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Identification of *Shigella sonnei* Form I Plasmid Genes Necessary for Cell Invasion and Their Conservation among *Shigella* Species and Enteroinvasive *Escherichia coli*

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A series of Tn*1* insertions in pSS120, the 120-megadalton form I plasmid of *Shigella sonnei*, were constructed by a Tn*1*-mediated conduction system previously described (H. Watanabe and A. Nakamura, Infect. Immun. 48:260–262, 1985) and screened for cell invasion in a tissue culture assay. The analysis of Tn*1* insertion sites of seven noninvasive mutants suggested that four separate *Hin*dIII fragments were necessary for cell invasion. *Hin*dIII fragments including Tn*1* of mutant plasmids were cloned into a vector plasmid, pACYC184. The DNA was used as a DNA probe to identify the corresponding, parental *Hin*dIII fragments. We identified one contiguous molecule of 2.6- and 4.1-kilobase pair (kb) *Hin*dIII fragments as being responsible for restoring cell invasiveness to the three mutant plasmids, pHW505, pHW510, and pHW511. Polypeptide analysis in minicells demonstrated that the contiguous *Hin*dIII fragments of 2.6 and 4.1 kb coded for at least four polypeptides, of 38, 41, 47, and 80 kilodaltons (kDa). A comparison of polypeptides synthesized by parental and mutant plasmids strongly suggested that the 38-kDa protein was essential for cell invasion. The 4.1-kb DNA which encoded the 38-kDa protein was conserved among plasmids of *Shigella* species and enteroinvasive *Escherichia coli*.

The essential virulence property of dysentery-producing bacteria is the ability to penetrate, multiply within, and kill epithelial cells of the colon (15, 18). The invasiveness phenotype of the four *Shigella* species is associated with the presence of a large, 120- to 140-megadalton (MDa) plasmid (20, 23, 24, 30). Large-plasmid-cured derivatives are nonpathogenic and are not able to invade tissue culture cells (23, 24, 30). Complete restoration of the invasiveness phenotype is accomplished by transfer of the plasmid back into the plasmid-cured cells (23, 24, 30). The virulence-associated large plasmids are interchangeable among *Shigella* species and have in common the function for cell invasion (30). When the plasmid is transferred to the noninvasive laboratory strain *Escherichia coli* K-12, this bacterium acquires the ability to invade and multiply within tissue culture cells (22, 30). Thus, the large plasmid carries determinants that are essential and sufficient for bacterial invasion of mammalian cells.

A large plasmid of *Shigella flexneri* encoded over 40 polypeptides (7). Hale et al. (7) demonstrated that seven of these polypeptides were unique to invasive strains of *S. flexneri* and *E. coli*. Maurelli et al. (17) have recently cloned an *S. flexneri* plasmid DNA sequence of ca. 37 kilobases (kb) which was sufficient to enable avirulent, plasmidless *S. flexneri* cells to invade HeLa cells. An *S. flexneri* strain having the 37-kb DNA sequence (17) expressed the same four polypeptides, of 38, 43, 62, and 78 kilodaltons (kDa), as those described by Hale et al. (7). However, whether these polypeptides are essential for cell invasion is not known.

We have recently developed a method to efficiently transfer a nonconjugative plasmid from one strain to another by Tn*1*-mediated conduction (30). This method allowed us to construct Tn*1* transposon insertions of the *Shigella* virulence-associated large plasmid. In this study, we describe the identification of a 6.7-kb DNA fragment of *S. sonnei* plasmid pSS120 as a DNA molecule restoring cell invasiveness to noninvasive mutants. Minicells with the 6.7-kb DNA synthesized four polypeptides; at least one of them, the 38-kDa protein, was suggested to be essential for cell invasion. The DNA fragment which encoded the 38-kDa protein was conserved among *Shigella* and enteroinvasive *E. coli* plasmids.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The bacterial strains and plasmids used in this study are listed in Table 1. Bacteria were grown in L broth or on plates of L agar or antibiotic medium number 3 (Difco Laboratories, Detroit, Mich.) solidified with 1.5% agar. Antibiotics were used at the following concentrations (in micrograms per milliliter): ampicillin, 50; chloramphenicol, 50; kanamycin, 50; streptomycin, 200; and tetracycline, 6.

Tissue culture infection. Infection of tissue culture with bacteria was carried out by the procedure described previously (32). Nonconfluent monolayers of LLC-MK$_2$ cells (30) were used for tissue culture assay. After infection, extracellular bacteria were killed by the addition of kanamycin (200 μg/ml) and gentamicin (100 μg/ml).

Tn*1* insertion mutagenesis. The strategy of Tn*1* insertion mutagenesis was described previously as the Tn*1*-mediated conduction system (30). This method is based on the facts that Tn*1*-related ampicillin transposons transpose onto the plasmid DNA more readily than onto the cellular chromosome (13) and that a cointegrate between Tn*1* donor and recipient is formed as an intermediate of Tn*1*-mediated transposition (25). Plasmid pTH10 (8), used as the Tn*1* donor, is a derivative of RP4 that is temperature sensitive for maintenance and confers upon its host resistance to kanamycin, tetracycline, and ampicillin (Km^r, Tc^r, and Ap^r, respectively). Plasmid pSS120 (30) has genes necessary for form I antigen production and sufficient to allow *E. coli* K-12 to invade culture cells in vitro. *S. sonnei* HW436(pSS120,

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TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Relevant characteristics[a]	Invasiveness[b] in LLC-MK$_2$ cells	Source or reference
S. sonnei			
HW383	*nad*, pSS120, cryptic plasmids	+	30
HW436	HW383(pTH10)	+	30
S. dysenteriae			
HW257	Serotype 1, a large plasmid, cryptic plasmids	+	30
S. boydii			
pp343	Serotype 1, a large plasmid	+	N. Okamura
S. flexneri			
HW283	Serotype 2a, *nad*, pSF140, cryptic plasmids	+	30
HW1002	Serotype 1b, no large plasmid, cryptic plasmids	−	30
E. coli			
HW906	0124, a large plasmid, cryptic plasmids	+	M. Ohashi
HW907	0136, a large plasmid	+	M. Ohashi
HB101	*ara-14 leu proA2 lacY1 glnV44 galK2 recA13 rpsL20 xyl-5 mtl thi hsdS20*	−	2
P678-54	*thr leu lacY minA minB gal str thi*	−	1
pSS120	Virulence plasmid from *S. sonnei* HW383, nonconjugative, form I antigen		30
pSF140	Virulence plasmid from *S. flexneri* 2a HW283, nonconjugative		30
PTH10	Temperature-sensitive derivative of RP4, conjugative, Ap^r (Tn*1*) Km^r Tc^r		8 T. Iino
pACYC184	Cloning vector, Cm^r Tc^r		3
pHSG415^r	Cloning vector, Ap^r Km^r Cm^r, temperature-resistant derivative of pHSG415		T. Hashimoto
pSC101::Tn*1*	Tn*1* probe, Tc^r Ap^r (Tn*1*)		pTH10-derived Tn*1* insert of pSC101

[a] Abbreviations and nomenclature are essentially those of Demerec et al. (4).

[b] +, Invasive; −, noninvasive.

pTH10) was cultured in the medium containing kanamycin and ampicillin at the nonpermissive temperature (42°C) for maintenance of plasmid pTH10 to select cointegrates of pTH10 and pSS120. Resultant colonies were mixed with *E. coli* HB101. The mating mixture was spread on the plate containing streptomycin, ampicillin, and kanamycin for selection of transconjugants. One transconjugant was selected from a mating experiment, and possession of form I antigen in the transconjugant was determined by the slide agglutination test with form I antigen-specific rabbit antiserum. Form I-positive transconjugants were examined for cell invasion by tissue culture assay.

Isolation and characterization of plasmid DNA. Rapid screening of plasmid DNA was done by the procedure of Kado and Liu (11). For the preparation of large quantities, DNA was isolated by a modification of this procedure. Stationary phase cells from a 200-ml L broth culture were harvested and suspended in 50 ml of 1% sodium dodecyl sulfate–10 mM Tris (pH 12.6). Lysates were heated at 65°C for 1 h, neutralized by the addition of 15 ml of 2 M Tris hydrochloride (pH 7.0), and centrifuged at 15,000 rpm for 30 min. DNA was concentrated with 10% (wt/vol) polyethylene glycol (Carbowax [Union Carbide Corp., New York, N.Y.] 6000) and further purified by centrifugation in a CsCl density gradient containing ethidium bromide. Restriction endonuclease digestions of plasmid DNA were performed by the procedures recommended by the manufacturers. Plasmid DNA was characterized by electrophoresis on 0.8% agarose gels with Tris-borate (89 mM Tris base, 2.5 mM disodium EDTA, 89 mM boric acid [pH 8.0]) as a running buffer.

Cloning procedure. The methods used for constructing recombinant DNA molecules were essentially those described previously (28, 31). Plasmid DNA was partially or completely digested with restriction enzymes. The cloning vector pACYC184 (3) or pHSG415^r (provided by T. Hashimoto) was digested with *Hin*dIII and dephosphorylated. Donor and vector DNAs were ligated, and the ligated DNA was transformed into *E. coli* HB101 with or without plasmid as previously described (14).

Preparation of DNA probes and hybridization. The DNA fragment to be used as a probe was extracted from a 0.8% agarose gel by the procedure of Vogelstein and Gillespie (29). DNA probe was labeled with [^{32}P]dCTP by nick translation (19). Before transfer, digested DNAs or covalently closed circular DNAs were treated with 0.1 N HCl for 10 min, denatured, and neutralized by the method of Southern (27). Treated DNA molecules were transferred from an agarose gel to a nitrocellulose filter (BA85; Schleicher & Schuell, Inc., Keene, N.H.) and hybridized to a radioactive probe under the stringent conditions used by Southern (27). Colony hybridization was carried out by the procedure of Grunstein and Hogness (6).

Analysis of protein expression in minicells. Suspensions of minicells were prepared and radiolabeled with 40 μCi of [^{35}S]methionine in sulfur-free Hershey medium as previously described (31). Labeled minicells were suspended in sample buffer containing 3% sodium dodecyl sulfate, 5% 2-mercaptoethanol, 10% glycerol, and 0.01% bromphenol blue, boiled for 3 min, and loaded onto a 13% (wt/vol) acrylamide gel containing sodium dodecyl sulfate. Electrophoresis was carried out at a constant current of 20 or 25 mA with a discontinuous buffer system (16). Fixation and fluorography of dried gels were performed as previously described (31).

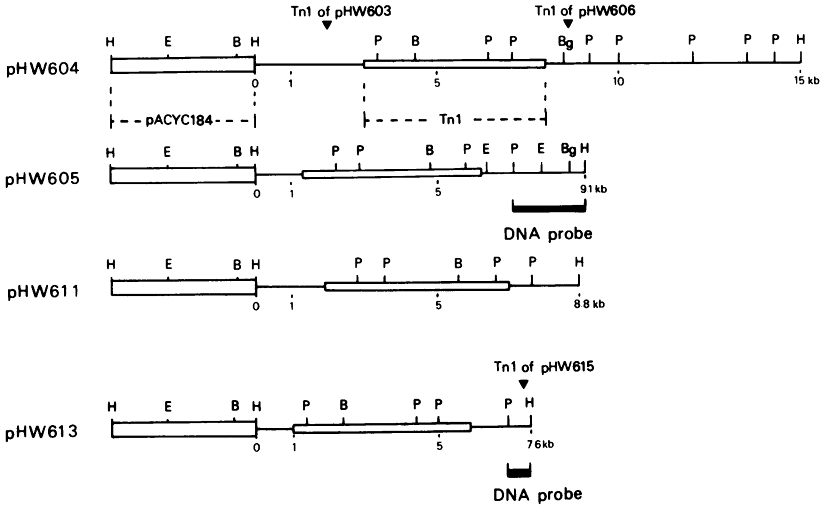

FIG. 1. Recombinants of Tn*1*-including *Hin*dIII fragments of seven noninvasive plasmids with the vector plasmid pACYC184. Plasmids pHW603, pHW604, and pHW606 were pACYC184-ligated recombinants of the 15.0-kb *Hin*dIII fragment of pHW502, pHW503, and pHW504, respectively. Plasmid pHW605 was a pACYC184-ligated recombinant of the 9.1-kb *Hin*dIII fragment of pHW505. Plasmid pHW611 was a pACYC184-ligated recombinant of the 8.8-kb *Hin*dIII fragment of pHW506. Plasmids pHW613 and pHW615 were pACYC184-ligated recombinants of the 7.6-kb *Hin*dIII fragment of pHW510 and pHW511, respectively. H, E, B, P, and Bg, Restriction sites of *Hin*dIII, *Eco*RI, *Bam*HI, *Pst*I, and *Bgl*II, respectively. The plasmids are linearized at a *Hin*dIII junction of pACYC184 and the insert. ■, DNA sequences used as DNA probes.

RESULTS

Insertion mutagenesis of pSS120 and identification of Tn*1* insertion sites of noninvasive mutants. To identify regions of plasmid pSS120 which were required for cell invasion, we constructed independent Tn*1* insertion sites by the procedure described in Materials and Methods. *S. sonnei* HW436 was cultured in the medium containing kanamycin and ampicillin at 42°C. Resultant colonies were mixed with *E. coli* HB101. A total of 500 form I-positive transconjugants of *E. coli* HB101 were selected and examined for the ability to penetrate LLC-MK$_2$ cells. We found that seven strains were noninvasive. Plasmid pTH10 was cured from the mutant strains by culturing at 42°C. The pTH10-cured strains still retained ampicillin resistance and form I antigen. Because a 120-MDa form I plasmid of *S. sonnei* is physically unstable (12), we could easily obtain form I-negative mutants from the pTH10-cured, ampicillin-resistant, form I-positive strains. The mutants always lost ampicillin resistance and the plasmid similar in size to plasmid pSS120, designated pSS120::Tn*1*. These results strongly suggested that the ampicillin resistance transposon Tn*1* of plasmid pTH10 inserted onto the 120-MDa form I plasmid, pSS120. Resulting pSS120::Tn*1* plasmids of seven noninvasive strains are referred to as pHW502, pHW503, pHW504, pHW505, pHW506, pHW510, and pHW511.

Tn*1* insertion sites of these plasmids were determined by hybridization with a Tn*1* probe, pSC101::Tn*1*, after cleavage of plasmid DNAs with *Hin*dIII restriction enzyme, which does not cut Tn*1* (10). The Tn*1* probe hybridized with *Hin*dIII fragments of four different sizes: the 15.0-kb *Hin*dIII fragments of pHW502, pHW503, and pHW504; the 9.1-kb fragment of pHW505; the 8.8-kb fragment of pHW506; and the 7.6-kb fragments of pHW510 and pHW511. These *Hin*dIII fragments were cloned into the *Hin*dIII site of a cloning vector, pACYC184, and selection was made for ampicillin resistance. The resultant recombinant plasmids were cleaved with various restriction enzymes. Restriction cleavage maps are shown in Fig. 1. Restriction cleavage comparison of DNA sequences including Tn*1* demonstrated that four *Hin*dIII fragments of pSS120 were different from each other. Four distinct *Hin*dIII fragments of pSS120 seemed to be necessary for cell invasion.

Isolation of DNA sequence of pSS120 corresponding to the pHW605 and pHW613 DNAs. For a mutation of the invasiveness gene to occur by Tn*1* insertion, it is necessary to clone DNA fragments which rescue the mutation. We further analyzed pHW605 and pHW613, which were pACYC184-ligated recombinants of the 9.1-kb *Hin*dIII fragment of pHW505 and the 7.6-kb *Hin*dIII fragment of pHW510, respectively (Fig. 1). We tried to identify parental *Hin*dIII fragments corresponding to the Tn*1*-containing *Hin*dIII fragments of pHW605 and pHW613. A part of the DNA sequence of pHW605 or pHW613 shown in Fig. 1 was used as a DNA probe to isolate the corresponding sequences. Plasmid pSS120 was partially digested with *Hin*dIII restriction enzyme and cloned into the *Hin*dIII site of a vector plasmid, pHSG415^{r}. A total of 300 recombinants were screened for homology with the DNA probe by colony hybridization (6). We isolated six recombinants, pHW625, pHW626, pHW627, pHW629, pHW635, and pHW641, which showed homology with the DNA probe of pHW605. *Hin*dIII-digested DNA fragments of these recombinants are shown in Fig. 2 (lanes 6 to 10) and 3. The cloned inserts all contained a common core of 4.1-kb *Hin*dIII fragment, which hybridized with the DNA probe from pHW605 (Fig. 2 and 3). With the DNA probe of pHW613, three recombinants, pHW626, pHW641, and pHW647, were isolated. The cloned inserts all contained a common core of 2.6-kb *Hin*dIII fragment (Fig. 3), which hybridized with the DNA probe from pHW613 (data not shown). Plasmids pHW626 and pHW641 shared the 2.6- and 4.1-kb *Hin*dIII fragments (Fig. 3). We constructed a restriction cleavage map of a part of the plasmid pSS120 with those inserts of recombinants (Fig. 3).

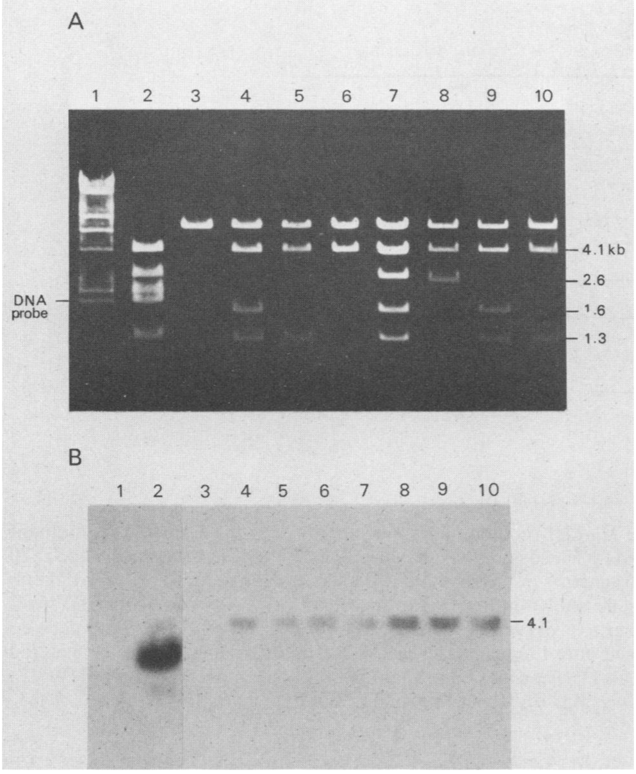

FIG. 2. *Hin*dIII digestions of recombinant plasmids and hybridization with a ^{32}P-labeled DNA probe of pHW605. Lanes: 1, *Hin*dIII-digested phage λ DNA; 2, *Hin*dIII- and *Pst*I-digested pHW605; 3 to 10, *Hin*dIII-digested pHSG415^{r}, pHW628, pHW636, pHW629, pHW635, pHW626, pHW625, and pHW627, respectively. Inserts of pHW628 and pHW636 were derived from pSF140. Inserts of pHW629, pHW635, pHW626, pHW625, and pHW627 were derived from pSS120. (A) Digested DNA. (B) Southern hybridization of the digested DNA with a ^{32}P-labeled DNA probe of pHW605.

The parental 4.1- and 2.6-kb *Hin*dIII fragments corresponding to the Tn*1*-containing *Hin*dIII fragments of pHW605 and pHW613, respectively, existed as one contiguous molecule.

Restoration of cell invasiveness. We examined whether the recombinant plasmids could restore invasiveness to *E. coli* HB101 harboring pHW505, pHW510, or pHW511. A recombinant plasmid was transformed into *E. coli* HB101 with or without a mutant plasmid by selection for chloramphenicol resistance (Cm^{r}), and chloramphenicol-resistant transformants were examined for cell invasion. The invasiveness phenotype of transformants is summarized in Table 2. None of the parental *Hin*dIII fragments were capable of restoring invasiveness to *E. coli* without mutant plasmids. All recombinants containing the 4.1-kb *Hin*dIII fragment (e.g., pHW626 and pHW629) restored invasiveness to *E. coli* HB101(pHW505). A recombinant containing only the 2.6-kb *Hin*dIII fragment (e.g., pHW647) could not rescue the mutation(s) of pHW510 and pHW511. To rescue the mutation(s) of pHW510 and pHW511, one contiguous molecule of 2.6- and 4.1-kb *Hin*dIII fragments (e.g., pHW626) was required. These results indicated that the 6.7-kb DNA sequence containing 2.6- and 4.1-kb *Hin*dIII fragments carried genes necessary to complement the mutations of pHW505, pHW510, and pHW511 and that other DNA sequences, in addition to the cloned DNA sequences, were also required for expression of invasiveness. Invasiveness of the Tn*1* insertion mutants was not fully restored by the recombinant plasmids. A total of 5- to 10-fold fewer cells were infected by strains with noninvasive Tn*1*-inserted plasmids and the corresponding recombinant plasmids compared with the rate of infection by a strain with a parental large plasmid.

S. sonnei form I plasmid is known to be unstable (12, 23). A single colony purified with or without selective pressure was spread onto L agar, and the resultant colony types were examined for the presence of form I pSS120 plasmid by the slide agglutination test using anti-form I serum or were examined for drug resistance after 24 h of incubation at 37°C. Plasmids pHW505, pHW510, and pHW511 were maintained in ca. 95% of the HB101 cells. Recombinant clones containing the 4.1-kb *Hin*dIII fragment (e.g., pHW626 and pHW629) were unstable when purified without chloramphenicol; approximately 30% of the cells lost chloramphenicol resistance. Plasmids pHSG415^{r} and pHW647, the latter a recombinant clone containing the 2.6-kb *Hin*dIII fragment, were more stable; more than 98% of the cells maintained chloramphenicol resistance. When a strain harboring pHW505 and a recombinant clone containing the parental 4.1-kb *Hin*dIII fragment (e.g., pHW629) or a strain harboring pHW505, pHW510, or pHW511 and a recombinant clone containing both parental 4.1- and 2.6-kb *Hin*dIII fragments (e.g., pHW626) was cultured under the selective pressure of chloramphenicol, only ca. 20% of the cells retained form I pSS120 plasmid. However, pHW505, pHW510, or pHW511 coexisting with a recombinant clone containing only the 2.6-kb *Hin*dIII fragment (e.g., pHW647) or the vector plasmid pHSG415^{r} was as stable as pHW505, pHW510, or pHW511 itself. We do not know why pHW505, pHW510, and pHW511 were much more unstable after the coexistence of certain recombinants. The instability of pHW505, pHW510, and pHW511 may, however, reflect the lower infective rate of HB101 derivatives with noninvasive Tn*1*-inserted pSS120 which were transformed by the recombinant plasmids.

Identification of plasmid-coded polypeptides essential for cell invasion. Identification of plasmid-coded polypeptides essential for cell invasion was carried out by the analysis of products expressed in minicells. The 4.1-kb *Hin*dIII fragment was able to complement the mutation of plasmid pHW505 (Table 2). We then cloned the 4.1-kb *Hin*dIII fragment of pHW629 into the *Hin*dIII site of a vector plasmid, pACYC184, which was designated pHW655, and compared polypeptides of pHW655 expressed in minicells with those of pHW605. Plasmid pHW655 synthesized 38 (Fig. 4, lane 1, a)- and 39-kDa proteins in addition to those of the vector plasmid, pACYC184 (Fig. 4, lanes 1 and 3). The 38-kDa protein synthesized in pHW655 disappeared in the Tn*1* insertion mutant plasmid pHW605 (Fig. 4, lanes 1 and 2). Plasmid pHW605 synthesized a 36.5-kDa protein (Fig. 4, lane 2, a′) which seemed to be a truncated product of the 38-kDa protein. This result strongly suggested that the 38-kDa protein was a determinant essential for cell invasion.

A pACYC184-ligated recombinant of the 2.6-kb *Hin*dIII fragment of pHW647, designated pHW656, did not synthesize any polypeptides other than those of pACYC184 (Fig. 4, lane 4). This may explain why the 2.6-kb *Hin*dIII fragment could not rescue the mutation of pHW510 and pHW511 (Table 2). A plasmid containing 2.6- plus 4.1-kb *Hin*dIII fragments as one contiguous molecule, pHW626, encoded 41-, 47-, and 80-kDa proteins in addition to the 38-kDa protein (Fig. 4, lane 7). This indicated that both 2.6- and 4.1-kb *Hin*dIII fragments were required for the expression of 41-, 47-, and 80-kDa proteins. The 39-kDa protein synthesized in pHW655 was not found in pHW626. The 39-kDa

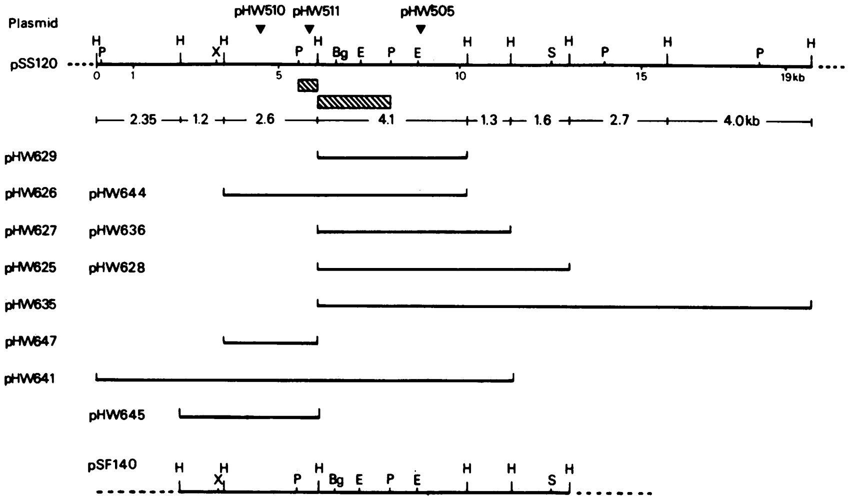

FIG. 3. Restriction maps of a part of plasmids pSS120 and pSF140 and of *Hin*dIII-recombinant clones. From various *Hin*dIII-recombinant clones of plasmids pSS120 and pSF140, restriction maps of a part of plasmids pSS120 and pSF140 were constructed. Dotted lines of pSS120 and pSF140 represent the parts of pSS120 and pSF140 not shown. *Hin*dIII fragments of pSS120 and pSF140 were ligated into the *Hin*dIII site of the vector plasmid pHSG415^r. Only *Hin*dIII inserts of the vector plasmid are shown in this figure. Inserts of pHW625, pHW626, pHW627, pHW629, pHW635, pHW641, and pHW647 were derived from pSS120. Inserts of pHW628, pHW636, pHW644, and pHW645 were derived from pSF140. ▼ (above the map of pSS120), Insertion sites of Tn*1* transposed onto the plasmid pSS120. Resultant plasmids were designated pHW510, pHW511, and pHW505 (see the text). ▨, DNA sequences used for DNA probes corresponding to those of pHW605 and pHW613, as shown in Fig. 1. Restriction endonuclease sites are abbreviated as follows: Bg, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; S, *Sal*I; X, *Xho*I.

protein would probably be a truncated product of the 41-, 47-, or 80-kDa protein. One or more of the 41-, 47-, and 80-kDa proteins seemed to be necessary for complementation of the mutation(s) of plasmids pHW510 and pHW511.

A 1.6-kb *Eco*RI fragment (Fig. 3) within the 4.1-kb *Hin*dIII fragment of pHW626 was deleted. The plasmid, designated pHW651, could not rescue the mutation of pHW505 (data not shown) and did not synthesize any of the four polypeptides of 38, 41, 47, and 80 kDa (Fig. 4, lane 8). The 1.6-kb *Eco*RI region was important for the expression of the genes essential for cell invasion.

Conservation of the 4.1- and 2.6-kb DNA sequences among plasmids of *Shigella* species and enteroinvasive *E. coli*. We isolated the DNA sequence of plasmid pSF140, the 140-MDa plasmid of *S. flexneri* 2a, corresponding to the 4.1- and 2.6-kb *Hin*dIII fragments of pSS120, from 300 recombinants of partially *Hin*dIII-digested pSF140 with the vector plasmid pHSG415^r. Plasmids pHW628, pHW636, and pHW644, which hybridized with a probe of pHW605, had a common core of 4.1-kb *Hin*dIII fragment (Fig. 2 [lanes 4 and 5] and 3). Plasmids pHW644 and pHW645, which hybridized with a probe of pHW613, had a common core of 2.6-kb *Hin*dIII fragment (Fig. 3). A restriction cleavage map of a part of the plasmid pSF140 was constructed with those inserts from recombinants. Comparison of the cleavage maps of plasmids pSS120 and pSF140 showed that they shared at least *Hin*dIII fragments of 1.2, 2.6, 4.1, 1.3, and 1.6 kb and that the order of these fragments in pSS120 was the same as for pSF140 (Fig. 3). Further, pSF140 and pSS120 shared a function for cell invasion. Plasmid pHW628 restored cell invasiveness to a noninvasive strain with pHW505 (Table 2). Plasmid pSF140 had the same 4.1- and 2.6-kb *Hin*dIII fragments as those of pSS120.

TABLE 2. Invasiveness of *E. coli* HB101 with or without pHW505, pHW510, or pHW511 after acquisition of recombinants

E. coli HB101 harboring plasmids:		% LLC-MK_2 cells invaded
Resident	Incoming	
		<0.01
pHW505		<0.01
pHW510		<0.01
pHW511		<0.01
	pHW626	<0.01
	pHW629	<0.01
	pHW647	<0.01
	pHW641	<0.01
	pHW635	<0.01
	pHW628	<0.01
pHW505	pHW629	17.5
pHW505	pHW626	18.5
pHW505	pHW628	12.5
pHW510	pHW626	10.4
pHW511	pHW626	8.5
pHW510	pHW647	<0.01
pHW511	pHW647	<0.01
pHW511	pHW629	<0.01
pHW501[a]		85.0

[a] Plasmid pHW501 is a Tn*1* derivative of pSS120; it maintained invasiveness (30).

We further examined conservation of the 4.1- and 2.6-kb DNA sequences among plasmids of other *Shigella* species and enteroinvasive *E. coli*. Plasmid DNAs prepared by the method of Kado and Liu (11) were hybridized with the ^{32}P-labeled 4.1- or 2.6-kb *Hin*dIII fragment. The 120- to 140-MDa invasive plasmids of *Shigella* species and enteroinvasive *E. coli* had homology with the probe DNA under stringent conditions, whereas avirulent *Shigella* and *E. coli* strains losing a large plasmid did not show any homology with the probe (Fig. 5). In the case of the 2.6-kb DNA probe, we found the same result (data not shown).

DISCUSSION

Large plasmids of *Shigella* species encode genes necessary for cell invasion (23, 24, 30). Since they are large in size

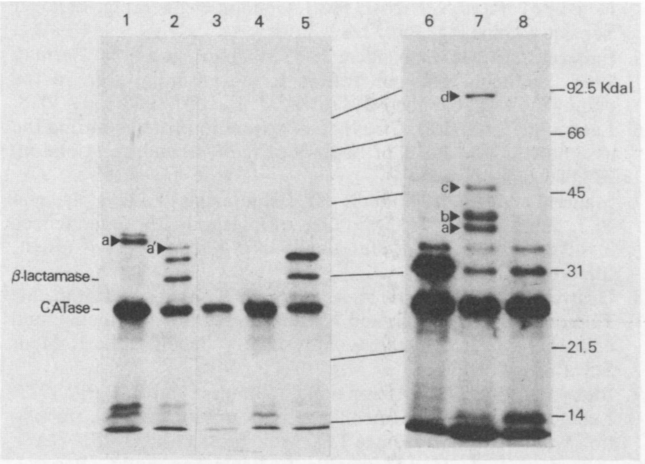

FIG. 4. Polypeptides expressed in minicells by recombinant clones. Polypeptides were labeled with $[^{35}S]$methionine in minicell preparations of strain P678-54 containing a plasmid, separated on 13% polyacrylamide-sodium dodecyl sulfate gels, and visualized by fluorography. Lanes: 1, pHW655; 2, pHW605; 3, pACYC184 (vector); 4, pHW656; 5, pHW613; 6, $pHSG415^r$ (vector); 7, pHW626; 8, pHW651. Molecular weight standards were phosphorylase *b* (92,500), bovine serum albumin (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), and lysozyme (14,400), all from Bio-Rad Laboratories (Richmond, Calif.). Stained molecular weight standards on dried gels were marked with radioactive ink, and the gels were exposed to Fuji X-ray film (RXO-H) at −80°C for 1 to 4 days. Letters a, b, c, and d indicate 38-, 41-, 47-, and 80-kDa polypeptides, respectively, as referred to in the text. The positions of β-lactamase (5) and chloramphenicol acetyltransferase (CATase) (26) were estimated from a comparison with the molecular weight standards. The approximately 34-kDa protein (lanes 2 and 5) shown above β-lactamase might be derived from Tn*1* or might be a result of Tn*1* insertion. The polypeptide indicated by a' seemed to be a truncated product of a.

and non-self-conjugative and have no distinct markers to allow positive selection, it has been difficult to analyze genetically the virulence-associated plasmids. The Tn*1*-mediated conduction system that we developed proved to be useful and convenient for constructing a series of Tn*1* insertions in the nonconjugative plasmid, as well as for transferring the plasmid into another strain.

Using the Tn*1*-mediated conduction system and Tn*1* insertion mutagenesis, we found that Tn*1* inserted into four different *Hin*dIII fragments of pSS120 in mutants losing cell invasiveness. One such *Hin*dIII fragment, the 4.1-kb *Hin*dIII fragment of pSS120, was able to restore invasiveness to strain HB101(pHW505), a Tn*1* insertion mutant which had lost cell invasiveness. This shows that the 4.1-kb DNA fragment encodes a *trans*-acting substance. A comparison of proteins expressed by parental and Tn*1*-inserted mutant plasmids strongly suggested that a 38-kDa protein encoded by the 4.1-kb DNA was the *trans*-acting substance and a determinant necessary for cell invasion. The *Hin*dIII fragment of the same size of pSF140 was also able to restore invasiveness to strain HB101(pHW505). The 4.1-kb *Hin*dIII fragment was conserved among plasmids of not only *S. sonnei* and *S. flexneri* but also other *Shigella* species and enteroinvasive *E. coli*. These results support the possibility that the 4.1-kb DNA sequence was important for cell invasion.

One contiguous molecule of 2.6- and 4.1-kb *Hin*dIII fragments complemented the mutations of pHW505, pHW510, and pHW511 and synthesized four polypeptides of 38, 41, 47, and 80 kDa in minicells. Both 4.1- and 2.6-kb *Hin*dIII fragments were necessary for the expression of 41-, 47-, and 80-kDa polypeptides. Deletion of the 1.6-kb *Eco*RI fragment within the 4.1-kb *Hin*dIII fragment caused the loss of all four polypeptides. These results probably suggest that polypeptides of 41, 47, and 80 kDa in addition to that of 38 kDa also were involved in the cell invasion of bacteria and that the 1.6-kb *Eco*RI fragment contains DNA sequences important for the expression of all four polypeptides, i.e., a promotor region, an early part of an operon encoding genes for four polypeptides, or a regulatory gene. To test them, we are making a series of Tn*5* insertions in 2.6- and 4.1-kb *Hin*dIII fragments and analyzing the products of insertions.

Maurelli et al. (17) have recently cloned ca. 37-kb DNA sequences of *S. flexneri* 5 which are sufficient to enable an avirulent, plasmidless mutant to invade HeLa cells. An *S. flexneri* 5 strain with the cloned 37-kb DNA (17) expressed the same 38-, 43-, 62-, and 78-kDa polypeptides as those

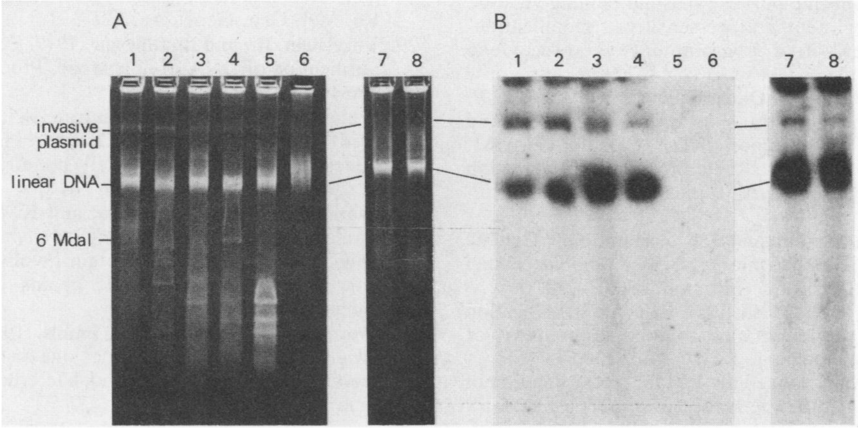

FIG. 5. Plasmids of *Shigella* species and enteroinvasive *E. coli* and hybridization with a ^{32}P-labeled 4.1-kb DNA fragment. Lanes: 1, *S. boydii* pp343; 2, *S. sonnei* HW383; 3, *S. flexneri* HW283; 4, *S. dysenteriae* HW257; 5, *S. flexneri* HW1002; 6, *E. coli* HB101; 7, enteroinvasive *E. coli* HW906; 8, enteroinvasive *E. coli* HW907. (A) Plasmid profiles prepared by the method of Kado and Liu. (B) Southern hybridization of the plasmids with ^{32}P-labeled probe under stringent conditions (27). A 6-MDa plasmid of *S. dysenteriae* HW257 was described previously (32).

described by Hale et al. (7), which were recognized by serum from a monkey immunized against *S. flexneri*. It is unknown whether these four polypeptides are involved in cell invasion. The 38-, 41-, and 80-kDa polypeptides encoded by the 6.7-kb DNA of pHW626 are of the same or similar electrophoretic mobility as the polypeptides described by Hale et al. (7) and Maurelli et al. (17). The polypeptides we detected might be the same as those. Studies are under way to determine the antigenic relationship between them.

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