase activity confirmed the functionality of a classical signal sequence detected in the sequence of IpgF. The *ipgD* and *ipgF* genes were each inactivated on the large virulence plasmid by insertion of a nonpolar cassette; each of the *ipgD* and *ipgF* mutants thus constructed showed the same invasive phenotype as the wild-type strain and was also able to provoke keratoconjunctivitis in guinea pigs. It thus appears that two genes located at the *ipa*-proximal part of the *mxi* locus are not directly involved in invasion. Analysis of concentrated culture supernatants of the wild-type and *ipgD* strains indicated that secretion of one polypeptide, whose size was consistent with that predicted for the IpgD protein (60 kDa), was abolished in the *ipgD* mutant.

Shigella spp. are gram-negative microorganisms that cause bacillary dysentery in humans by invading epithelial cells of the colonic mucosa (20). The *S. flexneri* invasion process (reviewed in references 15 and 39) consists of entry of the bacteria into epithelial cells by a mechanism similar to phagocytosis (11), lysis of the membrane of the phagocytic vacuole (41), movement of the bacteria within the cytoplasm of infected cells (23, 33, 34), formation of protrusions by which bacteria pass into adjacent cells through an actin-based movement (10, 18), and lysis of the cellular membranes of the protrusions, which releases the bacteria into the cytoplasm of adjacent cells and completes the process of intercellular spread (2).

The virulence properties of *S. flexneri* are associated with the presence of a 220-kb plasmid (40). The IpaA, IpaB, IpaC, and IpaD polypeptides (invasion plasmid antigens), which are dominant antigens in the humoral immune response during shigellosis (31), are encoded on this virulence plasmid. Subcloning into a cosmid and Tn*5* mutagenesis allowed the definition of a 37-kb fragment of the virulence plasmid that includes the *ipa* genes and that is necessary and sufficient for expression of invasive functions (26). Subsequent analysis indicated that the *ipaB*, *ipaC*, *ipaD*, and *ipaA* genes are clustered in an operon and that the IpaB, IpaC, and IpaD proteins are essential for the entry process (7, 8, 16, 42, 43, 49, 50). The region necessary for invasion that is located upstream from the *ipa* locus (26, 43) is involved in export and secretion of the Ipa proteins (3–5, 17, 51). Transposon insertions in the *mxiA* gene (membrane expression of invasion plasmid antigens) and in the *spa* locus (surface presentation of invasion plasmid antigens), which is located downstream from *mxiA*, abolish surface presentation and secretion of IpaB and IpaC (5, 51). We have recently characterized the *mxiD* and *mxiJ* genes, which are located between the *ipa* locus and *mxiA* and whose products are necessary for secretion of the Ipa proteins (3, 4).

We report here the characterization of the *ipgD*, *ipgE*, and *ipgF* genes, which are located on the virulence plasmid pWR100 at the 5′ end of the *mxi-spa* locus. The three genes are very close to one other, which suggests that they might belong to the same operon. Examination of the deduced amino acid sequence of IpgF suggested that IpgF was exported, which was confirmed by analysis of IpgF-PhoA fusion proteins endowed with high levels of alkaline phosphatase activity. An *ipgD* mutant and an *ipgF* mutant, constructed by insertion of a nonpolar cassette in the *ipgD* and *ipgF* genes, respectively, showed the same invasive phenotype as the wild-type strain and were also able to provoke keratoconjunctivitis in guinea pigs. Analysis of concentrated culture supernatants of the wild-type and *ipgD1* strains indicated that secretion of one polypeptide, whose size was consistent with that predicted for the IpgD protein, was abolished in the *ipgD* mutant.

MATERIALS AND METHODS

Bacterial strains and growth media. *S. flexneri* M90T (wild type), its plasmidless derivative BS176, and M90T-Sm, a streptomycin-resistant derivative of M90T, have been previously described (2, 40). *Escherichia coli* DH5α (53) was used for plasmid constructions, *E. coli* SM10λ*pir* (30) was used to transfer plasmids to *S. flexneri*, and *E. coli* CC118 (25) was used for Tn*phoA* mutagenesis. Bacteria were grown in Luria-Bertani medium or tryptic soy broth. Antibiotics were used at the following concentrations: ampicillin, 50 μg/ml; kanamycin, 30 μg/ml; and streptomycin, 100 μg/ml.

Molecular cloning procedures. DNA analysis and transformation of *E. coli* strains were performed according to standard methods (24). Nucleotide sequences were determined by the dideoxy chain termination procedure (38) on

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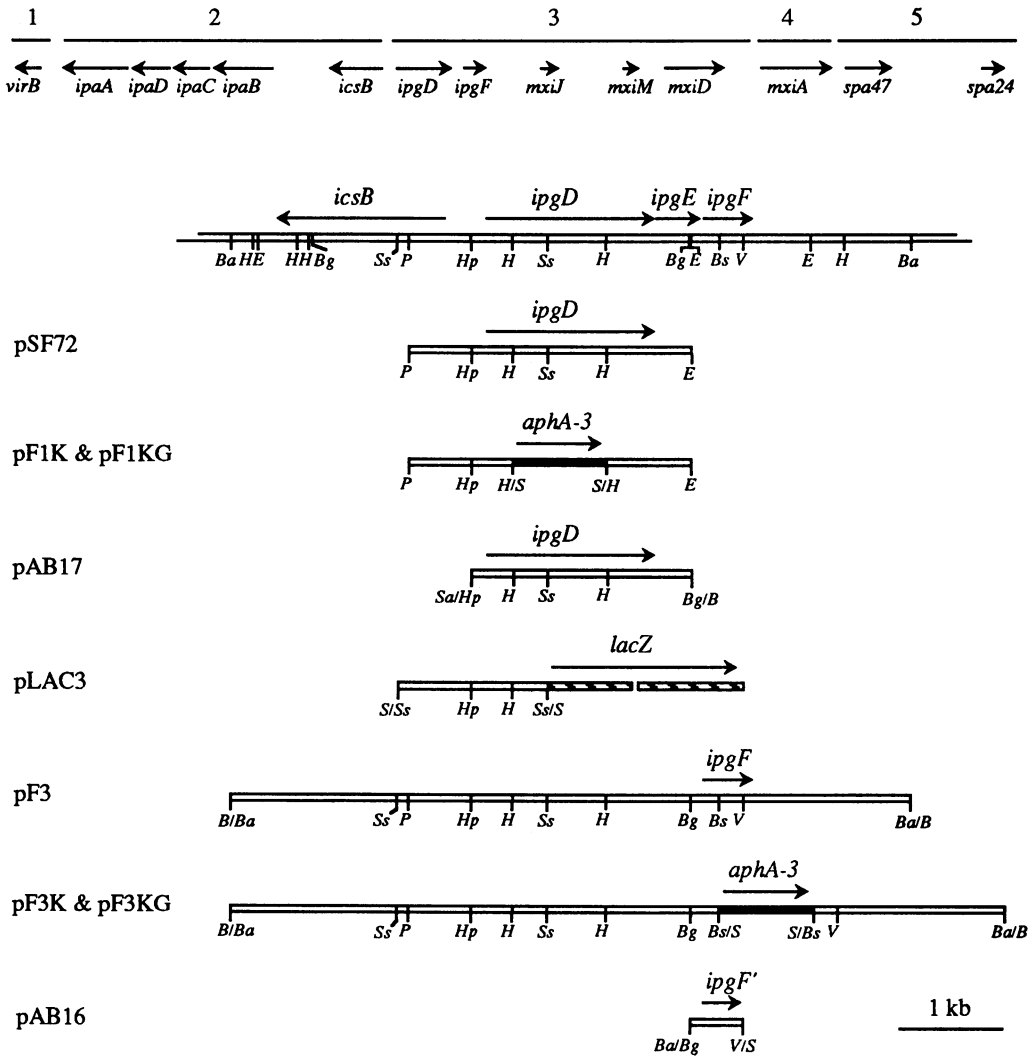

FIG. 1. Structures of plasmids carrying the *ipgD*, *ipgE*, and *ipgF* genes. The relative positions and orientations of some of the genes identified in the five regions of the virulence plasmid fragment that is necessary for invasion are indicated by arrows on the first line. Below is shown a schematic restriction map of the region encompassing the *icsB*, *ipgD*, *ipgE*, and *ipgF* genes: *B*, *Bam*HI; *Ba*, *Bal*I; *Bg*, *Bgl*II; *Bs*, *Bsp*EI; *E*, *Eco*RI; *H*, *Hin*dIII; *Hp*, *Hpa*I; *P*, *Pvu*II; *V*, *Eco*RV; *S*, *Sma*I; *Sa*, *Sal*I; *Ss*, *Ssp*I. The *S. flexneri* DNA carried by each plasmid is shown by an open bar, the 850-bp fragment carrying the *aphA-3* gene is indicated by a solid bar, and the 3-kb DNA carrying the *lacZ* gene is indicated by a hatched bar (not shown to scale). Arrows indicate the positions and extent of the genes.

single-stranded M13 DNA or on alkaline-denatured plasmid DNA. The two DNA strands were completely sequenced.

Construction of an *ipgD-lacZ* transcriptional fusion. The plasmid vector pLAC1 (2) contains the *lacZ* reporter gene from Tn*917-lac* (36) inserted into the *Eco*RV site of pGP704, a derivative of the suicide vector pJM703.1 that confers resistance to ampicillin (30). Plasmid pLAC3 (Fig. 1) was constructed by cloning a 1.4-kb *Ssp*I fragment, extending up to bp 840 in Fig. 2, into the *Sma*I site located upstream from the *lacZ* reporter gene in pLAC1. pLAC3 was then transferred by conjugation into *S. flexneri* M90T-Sm, with transconjugants selected on plates that contained streptomycin and ampicillin. Since pLAC3 does not replicate in *S. flexneri*, the Ap^r clones arose through homologous recombination between the identical sequences carried by the large virulence plasmid pWR100 and the recombinant plasmid pLAC3, thereby placing the *lacZ* reporter gene under the control of the *ipgD* promoter. Integration of pLAC3 into pWR100 also led to a duplication of the *icsB-ipgD* intergenic region. Southern analysis confirmed the structure of the pWR100 derivative carrying the *ipgD-lacZ* transcriptional fusion at the *ipgD* locus; this strain was designated SF134.

β-Galactosidase and alkaline phosphatase assays. β-Galactosidase activity was assayed by using the substrate *o*-nitrophenyl-β-D-galactoside as described by Miller (29); alkaline phosphatase activity was assayed by using the substrate *p*-nitrophenyl phosphate as described by Manoil and Beckwith (25). Both activities are expressed in milli-optical density units at 420 nm per minute per optical density unit at 600 nm (29).

Preparation of protein extracts, SDS-PAGE, and immunoblotting. Bacteria in exponential growth phase were harvested by centrifugation; after filtration through 0.22-μm-pore-size filters, proteins present in the culture supernatant were precipitated by the addition of 1/10 (vol/vol) trichloroacetic acid, as previously described (2). Electrophoresis in

10% polyacrylamide gels in the presence of sodium dodecyl sulfate (SDS-PAGE) was performed as described by Laemmli (21). After electrophoresis, proteins were either stained with Coomassie brilliant blue or transferred to a nitrocellulose membrane (47). Immunoblotting procedures were carried out using either rabbit serum raised against purified *E. coli* alkaline phosphatase (kindly provided by A. Pugsley) or serum from a monkey experimentally infected with M90T.

Virulence assays. Infection of HeLa cells was performed as previously described (41). The virulence properties of the strains were also evaluated using the plaque assay, as described by Oaks et al. (32), and the Serény test (45).

Nucleotide sequence accession number. The nucleotide sequence reported here has been submitted to GenBank and assigned the accession number L04309.

RESULTS

Regulation of *ipgD* expression by temperature. We have previously observed a high level of expression of β-galactosidase when a fragment carrying the *icsB* promoter (2) was inserted in the opposite orientation upstream from a *lacZ* reporter gene; this result suggested that a promoter was located upstream from and in the opposite orientation to *icsB* (Fig. 1). Construction of strain SF134 carrying this transcriptional fusion is described in Materials and Methods; the gene which was fused to the *lacZ* reporter gene was designated *ipgD* (invasion plasmid gene). Since Tobe et al. (46) have described a thermoregulated promoter (P4) located upstream from and in the opposite orientation to *icsB*, we examined the expression of the *ipgD-lacZ* fusion after growth at 30 and 37°C. β-Galactosidase activity was 30-fold higher after growth of SF134 at 37°C than at 30°C (640 U versus 25 U), confirming that expression of *ipgD*, like the invasive phenotype of *S. flexneri* (27), is regulated by temperature.

Characterization of the *ipgD*, *ipgE*, and *ipgF* genes. To characterize the role of *ipgD* in the virulence of *S. flexneri*, we first determined the complete nucleotide sequence of the corresponding wild-type locus. The sequence of the 2,880-bp DNA fragment located upstream from the *icsB* gene is shown in Fig. 2. Sequence analysis revealed the presence of three open reading frames (ORFs), the first (*ipgD*) from bp 372 to 1985, the second from bp 1989 to 2357, and the third from bp 2333 to 2815; the last two ORFs were designated *ipgE* and *ipgF*, respectively. The first ATG codon (bp 372) of the *ipgD* ORF, which is preceded by the sequence 5′-GAGGA-3′, is likely to be the *ipgD* translation start site. The *ipgD* gene is predicted to encode a 538-amino-acid-residue polypeptide with a calculated M_r of 59,764. The first ATG codon within the *ipgE* ORF (bp 1998) is preceded by a potential ribosome binding site (5′-GAGGA-3′). The *ipgE* gene is predicted to encode a 120-amino-acid-residue polypeptide with a calculated M_r of 13,717. The first ATG codon within the *ipgF* ORF (bp 2360) is also preceded by a potential ribosome binding site (5′-AGGGG-3′); *ipgF* is predicted to encode a 152-amino-acid-residue polypeptide with a calculated M_r of 17,560.

The intergenic region between *icsB* (2) and *ipgD* consists of 315 bp and should contain the two divergent promoters involved in the thermoregulated expression of the *icsB* and *ipgD* genes. There are 9 bp between the *ipgD* stop codon and the *ipgE* start codon and a 1-bp overlap between the *ipgE* stop codon and the *ipgF* start codon. This organization suggests that *ipgD*, *ipgE*, and *ipgF* belong to the same transcriptional unit.

Construction and characterization of an *ipgD* mutant. To investigate the role of *ipgD*, we constructed strain SF701, in which *ipgD* has been inactivated in the large plasmid pWR100. Plasmid pSF72 (Fig. 1) was first constructed by cloning a 2.7-kb *Pvu*II-*Eco*RI fragment of pHS5103 (8), encompassing the *ipgD* gene, between the *Pvu*II and *Eco*RI sites of pBR322. Plasmid pF1K (Fig. 1) was then constructed by replacing the 900-bp *Hin*dIII fragment of pSF72, internal to *ipgD* (from bp 551 to 1437 in Fig. 2), by an 850-bp *Sma*I fragment carrying the *aphA*-3 gene, which confers resistance to kanamycin (28). This mutation in *ipgD* was designated *ipgD1*. The filled-in *Pvu*II-*Eco*RI fragment of pF1K was then cloned into the *Sma*I site of pLAC2, a derivative of the suicide vector pJM703.1 that confers resistance to ampicillin (30), to give rise to pF1KG (Fig. 1). Finally, plasmid pF1KG was transferred to *S. flexneri* M90T-Sm by conjugal mating, with selection for transconjugants on plates that contained streptomycin and kanamycin. Clones in which a double recombinational event had exchanged the wild-type *ipgD* gene with the mutated copy carried by the recombinant plasmid pF1KG were identified by their sensitivity to ampicillin. The structure of the resultant large plasmid carrying the *ipgD1* mutation was confirmed by Southern analysis, and the corresponding strain was designated SF701.

The virulence properties of the *ipgD* mutant were studied by using the HeLa cell invasion test, the plaque assay, and the Serény test. The *ipgD* mutant showed the same ability as the wild-type strain to invade HeLa cells, to induce the formation of plaques on confluent monolayers of HeLa cells, and to provoke keratoconjunctivitis in guinea pigs. In addition, the *ipgD* mutant was still able to bind the dye Congo red, a property that has been associated with the invasive phenotype of *Shigella* species (27).

Previous analysis of the protein content in the culture supernatant of M90T-Sm (wild type) has shown that *S. flexneri* secretes nine proteins of 110, 95, 70, 62, 60, 58, 41, 39, and 35 kDa (3, 4). The 70-, 62-, and 41-kDa polypeptides have been identified as IpaA, IpaB, and IpaC, respectively, and the 95-kDa polypeptide has been identified as a matured form of IcsA (4, 13). Comparison of the protein contents in the culture supernatants of the wild-type and *ipgD* strains indicated that the protein of 58 kDa was missing in the culture supernatant of the *ipgD* mutant (Fig. 3). To ascertain that the lack of this protein was due to the *ipgD* mutation, plasmid pAB17 (Fig. 1) was constructed by cloning the 2-kb *Hpa*I-*Bgl*II fragment (from bp 153 to 2249 in Fig. 2), which encompasses the *ipgD* gene, between the *Sal*I and *Bam*HI sites of pUC19 (54) after filling in the *Sal*I site. Secretion of the 58-kDa protein was restored in the *ipgD* mutant harboring plasmid pAB17 (data not shown). The size of this protein (58 kDa) is in agreement with that expected for the *ipgD* gene product (60 kDa), which suggests that it corresponds to IpgD. In contrast to the Ipa proteins, which are consistently recognized by the sera of people convalescing from shigellosis (31), the secreted 58-kDa protein was not recognized by serum from a monkey experimentally infected with M90T (data not shown).

Isolation of IpgF-PhoA protein fusions. Analysis of the predicted N-terminal sequence of IpgF revealed the presence of a classical signal sequence, suggesting that IpgF is exported. To test this hypothesis, we constructed plasmid pAB16, which carries most of the *ipgF* gene on a 0.5-kb *Bgl*II-*Eco*RV fragment (from bp 2249 to 2756 in Fig. 2) inserted between the *Bam*HI and *Sma*I sites of pUC19, i.e., in proper orientation downstream from the *lac* promoter of the vector (54). pAB16 was then mutagenized in vivo by using the transposon Tn*phoA*, which has been widely used

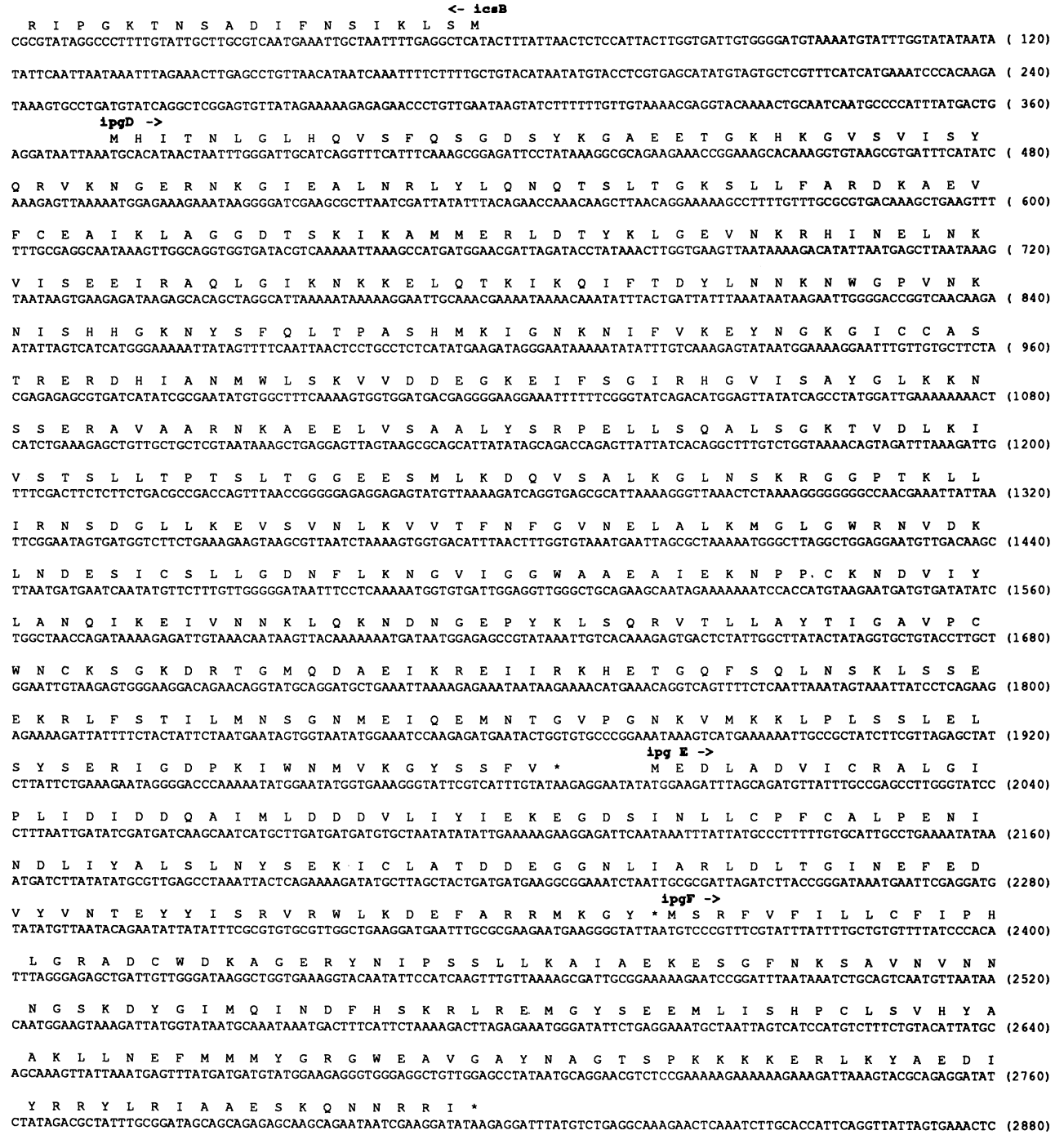

FIG. 2. Nucleotide sequence of the *ipgD*, *ipgE*, and *ipgF* genes. The nucleotide sequence of the 2,880-bp fragment located upstream from *icsB* is shown along with the deduced amino acid sequences of IpgD, IpgE, and IpgF and of the N-terminal portion of IcsB. Since *icsB* is in the opposite orientation to the *ipg* genes, the IcsB deduced amino acid sequence reads from right to left. Stars indicate the positions of the *ipgD*, *ipgE*, and *ipgF* stop codons. The sequence from bp 1 to 158 has been previously published (2).

to identify genes that encode membrane or secreted proteins (25). After Tn*phoA* mutagenesis, restriction analysis of plasmids carried by *E. coli* clones that exhibited a blue color on plates containing 5-bromo-4-chloro-3-indolyl phosphate (the chromogenic substrate for alkaline phosphatase) allowed the identification of four different Tn*phoA* insertions within *ipgF*. The sequence of the Tn*phoA* insertion sites was determined by using an oligonucleotide that hybridizes to the 5′ end of *phoA*. A junction between *ipgF* and *phoA* was found after codon 23 (pAB40), codon 64 (pAB44), codon 80 (pAB45), and codon 110 (pAB46) of *ipgF*. The alkaline phosphatase activity expressed by each of the plasmids carrying an *ipgF-phoA* fusion was assayed in *E. coli* CC118 (Δ*phoA*) and *S. flexneri* M90T; similar high levels of alkaline

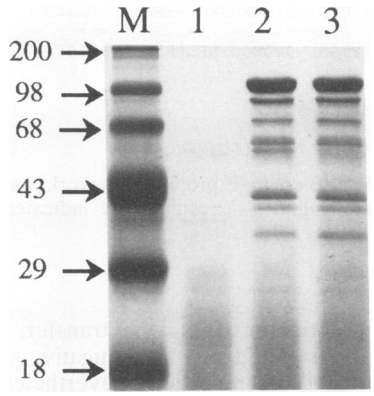

FIG. 3. Analysis of the secreted proteins in the *ipgD* mutant. Concentrated culture supernatants of *S. flexneri* BS176 (lane 1), M90T-Sm (lane 2), and SF701 (*ipgD*) (lane 3) were separated by SDS-PAGE and stained with Coomassie brilliant blue. The positions and sizes (in kilodaltons) of the protein standards (lane M) are indicated by arrows.

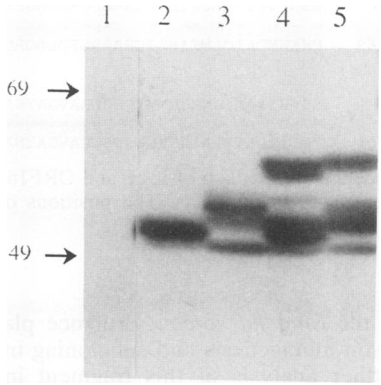

FIG. 4. Immunoblot analysis of the IpgF-PhoA hybrid proteins. Extracts of *E. coli* CC118 (Δ*phoA*) carrying each of several plasmids that express an *ipgF-phoA* fusion were separated by SDS-PAGE, transferred onto nitrocellulose, and reacted with a serum directed against alkaline phosphatase. Lane 1, pUC18; lane 2, pAB40; lane 3, pAB44; lane 4, pAB45; lane 5, pAB46. The positions and sizes (in kilodaltons) of protein standards are indicated by arrows.

phosphatase activity were obtained in both strains (Table 1), indicating that the PhoA moiety of the hybrids was exported. In addition, immunoblot analysis (Fig. 4), using an antiserum directed against *E. coli* alkaline phosphatase, indicated that the sizes of the hybrid proteins were consistent with the transposon insertion sites in *ipgF*, thereby confirming the sequence and proposed translation start site of *ipgF*. Additional polypeptides, ranging in size between those of the hybrid IpgF-PhoA fusions and that of mature PhoA (48 kDa), were also detected by the anti-PhoA serum and are likely to be degradation products of the hybrid proteins.

Construction and characterization of an *ipgF* mutant. To investigate the role of *ipgF*, we constructed strain SF702, in which the *ipgF* gene has been inactivated in the large plasmid pWR100, using the same strategy as that described for the construction of the *ipgD* mutant (see above). Briefly, plasmid pF3 (Fig. 1) was constructed by inserting the 6.4-kb *Bal*I fragment of pHS5103 (8) into the filled-in *Bam*HI site of pUC18 (54); in pF3, the *Bam*HI sites flanking the insert were reconstituted. Plasmid pF3K was then constructed by inserting an 850-bp *Sma*I fragment carrying the *aphA-3* gene (28) into the *Bsp*EI site of pF3 (internal to *ipgF*, bp 2486 in Fig. 2), and the 7.3-kb *Bam*HI fragment of pF3K was subsequently cloned into the *Eco*RV site of pGP704, a derivative of the suicide vector pJM703.1 that confers resistance to ampicillin (30), to give rise to pF3KG (Fig. 1). A double recombinational event between the wild-type *ipgF* gene carried by pWR100 and the mutated copy (*ipgF1*) carried by pF3KG yielded *S. flexneri* SF702. Southern blot analysis confirmed the structure of the large plasmid carrying the *ipgF1* mutation.

The *ipgF* mutant showed the same ability as the wild-type strain to invade HeLa cells, to form plaques on confluent monolayers of HeLa cells, and to provoke keratoconjunctivitis in guinea pigs. In addition, the *ipgF* mutant was still able to bind the dye Congo red, and no difference was detected between the protein contents of the supernatants prepared from the wild-type strain and the *ipgF* mutant (data not shown).

Sequence comparisons. The sequences of the IpgD, IpgE, and IpgF proteins were compared with the protein sequences translated from the GenBank library by using the FASTA program (35). This comparison revealed an extensive sequence similarity between IpgF and a 169-residue protein (ORF169) encoded by the leader region of the F plasmid (22) and homologous proteins encoded by plasmids R100 (12) and R1 (14). As shown in Fig. 5, the IpgF and ORF169 sequences are homologous over their entire length, with 52 (34%) identical residues. No protein homologous to IpgD or IpgE was detected in the protein sequence library.

TABLE 1. Alkaline phosphatase activity expressed by *ipgF-phoA* fusions in *E. coli* and *S. flexneri*

Plasmid (fusion)[a]	PhoA (U)[b]	
	E. coli	*S. flexneri*
pAB40 (aa 23)	4,620	3,000
pAB44 (aa 64)	3,080	2,020
pAB45 (aa 80)	2,600	1,770
pAB46 (aa 110)	2,190	2,670

[a] For each plasmid, the last amino acid (aa) of the IpgF portion of the hybrid protein is indicated in parentheses.

[b] Alkaline phosphatase activity in transformants of *E. coli* CC118 (Δ*phoA*) or *S. flexneri* M90T was assayed as described previously (25).

DISCUSSION

The fragment of the *Shigella* virulence plasmid that is sufficient for the invasive phenotype was initially identified by constructing cosmids which were able to confer the capacity to invade mammalian cells to an *S. flexneri* strain lacking the virulence plasmid. The inserts present in these cosmids contained a common region of 37 kb, into which were also mapped the Tn*5* insertion sites of six Tn*5*-induced noninvasive mutants (26). Over 300 independent Tn*5* insertion mutations affecting the virulence plasmid pMYSH6000 were isolated from *S. flexneri* 2a, and the transposon insertion site for 134 of the mutants was characterized, thereby allowing the identification of five contiguous regions (designated regions 1 to 5) which covered a 31-kb fragment that was necessary for invasion (43, 44). The restriction map of this fragment was almost identical to that of the fragment cloned in the cosmids constructed from the virulence plasmid pWR100 of *S. flexneri* 5. A similar region has also been

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IpgF     MSRFVFIL-LCFIPHLGR-ADCWDKAGERYNIPSSLLKAIAEKESGFNKSAVNVNNNGSKDYGIMQINDFHSKRLREMGYSEEMLISHPC (88)
ORF169   MKKWMLAICLMFINGICEAADCFDLAGRDYKIDPDLLRAISWKESRYRVNAIGINPVTGYGSGLMQVDSQHFNELARYGIKPEHLTTDPC (90)

IpgF     LSVHYAAKLLNEFMMMYGRGWEAVGAYNAG--TSPKKKKERLKYAEDIYRRYLRIAAESKQNNRRI                          (152)
ORF169   MNIYTGAYYLAIAFKKWGVSWEAVGAYNAGFRKTERQNQRRLAYASEVYRIYTWIKSSKGIRVPTTKKSLSQINSVQKN            (169)
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FIG. 5. Sequence comparison of IpgF and ORF169. The complete sequence of the *S. flexneri* IpgF protein (this work) has been aligned with the sequence of ORF169 (22). The positions of identical (=) and functionally equivalent (—) residues are indicated between the sequences.

identified on the *Shigella sonnei* virulence plasmid, using both transposon mutagenesis and subcloning into a cosmid (19, 52). Further analysis of this fragment in *S. flexneri* indicated that region 1 corresponds to the regulatory gene *virB* (1), region 2 corresponds to the *ipa* locus (7, 8, 42, 49, 50), region 4 corresponds to the *mxiA* gene (5, 6), and region 5 corresponds to the *spa* locus (51). The *mxiA* and *spa* genes, as well as the *mxiJ* and *mxiD* genes, which are located in region 3, are involved in the surface presentation and secretion of the Ipa proteins (3–5, 17, 51), whose N-terminal sequences do not exhibit the features characteristic of a signal sequence. Several genes in regions 3, 4, and 5 thus appear to encode proteins necessary for export of the Ipa proteins.

We have reported here the characterization of the portion of the *mxi-spa* locus that is proximal to the *ipa* locus and have shown that it consists of three genes, designated *ipgD*, *ipgE*, and *ipgF*. These three *ipg* genes, which are transcribed in the same direction as the downstream *mxi* and *spa* genes, are very close to one other, which, together with the comparison of the phenotypes induced by polar and nonpolar mutations in this region (see below), strongly suggests that they belong to the same operon. This operon might also include genes located downstream from *ipgF*, such as *mxiJ* (see Fig. 1 and reference 3). Expression of an *ipgD-lacZ* transcriptional fusion constructed in the *ipgD* locus on pWR100 is regulated by the temperature of growth, which is consistent with the previously reported identification of a thermoregulated promoter (P4) in the *icsB-ipgD* intergenic region (46).

Examination of the IpgF amino acid sequence revealed the presence of a characteristic signal sequence (reviewed in reference 37), which suggested that IpgF is exported. Using Tn*phoA* mutagenesis (25), we have constructed *ipgF-phoA* fusions that express hybrid proteins endowed with high levels of alkaline phosphatase activity, which confirms the functionality of the proposed IpgF signal sequence. Moreover, the high activity of the fusions constructed after amino acids 23, 64, 80, and 110 of IpgF indicates that most, if not all, of IpgF is exported. These results, together with the hydrophilic nature of the protein, suggest that IpgF might be periplasmic. Three other proteins encoded by region 3, MxiJ, MxiM, and MxiD, also have an N-terminal signal sequence, which suggests that they are exported by the general export pathway (3, 4).

Sequence comparisons have revealed an important similarity between IpgF and ORF169, a protein encoded by the leading region adjacent to the origin of transfer of plasmids F (22), R100 (12), and R1 (14). The similarity detected between these proteins encompasses the entire length of their sequences, strongly suggesting that they derive from a common ancestor and might be endowed with similar functions. The leading region is the first portion of the plasmid to enter the recipient bacteria during conjugation. Although this region is not essential for conjugative transfer, its conservation among various F and non-F conjugative plasmids suggests that it might be important; nevertheless, its exact function has not yet been elucidated (22).

To investigate the roles of *ipgD* and *ipgF* in the virulence of *S. flexneri*, we constructed the *ipgD1* mutant, in which an *aphA-3* cassette is inserted after codon 60 of the 538-codon-long *ipgD* gene, and the *ipgF1* mutant, in which the same *aphA-3* cassette is inserted after codon 43 of the 152-codon-long *ipgF* gene; these two mutants showed the same ability as the wild-type strain to secrete the Ipa proteins, invade HeLa cells, induce the formation of plaques on confluent monolayers of HeLa cells, and provoke keratoconjunctivitis in guinea pigs. This is in contrast with the noninvasive phenotype of the *S. flexneri* and *S. sonnei* mutants carrying Tn*5* and Tn*3-lac* insertions, respectively, in this region of the large virulence plasmid (43, 52). Since insertion of transposons such as Tn*5* into genes that belong to an operon is known to introduce a polar effect on the expression of the genes located downstream from the transposon insertion site (9), this difference between the phenotypes of the two types of mutants is likely to be a reflection of the polar and nonpolar nature of the mutations. The *aphA-3* cassette that was used in this study to inactivate both the *ipgF* and *ipgD* genes was amplified by a polymerase chain reaction from plasmid pAT21 (48) and contains almost exclusively the *aphA-3* coding sequence, without any promoter or transcription termination site; when inserted into an operon, this cassette does not introduce a polar effect (28). The proximal part of the *mxi* locus thus contains two genes, *ipgD* and *ipgF*, which are not necessary for invasion of cultured cells.

It has been previously reported that inactivation of *ipaA* does not lead to a defect in virulence (42), and we have recently identified the 70-kDa protein secreted by wild-type *S. flexneri* as IpaA (4). One protein with an estimated mass of 58 kDa was absent in the supernatant of the *ipgD1* mutant; since the size of this protein is in agreement with that calculated for the *ipgD* gene product (60 kDa), this protein might correspond to IpgD. Alternatively, IpgD might be involved in the expression or secretion of this 58-kDa protein. Since this protein, like the Ipa proteins, is not secreted in *mxiD* and *mxiJ* mutants (3, 4), its secretion appears to be dependent on the *mxi* gene products. The absence of the 58-kDa protein in the supernatant of the *ipgD* mutant does not correlate with a defect in virulence, whether tested in vitro by invasion of HeLa cells or in vivo by the Serény test. It thus appears that two secreted proteins, IpaA and possibly IpgD, are not involved in the virulent phenotype of *S. flexneri*. This is very surprising, especially considering the positions of the *ipaA* and *ipgD* genes in the invasion region of the virulence plasmid and the presence of the encoded proteins among the few proteins that are secreted into the culture supernatant. This raises the question of the sensitivity of the virulence tests that are available for

S. flexneri. Nothing is known about the determinants of *Shigella* host specificity, which is restricted to human beings and primates. Whether IpaA and IpgD are necessary for full expression of the pathogenic potential of *S. flexneri* in its natural hosts remains to be evaluated.

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