JOURNAL OF BACTERIOLOGY, Mar. 1992, p. 1990–2001
0021-9193/92/061990-12$02.00/0

Vol. 174, No. 6

Surface Presentation of *Shigella flexneri* Invasion Plasmid Antigens Requires the Products of the *spa* Locus

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Received 2 October 1991/Accepted 28 November 1991

An avirulent, invasion plasmid insertion mutant of *Shigella flexneri* 5 (pHS1059) was restored to the virulence phenotype by transformation with a partial *Hin*dIII library of the wild-type invasion plasmid constructed in pBR322. Western immunoblot analysis of pHS1059 whole-cell lysates revealed that the synthesis of the invasion plasmid antigens VirG, IpaA, IpaB, IpaC, and IpaD was similar to that seen in the corresponding isogenic *S. flexneri* 5 virulent strain, M90T. IpaB and IpaC, however, were not present on the surface of pHS1059 as was found in M90T, suggesting that the transport or presentation of the IpaB and IpaC proteins onto the bacterial surface was defective in the mutant. pHS1059 was complemented by pWR266, which carried contiguous 1.2- and 4.1-kb *Hin*dIII fragments of the invasion plasmid. pHS1059(pWR266) cells were positive in the HeLa cell invasion assay as well as colony immunoblot and enzyme-linked immunosorbent assays, using monoclonal antibodies to IpaB and IpaC. These studies established that the antigens were expressed on the surface of the transformed bacteria. In addition, water extraction of pHS1059 and pHS1059(pWR266) whole cells, which can be used to remove IpaB and IpaC antigens from the surface of wild-type M90T bacteria, yielded significant amounts of these antigens from pHS1059(pWR266) but not from pHS1059. Minicell and DNA sequence analysis indicated that several proteins were encoded by pWR266, comprising the *spa* loci, which were mapped to a region approximately 18 kb upstream of the *ipaBCDAR* gene cluster. Subcloning and deletion analysis revealed that more than one protein was involved in complementing the Spa$^-$ phenotype in pHS1059. One of these proteins, Spa47, showed striking homology to ORF4 of the *Bacillus subtilis flaA* locus and the *fliI* gene sequence of *Salmonella typhimurium*, both of which bear strong resemblance to the α and β subunits of bacterial, mitochondrial, and chloroplast proton-translocating F_0F_1 ATPases.

Shigella species and enteroinvasive *Escherichia coli* (EIEC) must be able to recognize, invade, multiply, and spread within epithelial cells of the colon to cause bacillary dysentery. Genetic determinants of these phenotypes have been located on the chromosome and a large (120- to 140-MDa) nonconjugative plasmid found in all virulent isolates of the shigellae (reviewed in reference 20). The *Shigella flexneri* 5 invasion plasmid (pWR100) contains a 37-kb section of DNA that carries the genes responsible for the invasion phenotype (32, 41). Within this highly conserved DNA segment are four invasion plasmid antigen (*ipa*) genes (*ipaA*, *ipaB*, *ipaC*, and *ipaD*) encoding the 70-kDa (IpaA), 62-kDa (IpaB), 42-kDa (IpaC), and 37-kDa (IpaD) polypeptides which, along with the *virG*-encoded 120-kDa protein, are the dominant protein antigens inducing a serum and secretory immune response during infection in humans and monkeys (9, 13, 30, 34). Extensive transposon mutagenesis and genetic complementation experiments have indicated that IpaB, IpaC, and IpaD are essential invasion determinants and that the VirG protein is pivotal in the spread of bacteria within and between colonic enterocytes (5, 20, 32, 41). Since the invasion and intercellular spreading phenotypes require the interaction of bacterial and host cell surfaces, it is not unreasonable to postulate that the Ipa and VirG antigens are surface-exposed proteins.

Recent experimental evidence supports the idea that the Ipa and VirG proteins are configured on the cell surface. Virulent *Shigella* and EIEC strains are recognized by plasmid antigen-specific convalescent monkey antisera in a whole-cell enzyme-linked immunosorbent assay (ELISA), whereas *Shigella* strains cured of the invasion plasmid are not recognized by such sera (33). Similarly, hyperimmune rabbit antiserum, raised against EIEC and absorbed with an isogenic avirulent derivative, detects virulence marker antigens found only in virulent *Shigella* and EIEC cells; further characterization of the virulence marker antigen showed that it consisted of the IpaB and IpaC antigens (36). Water extraction of plasmid-containing, virulent *S. flexneri* 5 cells and concentration of the supernatant provides a mix of antigens, primarily IpaB and IpaC, that are specifically recognized in an ELISA using either convalescent monkey or human antisera (34). Monoclonal antibodies (MAbs) raised against the IpaB and IpaC antigens specifically detect virulent *Shigella* and EIEC cells in both a whole-cell ELISA and colony immunoblot procedure (33). In addition, one IpaB-specific MAb (2F1) reduced the BHK cell plaque-forming capacity of *S. flexneri* 5 by >50%, suggesting that the corresponding IpaB epitope is involved in the invasion process, in agreement with the predicted hydrophilic nature of the 2F1 epitope determined from the nucleotide sequence of the *ipaB* gene (33, 51). Extrinsic labeling of *S. flexneri* 2a with ^{125}I has shown that the 120-kDa VirG protein is positioned on the cell surface (30). These data are congruent with the proposed surface location of the Ipa and VirG antigens, although the exact nature of their interaction with the outer membrane, if any, remains to be determined.

DNA sequence analysis of the *ipaBCDA* and *virG* genes revealed that these proteins do not contain recognizable signal sequences characteristic of many secreted and integral membrane polypeptides (4, 51), suggesting that the Ipa and VirG proteins are transported by an invasion plasmid anti-

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gen-specific pathway requiring auxiliary proteins. Recent genetic analysis of avirulent insertion and spontaneous deletion mutants of *S. flexneri* invasion plasmids indicates that genes responsible for the correct posttranslational processing, transport, or presentation of the Ipa antigens are clustered in a region 15 to 22 kb upstream of the *ipa* gene regulon (3, 49, 50).

In this study, we used a plasmid library of pWR100 to complement two Tn*5* insertion mutants of *S. flexneri* 5 (pHS1059 and pHS1060 [32]) that did not express the Ipa antigens on the bacterial surface. The associated Spa^+ phenotype (surface presentation of Ipa antigens) was expressed by the *spa* locus carried on recombinant plasmid pWR266. The *spa* locus genes were characterized by DNA sequence and minicell analysis, and the role of the *spa* genes in the surface presentation of the Ipa antigens was confirmed by cell fractionation studies of the IpaB, IpaC, and VirG proteins in pHS1059 and its Spa^+ transformants.

MATERIALS AND METHODS

Bacterial strains and media. pHS1059 and pHS1060 were obtained from T. L. Hale, Department of Enteric Diseases, Walter Reed Army Institute of Research. M90T containing invasion plasmid pWR100 has been described previously (9). Bacterial strains were grown at 37°C on LB media with appropriate antibiotics unless otherwise stated. Ampicillin and kanamycin were used at concentrations of 50 and 25 μg/ml, respectively. Bacteria were routinely streaked on Congo red plates, and the ability of the bacteria to bind the Congo red dye was used as a first measure of invasive and virulent strains, as has been described recently (8, 10).

DNA manipulations. pWR100 was isolated as previously described (8, 9). pBR322-derived plasmid clones were purified on CsCl and digested with restriction enzymes as described previously (51). Transformations of competent cells with plasmid DNA were carried out by using routine $CaCl_2$-derived competent cells as well as electroporation. Electroporation was done on cells grown at 37°C to an optical density at 600 nm of 0.5 and washed twice with 1 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 7.2) and twice with 10% glycerol. Cells were resuspended at 1/100 volume in 10% glycerol and transformed with 1 ng of DNA. The electroporation was carried out in a Gene Pulser (Bio-Rad) set at 2.5 kV, 25-μF capacitance, and 600 ohms of resistance. Immediately after delivery of the pulse, 0.9 ml of SOC (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM each $MgCl_2$ and $MgSO_4$, 20 mM glucose) medium was added, and the cells were allowed to recover at 37°C for 1 to 2 h.

Western immunoblots, colony immunoblots, and peptide sera. Whole-cell sodium dodecyl sulfate (SDS) lysates of *Shigella* strains were separated on polyacrylamide gels cross-linked with *N,N'*-diallyltartardiamide (DATD) in a discontinuous SDS-polyacrylamide gel electrophoresis system using Laemmli buffers and electroeluted onto nitrocellulose as previously described (9, 33, 51). Western blots were probed with human antisera to *S. flexneri* 1b. Colony immunoblots were carried out by streaking single colonies of M90T, pHS1060, pHS1059, and pHS1059 transformed with plasmid clones onto LB plates overlaid with nitrocellulose filters and immunoreacted with MAbs to IpaB and IpaC, as previously described (33). Antisera against the VirG protein was generated by immunizing rabbits with synthetic VirG peptides conjugated to either keyhole limpet hemocyanin or bovine serum albumin. Immunoblots were developed with alkaline phosphatase-labeled protein A (Kirkegaard & Perry, Gaithersburg, Md.) as described previously (33).

Membrane fractionations. The inner and outer membranes of invasive M90T, pHS1059, and *spa*-complemented pHS1059, grown in Penassay broth (Difco Laboratories, Detroit, Mich.) at 37°C, were prepared by sucrose gradient purification of French press-disrupted organisms (42). Extractions of whole organisms with water were performed by shaking the bacteria with water, followed by low- and high-speed (100,000 × *g*) centrifugation of the extracted material to remove whole bacteria and membrane fragments, as previously described (34). Total protein (10 μg) for each membrane preparation or water extract was electrophoresed on 13% polyacrylamide–DATD gels and blotted as described above. Protein concentrations were determined by the bicinchonic acid (Pierce Chemical Co.) procedure, using bovine serum albumin as the standard (44).

Mapping of Tn*5* insertions. Invasion plasmid DNA was isolated from M90T, pHS1059, and pHS1060 and cut with *Hin*dIII, and the fragments were isolated on a 0.7% agarose gel. After transfer to nitrocellulose, the DNA was probed with T4 polynucleotide kinase-derived ^{32}P-labeled oligonucleotides from the leftward boundaries of either the 1.2- or 4.1-kb *Hin*dIII fragment of pWR266. Primer *Hin*dIII 1.2_L was 5'-GGAAGTCTTCCGACATGTGAC-3', and primer *Hin*dIII 4.1_L was 5'-GATTGGAAATTCTCGTGGACTTT CAC-3'. Hybridizations were carried out at 37°C for 16 h in 5× SSPE (0.9 M NaCl, 50 mM $NaPO_4$ [pH 7.7], 5 mM EDTA)–1% SDS–5× Denhardt's solution–100 μg of sonicated calf thymus DNA per ml–0.01% $NaPP_i$. Filters were washed twice at room temperature for 15 min in 1× SSPE containing 1% SDS and 0.01% $NaPP_i$ and then twice for 15 min each time at 45°C.

DNA sequencing. DNA sequencing of pWR266 was carried out on CsCl-purified plasmid DNA, using ^{35}S-labeled dATP and a DNA sequencing kit (United States Biochemical Corp., Cleveland, Ohio) as described previously (51). Seventeen-base-pair oligonucleotides, homologous to sequenced stretches of DNA, were routinely synthesized (model 8600; Milligen-Biosearch, San Rafael, Calif.), purified on PD-10 columns (Pharmacia), and used as primers for DNA synthesis.

Minicell analysis. *E. coli* DS410 was transformed with various *spa* recombinant clones as well as with pBR322, which acted as a control. Minicells were prepared by two successive sucrose gradient centrifugations and labeled with [^{35}S]methionine as described previously (51).

Computer analysis of DNA sequence. Open reading frames (ORFs), amino acid compositions of encoded proteins, and hydropathy and antigenicity indices of the proteins derived from DNA sequence analysis were determined by using the MacGene and MacVector programs and a Macintosh SE computer. Homology searches with the NBRF protein data base were carried out with the GCG sequence analysis software program of the University of Wisconsin.

Nucleotide sequence accession number. The sequence shown in Fig. 5 has been assigned GenBank accession number M81458.

RESULTS

Characterization of pHS1059 and pHS1060. pHS1059 and pHS1060 were initially obtained as two of eight noninvasive mutants isolated from a set of 1,000 kanamycin-resistant mutants resulting from the random insertion of Tn*5* into pWR100 (32). Using *Eco*RI, which has no sites within Tn*5*,

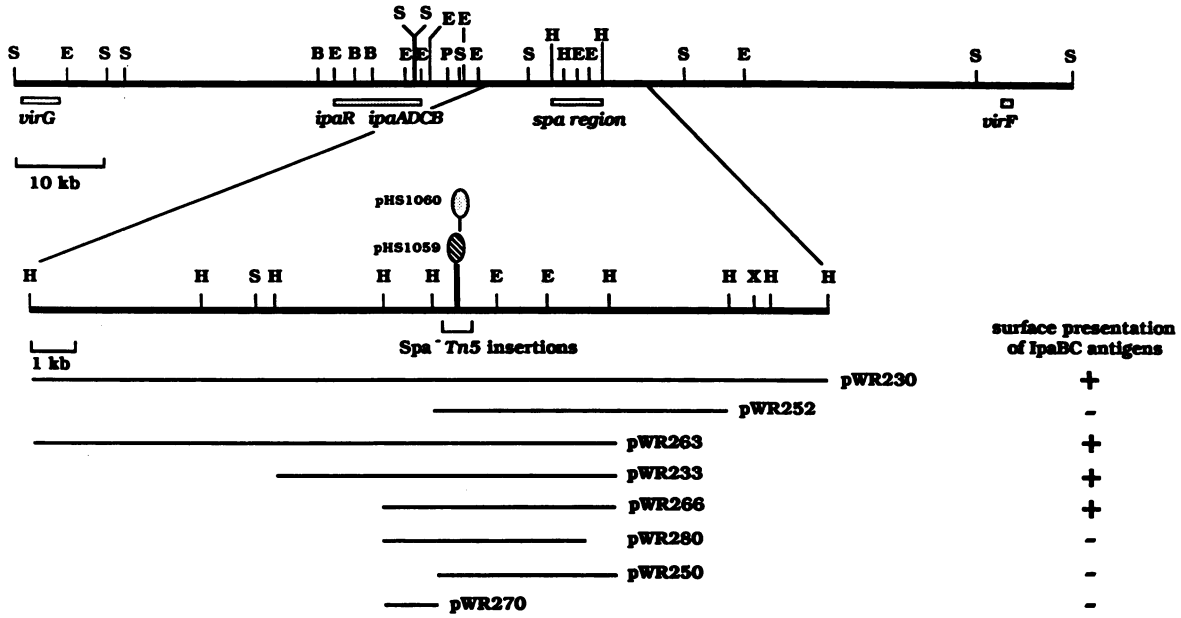

FIG. 1. Genetic map of pWR100 and mapping of the Tn*5* insertions. The top line represents a genetic and partial restriction map of virulence-associated genes of pWR100 showing the locations of the *ipaBCDAR* genes and the *virG*, *virF*, and *spa* loci relative to each other. An expanded view of the *spa* region is indicated in the center line along with the location of the Tn*5* insertions in pHS1059 and pHS1060. Restriction sites and genetic maps of pWR100 plasmid *spa* recombinants that complement pHS1059 are shown at the bottom. Their abilities to complement the mutation in pHS1059 are shown at the right; + indicates a reaction with IpaB and IpaC MAbs in the whole-cell colony blot assay, which correlated with HeLa cell invasiveness.

as the restriction enzyme and a ^{32}P-labeled Tn*5* probe, it was shown that the insertion of the transposon in pHS1059 and pHS1060 had occurred within an 11.5-kb *Eco*RI fragment of pWR100 (Fig. 1) (32). The noninvasive phenotype of the two mutants, as measured by their inability to penetrate cultured HeLa cells, was confirmed in our laboratory. Both pHS1059 and pHS1060 tested negative when plated on Congo red-containing agar plates. Western blot analysis of SDS lysates of the virulent parent strain (M90T) versus the two Tn*5* mutants (Fig. 2) indicated that relative to the parental control, the avirulent insertion mutants were not compromised in their ability to synthesize the Ipa or VirG antigen. Unlike the M90T parent, however, pHS1059 and pHS1060 cells did not give a positive signal in a whole-cell ELISA or colony blot assay using MAbs to IpaB and IpaC (see below). These results suggested that the insertions disrupt genes controlling the posttranslational modification and/or transport of the Ipa antigens from the intracellular compartment onto the bacterial surface, thereby affecting their surface presentation.

Complementation analysis of pHS1059 and pHS1060 and isolation of *spa* recombinants. To characterize the genes that could restore the wild-type phenotype to pHS1059 and pHS1060, a partial *Hin*dIII restriction digest of pWR100, containing 10- to 15-kb DNA fragments, was subcloned into pBR322 and the ligation mix was transformed into HB101 cells. Plasmid DNA was prepared from pools of ampicillin-resistant HB101 transformants and electroporated into kanamycin-resistant pHS1059 and pHS1060 cells. The resulting kanamycin- and ampicillin-resistant transformants were screened by the colony immunoblot assay for their ability to bind IpaB- and IpaC-specific MAbs. Recombinant pWR230 was identified as one clone that restored a positive immunoblot reaction in both mutants (Fig. 2). pHS1059(pWR230) cells were also wild type in their ability to bind Congo red dye and to invade cultured HeLa cells. Since essentially the same results were obtained with pHS1059 and pHS1060 (mapping data described below confirmed their similar origins), the former strain was used for all subsequent experiments.

Since pWR230 contained a fairly large segment of DNA (Fig. 1), an effort was made to determine the minimum size of the DNA that would complement the *spa* phenotype. Thus, pWR230 was subjected to another round of partial *Hin*dIII restriction enzyme digestion and subcloning into pBR322. Recombinants were tested as before for their ability to restore to pHS1059 a positive reaction in the colony immunoblot assay (Fig. 2). Restriction analysis of the various clones revealed that pWR266, which contained contiguous 1.2- and 4.1-kb *Hin*dIII fragments, represented the minimal DNA needed to restore the Spa$^+$ phenotype to pHS1059. Neither pWR270, which contained the 1.2-kb *Hin*dIII fragment, pWR250, which contained the 4.1-kb *Hin*dIII fragment, nor pWR252, which contained the 4.1-kb *Hin*dIII fragment in conjunction with a second fragment on its right side, could restore the mutant to the parental phenotype. Clones that restored the positive immunoblot reaction, such as pWR233, pWR263, and pWR266, also restored to pHS1059 the ability to bind Congo red and to invade HeLa cells. Deletion analysis showed that removal of 500 bp from the 3′ end of the 4.1-kb *Hin*dIII fragment in pWR266 (e.g., pWR280) resulted in an inability to complement the insertion mutant in pHS1059.

When pHS1059 cells transformed with individual *spa* clones were plated on Congo red plates, a high frequency of Congo red-negative colonies segregated from the Congo red-positive strains. These Congo red-negative colonies also tested negative on colony immunoblots and the HeLa cell

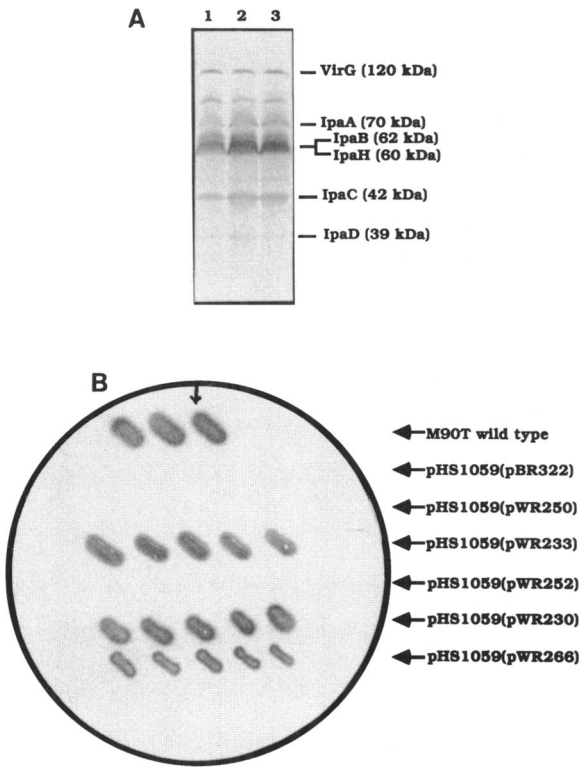

FIG. 2. Western blot and colony blot analysis of pHS1059, pHS1060, and Spa$^+$ transformants of pHS1059. (A) Whole-cell SDS lysates of pHS1059 (lane 1), pHS1060 (lane 2), and M90T (lane 3) were electrophoresed and immunoblotted against human convalescent antiserum to *S. flexneri* 1b. Identities of the antigens along with their molecular sizes are indicated at the right. (B) Whole-cell colonies of pHS1059 and transformants were lifted onto nitrocellulose filters and developed with MAbs to IpaB and IpaC as described in Materials and Methods.

invasion assay. Experiments are in progress to determine the cause of this instability (49).

Membrane fractionation studies. The loss of a positive reaction in whole-cell immunoblots exhibited by pHS1059 prompted a determination to physically locate the Ipa antigens in M90T, pHS1059, and the *spa*-complemented pHS1059 strains. Inner membranes, outer membranes, and water extract preparations were made, and their proteins were separated by polyacrylamide gel electrophoresis (Fig. 3). Western blots of these proteins were probed with IpaB and IpaC MAbs. IpaB was detected in the inner membranes but was not present in the outer membrane fraction of the wild-type, mutant, and restored strains. Water extract preparations of pHS1059 had a decreased quantity of IpaB in comparison with similar protein extracts from M90T and pHS1059 complemented with *spa* clones. IpaC, unlike IpaB, was present in all three fractions, with the relative quantities decreased in both outer membrane and water extract preparations of pHS1059 and subsequently restored to normal levels in pHS1059 complemented with the Spa$^+$ recombinants. Western blot analysis of growth medium from wild-type strains had indicated the presence of these antigens in the culture medium (3, 48).

The distribution of the 120-kDa VirG protein was not affected by the mutation in pHS1059. Figure 3C indicates that VirG was not present in the inner membrane but was located in the purified outer membrane of shigellae. The lack of VirG in the inner membrane indicated that these preparations were not contaminated with significant quantities of outer membrane, since previous work has documented the presence of VirG in the outer membrane (30). The size of the protein detected by the VirG-specific antisera varied according to the sample assayed; thus, the whole-cell lysate could detect predominantly a 120-kDa protein, while the fractionated outer membrane fraction and water extract contained a protein with a molecular size of 92 to 98 kDa.

Minicell analysis of Spa$^+$ region plasmid recombinants. Identification of gene products encoded on pWR266 was determined by introducing the plasmid DNA into *E. coli* minicell strain DS410 and analyzing the ^{35}S-labeled proteins on polyacrylamide gels. DS410(pWR266) revealed 10 radioactive bands with approximate sizes of 47, 34, 30, 22, 21, 19, 17, 12, and 8 kDa, respectively (Fig. 4). It is not clear how many of these labeled bands represent actual proteins encoded by pWR266. The band migrating with a molecular size of 34 kDa was actually composed of two protein bands, as observed during electrophoresis of the minicell-synthesized products on lower-percentage polyacrylamide gels (50). The distribution profile of proteins synthesized in strains DS410(pWR270), DS410(pWR250), and DS410(pWR266) (Fig. 4, lanes A to C) indicated that either the 47-kDa or the 8-kDa protein or both are essential for the Spa$^+$ activity. More recently, deletion of the pWR266 clone, along with minicell and complementation analysis of the deletions, has indicated that the restoration of the *spa* phenotype requires the participation of some of the other proteins that are encoded on pWR266 in conjunction with the 47-kDa and/or 8-kDa proteins (49).

Nucleotide sequence of pWR266. The 5.3-kb nucleotide sequence contained within the 1.2-kb *Hin*dIII and 4.1-kb *Hin*dIII fragments comprising the *spa* locus in pWR266 is shown in Fig. 5. Sequencing was carried out on CsCl-purified double-stranded plasmid templates, using 17-bp primers to successively walk through the sequence.

Analysis of the 5,308-bp nucleotide sequence indicated six complete ORFs, five of which are transcribed in the same direction, whose molecular sizes are 15.1, 47.5, 32.9, 33.4, and 24 kDa. These ORFs are referred to here as *spa15*, *spa47*, *spa32*, *spa33*, and *spa24*, respectively. ORF1, at the beginning of the sequenced region, remains open at its amino-terminal end and continues into the adjacent DNA fragment, while ORF2 encodes a 18.8-kDa protein on the complementary strand (Fig. 5). The intergenic regions were short (between 3 and 15 bases) except that between *spa47* and *spa32*, which was 421 bp in length; in one instance, between *spa32* and *spa33*, the reading frames appeared to overlap. Sequences corresponding to ribosomal binding sites (Shine-Dalgarno sequences; Fig. 5) were seen within 8 to 10 bases upstream of the ATG initiating codons of *spa15*, *spa32*, *spa33*, and *spa24*, while a similar sequence was observed 30 bp upstream of the *spa47* methionine-initiating codon. A recognizable Shine-Dalgarno sequence was not observed upstream of the ORF2 start site. The Spa proteins are moderately acidic, with calculated pI values of 4.2, 5.3, 5.6, 5.4, and 4.7 for Spa15, Spa47, Spa32, Spa33, and Spa24, respectively, while ORF2 encodes a fairly basic protein with a pI value of 9.3.

Computer-derived predictions of hydrophilicity and antigenic indices revealed that, overall, Spa15 is quite hydrophobic and Spa32 is mostly hydrophilic while Spa47 and Spa33 have a fairly uniform distribution of hydrophobic and hydrophilic amino acid residues. Spa24 has a centrally

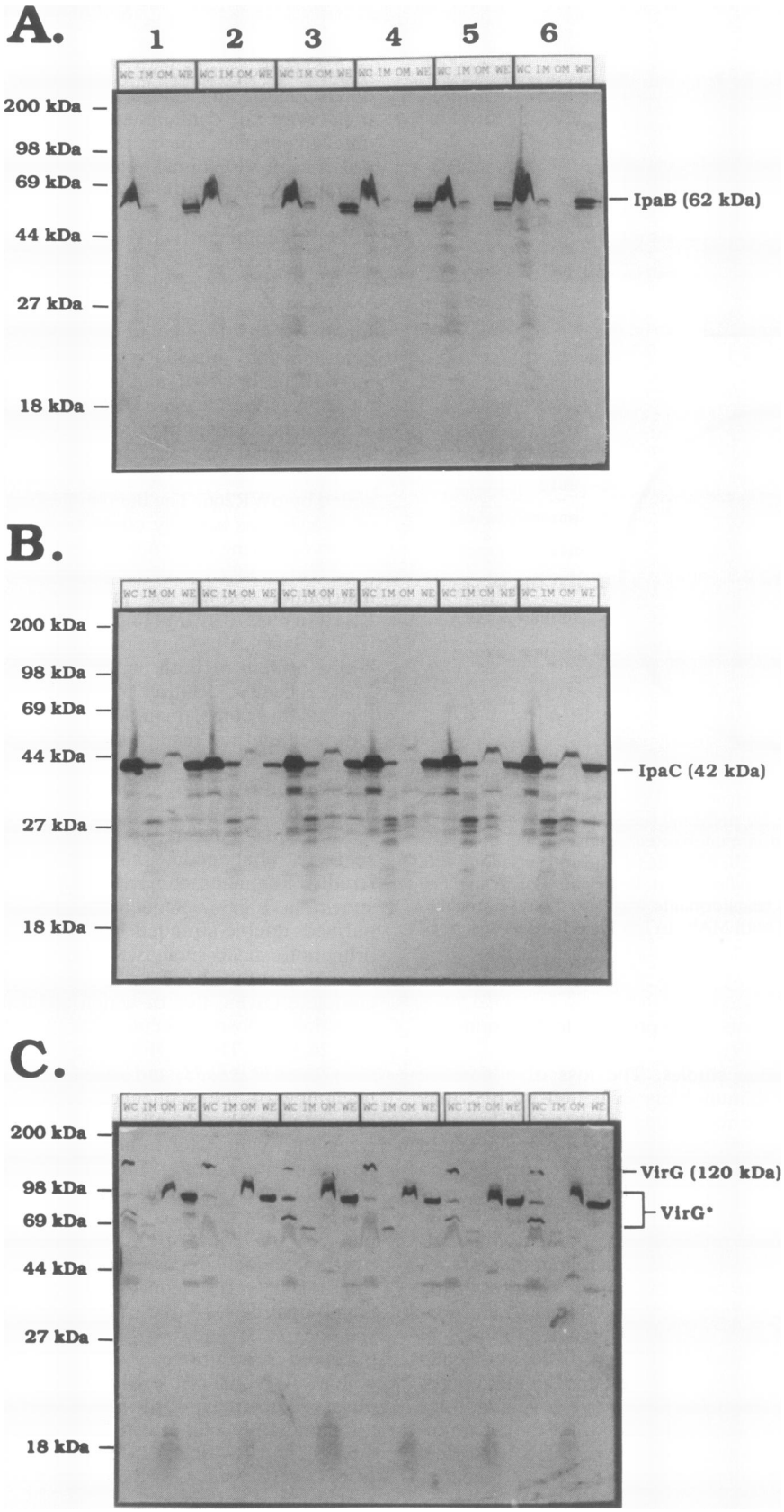

FIG. 3. Cell fractionation studies of Spa$^+$ and Spa$^-$ *S. flexneri* M90T derivatives. M90T (lane 1), pHS1059 (lane 2), pHS1059(pWR230) (lane 3), pHS1059(pWR233) (lane 4), pHS1059(pWR263) (lane 5), and pHS1059(pWR266) (lane 6) were fractionated to obtain inner membrane (IM), outer membrane (OM), whole-cell lysate (WC), and water extract (WE) preparations. The individual fractions were electrophoresed on polyacrylamide gels and blotted onto nitrocellulose filters. The filters were then immunoblotted with IpaB-specific MAb 2F1 (A), IpaC-specific MAb 2G2 (B), and anti-VirG rabbit antisera (C).

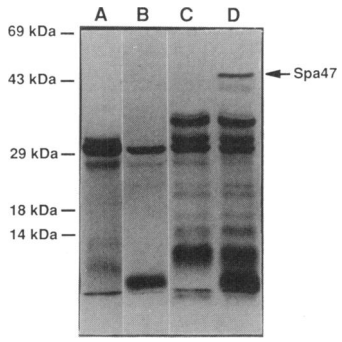

FIG. 4. Minicell analysis of *spa* region plasmid recombinants. [^{35}S]methionine-labeled proteins isolated from minicell strain DS410 transformed with *spa* recombinant plasmids were fractionated on an SDS–18% polyacrylamide gel and electroblotted to nitrocellulose, and the filter was exposed to Kodak Blue Brand X-ray film. The sizes of the protein markers are indicated at the left. The band corresponding to Spa47 is indicated by an arrow. Lanes: A, DS410 (pBR322); B, DS410(pWR270); C, DS410(pWR250); D, DS410 (pWR266).

charged hydrophilic region flanked by extensive stretches of nonpolar hydrophobic amino acids at its N- and C-terminal ends. The amino acid sequences of the Spa proteins do not indicate signal sequences at their amino-terminal ends, characteristic of many prokaryotic periplasmic and/or outer membrane proteins, and except for Spa24, they do not reveal transmembrane domains characteristic of integral membrane proteins. All of the Spa proteins have poor antigenic indices. Amino acid composition of the various Spa proteins revealed that Spa15 and Spa24 have one cysteine residue, while Spa47, Spa32, Spa33, and ORF2 have five, two, seven, and six cysteines, respectively.

It is clear that the number of protein bands synthesized in minicell strain DS410(pWR266) (Fig. 4) exceeded the number of ORFs predicted by DNA sequence analysis of pWR266. While the 47-kDa protein and the two protein bands migrating with molecular sizes of 34 kDa are easily accounted for in the nucleotide sequence (Fig. 5), the origin of the remaining proteins seen in minicells, in context with the ORFs of the DNA sequence, remains to be determined.

Mapping of the Tn*5* insertion in pHS1059 and pHS1060. A rough estimate of the Tn*5* insertion site in pHS1059 and pHS1060 was determined by using defined primers derived from the pWR266 DNA sequence. Since the oligomers hybridized the leftward end of their respective fragments and *Hin*dIII cuts Tn*5* 1,195 bp from the end of the transposon, the *Hin*dIII fragment carrying the transposon was smaller than its wild-type M90T counterpart. Mapping of the Tn*5* insertion in pHS1059 indicated that with primer *Hin*dIII 1.2_L there was no change in the size of the labeled fragment in pWR100, pHS1059, and pHS1060, while primer *Hin*dIII 4.1_L detected a 4.1-kb band in pWR100 and identical 1,750-bp bands in pHS1059 and pHS1060 (Fig. 6). This result positions the Tn*5* in these plasmids 555 bp from the leftward end of the 4.1-kb *Hin*dIII fragment (Fig. 1). It is interesting to note that both insertions appear to have occurred in the same region (but not necessarily the same site), accounting for their similar phenotypes. The Tn*5* insertion, therefore, occurred within the reading frame of the Spa47 protein at its carboxy-terminal end.

Similarities of Spa proteins with the proteins in the NBRF protein data base. The amino acid sequences of the Spa proteins, as deduced from the corresponding DNA sequences, were compared with entries in the NBRF-PIR protein data bases. The Spa47 protein showed significant similarity to the β subunits, and to a lesser extent to the α subunits, of the ATP-hydrolyzing F_1 portion of the H^+-transporting ATP synthases (F_0F_1 ATPases) from a large number of sources, including *E. coli*, *Saccharomyces cerevisiae*, bovine and human mitochondria, and chloroplasts from various plants (16, 17, 26, 35, 40, 45). With use of the FastP algorithm (37), the homology between the catalytic β subunit of *E. coli* F_0F_1 ATPase and Spa47 was 27.5% identity over a 408-amino-acid overlap (Fig. 7). The optimized score for the alignment was 419. No overwhelming similarities were seen between the rest of the Spa protein sequences of *S. flexneri* described here and those in the NBRF-PIR data bases.

The F_0F_1 ATPase complex, composed of a water-soluble component F_1 and a membrane bound F_0 sector, synthesizes ATP coupled with an electrochemical gradient of protons generated by the electron transfer chain (16). The α and β subunits of the F_1 sector contain both catalytic and regulatory sites that bind adenine nucleotides. Recently, two proteins which display homology to the α and β subunits of the F_0F_1 ATPase have been described, both of which are located within a cluster of flagellar genes and which, by virtue of their similarity to each other (49% identity), are considered functionally analogous (1, 52). These two proteins, designated FliI protein from the *fliGHIJK* locus of *Salmonella typhimurium* and ORF4 of the *flaA* locus from *Bacillus subtilis*, are strikingly similar to the Spa47 protein. With the FastP algorithm, the homology between Spa47 and the *flaA* ORF4 protein was 42.5% identity in a 398-amino-acid overlap, with an optimized alignment score of 810. The FliI protein, which is involved in the process of flagellum-specific export, has a 39.5% identity with Spa47 in a 403-amino-acid overlap, with an optimized alignment score of 749. The similarities between Spa47, FliI, and the β subunit of the *E. coli* F_0F_1 complex are indicated in Fig. 7, which shows that the homology between these proteins (and *flaA* ORF4) is distributed over their entire lengths, with greater conservation of amino acid residues in the central portion of the protein molecules than at the terminal ends. In addition, these four proteins are similar in size (Spa47 has 430 amino acids, *flaA* ORF4 has 440 amino acids, FliI has 456 amino acids, and the β subunit of *E. coli* F_0F_1 ATPase has 459 amino acids).

Homology of nucleotide-binding sites in Spa47 and other ATP-binding proteins. Sequence motifs, reflective of a common nucleotide-binding fold, were earlier described for the α and β subunits of *E. coli* ATP synthase, RecA, myosin, adenylate kinase, bovine mitochondrial ATPase, and phosphofructokinase (53). Since then, these residues have been described for a variety of proteins that are involved in transport phenomena (6, 18, 23, 25, 43). These motifs are also seen in Spa47, *flaA* ORF4, and FliI proteins (Fig. 8). That these motifs define a functional domain has been substantiated by a crystallographic and nuclear magnetic resonance study of the MgATP binding site of adenylate kinase, which has shown that these conserved sequences form part of the phosphate-binding region (Fig. 7) (12, 15). The sequence Gly-X-X-Gly-X-Gly forms a flexible loop structure followed by invariant lysine and threonine residues (Fig. 8). The lysine residue in adenylate kinase has been shown to interact with the α-phosphate of MgATP (15). Site-directed mutagenesis of segment A, using F_0F_1 from *E.*

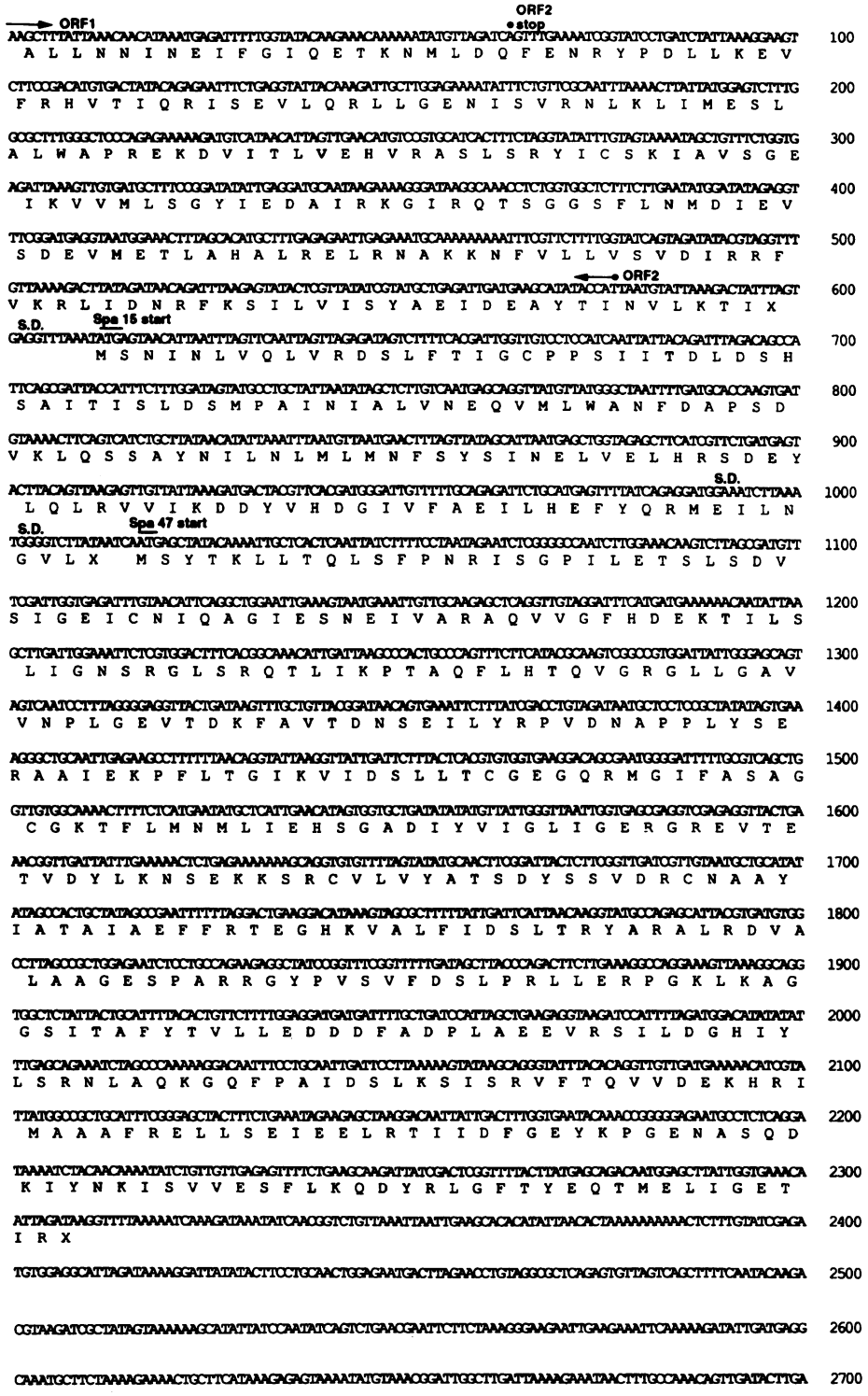

FIG. 5. Nucleotide sequence and deduced amino acid sequence of the *spa* region encoded in pWR266. Nucleotide residues are numbered in the 5′-to-3′ orientation beginning and ending with a *Hin*dIII site. The deduced amino acid sequence is indicated in one-letter code below the nucleotide sequence. The initiation codon for each Spa protein, along with those for ORF1 and ORF2, is indicated on top of the nucleotide sequence along with putative Shine-Dalgarno sequences (S.D.). ORF2 is transcribed on the complementary strand.

coli with mutationally altered subunits or wild-type F_0F_1 that is chemically modified or labeled with reactive nucleotide analogs, supports the idea that the conserved G-X-X-G-X-G-K-S/T is located close to the catalytic site and undergoes conformational movement essential for the catalysis step (15, 16). Sequence motif B, which forms a hydrophobic β-sheet structure approximately 100 amino acids distal to motif A, contains a conserved aspartic acid residue, which

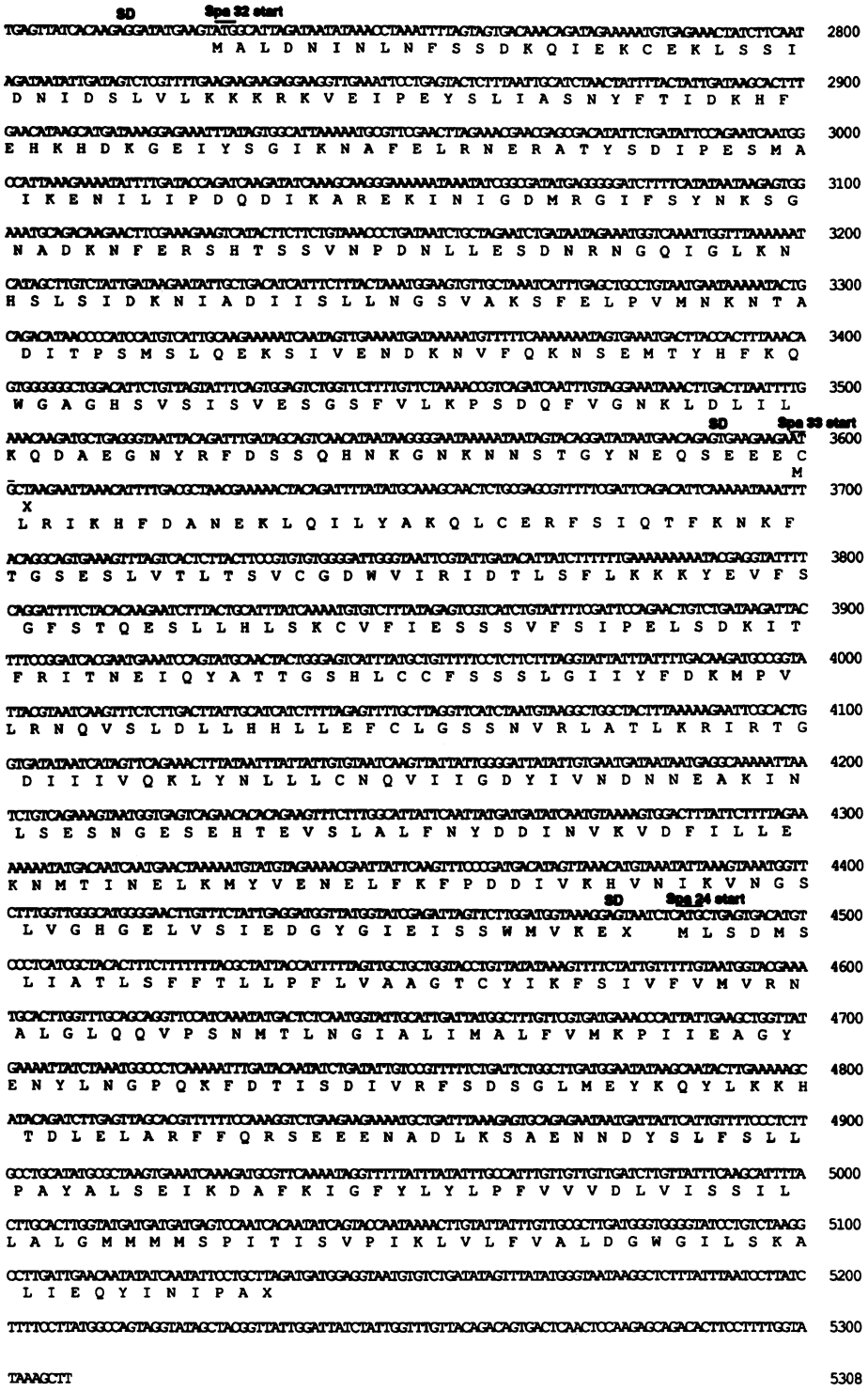

FIG. 5—*Continued.*

has been shown to flank the triphosphate chain of MgATP, including the reaction center (Fig. 8) (15). It is important to note that while most bacterial proteins that bind nucleotides, such as RecA, also retain these short consensus sequences, such proteins show no additional homology to *Shigella* Spa47 protein and its analogs.

DISCUSSION

Transposon mutagenesis of the invasion plasmids from different *Shigella* species has identified five contiguous regions that are associated with HeLa cell invasion (20, 41). These regions, encompassed by a 37-kb segment of the

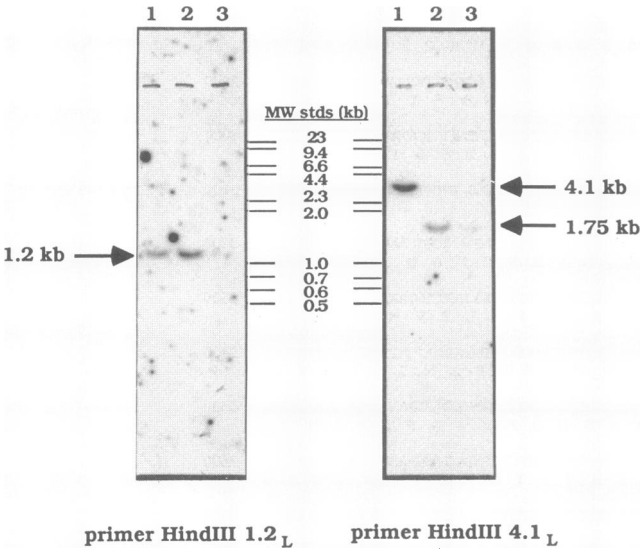

FIG. 6. Mapping of Tn*5* insertions in pHS1059 and pHS1060. Invasion plasmid DNAs, digested with *Hin*dIII, from M90T (lanes 1), pHS1059 (lanes 2), and pHS1060 (lanes 3) were separated electrophoretically, transferred to nitrocellulose, and hybridized with end-labeled primers as indicated. The sizes of the *Hin*dIII fragments that hybridize to each primer probe are shown by arrows. Sizes of molecular weight (MW) standards are indicated.

invasion plasmid, include *ipaR* (*virB*, *invE*), *ipaBCDA*, and virulence regions 3, 4, and 5 (9, 10, 32, 41). Restriction enzyme analysis indicates that the *spa* locus described in this report constitutes region 5 in *S. flexneri* 5. The restriction enzyme digestion patterns of various *spa* clones suggest that the *spa* locus may partially overlap the earlier-described *invA* region of *S. sonnei* (55). *invA* was characterized by constructing Tn*1* insertions into the *S. sonnei* invasion plasmid and identifying a contiguous molecule of 2.6- and 4.1-kb *Hin*dIII fragments that could restore cell invasiveness to the mutants. Minicell analysis of the *invA* clone demonstrated the synthesis of 38-, 41-, 47-, and 80-kDa products. The *invA* mutation was not characterized in terms of Ipa protein synthesis or the surface presentation of the Ipa antigens. Further characterization of the *invA* locus is necessary before the two loci can be adequately compared. A recent study, using random insertion of the transposable bacteriophage λ*plac*Mu53 in *S. flexneri* 2a, has identified two temperature-regulated noninvasive *inv*::*lacZ* fusion mutants (*mxiA* and *mxiB*) which mapped within the 11.5-kb *Eco*RI fragment (3, 24). The *mxiB* mutant, like pHS1059, was deficient in the surface expression of IpaB while maintaining intermediate levels of expression of IpaC. From restriction mapping analysis, the *mxiB* locus of *S. flexneri* 2a appears to overlap the *spa* locus in *S. flexneri* 5.

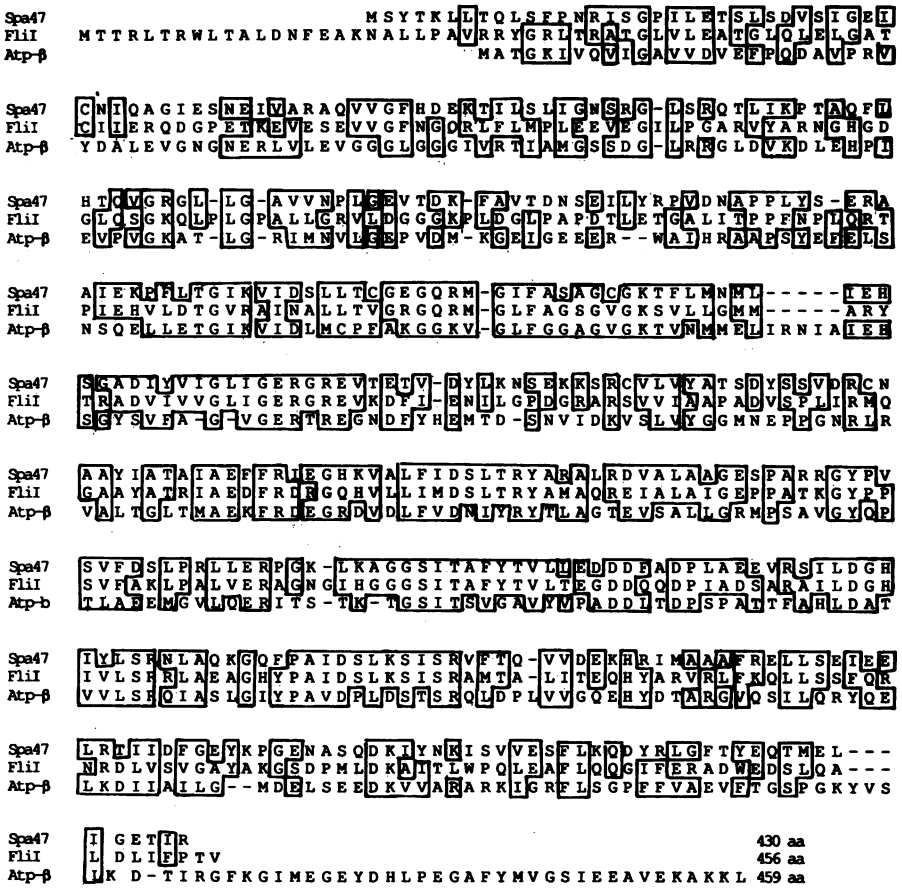

FIG. 7. Homology between the amino acid sequences of Spa47, FliI, and the β subunit of *E. coli* F_0F_1 ATPase (Atp-β). The amino acid sequences of the three proteins are aligned, with identical and/or conservative changes (I-L-V-M-F, A-G, F-Y, D-E, H-K-R, N-Q, and S-T) boxed to indicate homology. Gaps have been introduced to maintain alignment of homologous sequences in the primary structure. The total number of amino acid residues in each protein is indicated at the end of each sequence.

Sequence of amino acids in motif A	Residues#	Sequence of amino acids in motif B	Residues#	Protein	Total# am.ac.	Function
G Q R M G I F A S A G C G K T F	152-167	V A L F I D S L T R Y A	244-256	Spa47	430	transport of Ipa antigens
G Q R I G I F A G S G V G K S T	160-175	V M F M M D S V T R V A	252-263	ORF4	440	expo. path for flagellin.
G H F T A I I G P N G C G K S T	34-49	I M L L - D E P T T W L	164-176	FepC	269	enterobactin transport
G E V I G I V G R S G S G K S T	495-510	I L I F - D E A T S A L	626-638	HlyB	707	active hemolysin export
L A G C G D G G A L Q P G K R G	94-109			PapJ	193	P pilus chaperone
G E T V A I V G P T G A G K T T	360-375	I L V L - D E A T S A L	492-504	ChvA	588	export of β-1,2 glucan
G D V I S I I G S S G S G K S T	33-48	V L L F - D E P T S A L	172-190	HisP	258	uptake of histidine
G Q T V A L V G N S G C G K S T	419-434	I L L L - D E A T S A L	550-562	Mdr	1276	eff. pump for lipop.drug
G Q F T F I V G K S G S G K S T	385-400	P I L E L D E A V S A L	525-537	Ste6	1290	exp. of mating pheromn.
G Q R M G I F A S A G C G K T F	142-157	V L L F V D N I Y R Y T	237-248	E. c.β	459	proton-transloc. ATPase
G G T V A I P G A F G C G K T V	226-240	V A M M A D S S S R W A	163-180	Va. Atp	607	proton-transloc. ATPase
G G K I G L F G G A G V G K T V	360-375	T L I I Y D D L S K Q A	268-280	Chl. Atp	498	oxid. phophorylation
G G K I G L F G G A G V G K T V	188-203	V L F F I D N I F R F T	282-300	Mit. Atp	509	oxid. phosphorylation
S K I I F V V G G P G S G K G T	8-23	L L L Y V D A G P E T V	113-125	Ad. kin.	350	rev.trans.ofphosp.grp.
G R I V E I Y G P E S S G K T T	59-74	V I I V - D S V A A L T	140-152	RecA	352	recom/repair/phg.induc
N Q S I L I T G E S G A G K T V	191-206			Myosin	1939	muscle contraction
T E K L V V V G A R G V G K S A	2-17			Ras p21	385	oncogenic protein

FIG. 8. Alignment of conserved ATP-binding motifs observed in *S. flexneri* Spa47 and other proteins that bind ATP. The conserved sequences around Walker motifs A and B (53), which are separated in each protein by approximately 100 amino acid residues, are indicated in Spa47 as well as in other ATP-binding proteins. The amino acid sequences were from the following sources (listed in the same order as in the figure): Spa47 (this report), *flaA* ORF (1), FepC (43), HlyB (14), PapJ (46), ChvA (11), HisP (22), Mdr (9), Ste6 (28), F_0F_1 *E. coli* β subunit (40), *Neurospora crassa* vacuolar ATPase (7), chloroplast ATPase (26), β subunit of human mitochondrial ATPase (35), adenylate kinase (27), RecA (39), myosin (54), and $p21^{ras}$ (12).

The absence of IpaB and reduced levels of IpaC in water extracts of pHS1059, with concomitant loss of the HeLa cell invasion phenotype and subsequent restoration of both activities in *spa*-complemented pHS1059 strains, substantiate earlier observations that the surface localization of the Ipa antigens, and not just their synthesis, is crucial for the expression of the invasive phenotype (24, 33, 49, 50). The IpaB protein was detected in the inner membrane but not the outer membrane of all cell types tested. However, unlike what has been observed with the *mxiB* mutant, IpaB did not accumulate in the inner membrane of pHS1059, as might be expected if the cytoplasmic membrane is a staging area for transport of IpaB to the cell surface (3). The absence of IpaB and the low levels of IpaC from the outer membrane may result from a loose association of the proteins with this structure, especially if IpaB and/or IpaC are critical ligands for invasion of the host cells by the bacteria. The presence of IpaB and IpaC in culture supernatants, as described in this study and elsewhere (3, 24), as well as their presence in water extracts of *Shigella* cells, emphasizes the generally held notion that these antigens are excreted proteins or weakly associated with the cell periphery. The synthesis and expression of VirG were not affected in pHS1059. Although both the Ipa proteins and VirG lack typical signal peptide sequences, it appears that the transport and insertion of VirG into the outer membrane occurs by a mechanism different from the *spa*-associated expression of the Ipa antigens. This would not be unexpected, since the function of VirG (intra- and intercellular spreading) is independent of the Ipa proteins. Interestingly, a smaller version of VirG (VirG*) has been identified by using antisera specific for VirG. The source of VirG* has not been determined, but it likely arises either from a processing event of the larger VirG molecule or as the product of an in-frame internal initiation which has been previously proposed (30).

The *spa* locus described in this report encodes more than one protein that is required for the surface association of the Ipa antigens. Deletion analysis and mutagenesis of individual ORFs are in progress to determine how many of the encoded proteins are actually involved in maintaining the Spa^+ phenotype. What appears to be a common theme in many prokaryotic systems, including the *spa* locus, is the involvement of several contiguously located genes and gene products involved in the transport of amino acids, peptides, and bacterial antigens across the inner and outer membranes. For example, at least three of the genes encoded in the *fliGHIJK* loci of *S. typhimurium* are involved in a flagellin-specific export pathway. Similarly, multicomponent pathways exist for the synthesis and transport of the *E. coli* hemolysin, ferric enterobactin, and P pili, secretion of cyclic β-1,2-glucan by *Agrobacterium tumefaciens*, and the transport of amino acids and sugars such as histidine, arginine, and maltose (11, 18, 22, 29, 38, 43, 46). The observation that restoration of Spa47 protein synthesis alone does not restore the wild-type phenotype to pHS1059 indicates that the synthesis of more than one protein is disrupted by the Tn*5* insertion in the *spa47* gene.

The exact role of the Spa proteins in the surface presentation of the Ipa antigens remains to be elucidated. The Ipa antigens, unlike many *E. coli* proteins that reside in the periplasmic space or outer membrane, do not contain hydrophobic amino-terminal signal sequences that are proteolytically removed after membrane translocation. In *E. coli*, the translocation of periplasmic maltose-binding protein re-

quires the interaction of soluble factors with the maltose-binding protein precursor that promote or stabilize a specific prefolded conformation critical for export (29, 38). A well-characterized protein in this regard is the *E. coli secB* gene product; the export of several *E. coli* proteins, but not all, are defective in *secB* mutant cells. A similar role has been suggested for other proteins which function as molecular chaperones, such as trigger factor and the GroE proteins (29). Although the Spa proteins bear no resemblance to either SecB or GroE, it is conceivable that the assembly and/or maintenance of a particular folded conformation of the Ipa antigens precedes their export. The Spa proteins could then play a role in preserving the translocation competence of the Ipa antigens, and mutations in the *spa* loci would render the strain defective in the export of the plasmid antigens.

The significant sequence homology of Spa47 to *S. typhimurium* FliI and *B. subtilis flaA* ORF4 suggests that these proteins are analogous. Besides the conserved ATP-binding motifs that these three proteins share with the α and β subunits of the F_0F_1 ATPases, we were unable to achieve significant overall alignments between Spa47 and other ATP-utilizing proteins (listed in Fig. 8). This finding would suggest a common ancestral gene for *spa47*, *fliI*, *flaA* ORF4, and the α and β subunits of F_0F_1 ATPase that contained the ATP-binding motif, which then diverged to produce the particular DNA sequences of each gene. It is important to note that with the exception of Spa47, the products of none of the other *spa* genes encoded by pWR266 bear any resemblance to the remainder of the proteins encoded by the *flaA*, *fliGHIJK*, or *unc* operon. Thus, the presence of an ATP-binding protein may be an obligatory feature of different transport systems, the specificity of each being determined by the presence of other unique proteins within each system. The Spa proteins appear to be different from the periplasmic binding protein-dependent transport systems exemplified by HisP, which forms part of the ABC superfamily of transmembrane transporters involved in the movement of ions, small molecules, and peptides (23). In *E. coli* and *S. typhimurium*, the periplasmic transport system consists primarily of a periplasmic binding protein, two hydrophilic integral membrane proteins, and an ATP-binding peripheral membrane protein. None of the Spa proteins contain signal sequences, and their localization in different cell fractions remains to be determined.

The conserved nucleotide-binding domains seen in Spa47, FliI, FepC, HisP, etc., imply the role of ATP in these transport systems. Recent studies with HisP have shown that this protein can bind ATP and that hydrolysis of ATP is coupled to histidine transport both in vivo and in vitro (2). It remains to be determined whether Spa47 can bind ATP or whether hydrolysis of ATP is intrinsically linked to the export of the Ipa antigens. It is interesting to observe that the two conserved ATP-binding motifs are not seen in other ion-motive ATPases such as the plasma membrane Na^+-K^+ ATPase of sheep kidney, the *kdp* K^+ ATPase of *E. coli*, or the Ca^{2+} ATPase of rabbit sarcoplasmic reticulum, whose members are distinguished from the F_0F_1 family in forming a covalent β-aspartyl phosphate intermediate during catalysis (21).

ACKNOWLEDGMENTS

We thank P. J. Sansonetti and T. L. Hale for the generous donation of strains pHS1059 and pHS1060, J. A. Mills and K. Ross Turbyfill for help with Western blots, Ken Stover for help with the GCG DNA sequence analysis program, and T. L. Hale and E. A. Elsinghorst for reading the manuscript.

ADDENDUM IN PROOF

The ORF1 contained in pWR266 corresponds to the carboxy-terminal portion of a gene encoding a 76-kDa protein. Mutations in this gene, which overlaps both virulence region 4 and *mxiA* (3; C. Sasakawa, personal communication) on the *S. flexneri* invasion plasmid, result in a phenotype similar to that observed with pHS1059. The amino acid sequence of the 76-kDa protein, derived from the nucleotide sequence, indicates several membrane-spanning domains (C. Sasakawa, personal communication).

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