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

HARUO WATANABE* AND AKIKO NAKAMURA

Department of Bacteriology, National Institute of Health, Kamiosaki, Shinagawa-ku, Tokyo 141, Japan

Received 31 December 1985/Accepted 29 April 1986

A series of TnI insertions in pSS120, the 120-megadalan form I plasmid of *Shigella sonnei*, were constructed by a TnI-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 TnI insertion sites of seven noninvasive mutants suggested that four separate HindIII fragments were necessary for cell invasion. HindIII fragments including TnI of mutant plasmids were cloned into a vector plasmid, pACYC184. The DNA was used as a DNA probe to identify the corresponding, parental HindIII fragments. We identified one contiguous molecule of 2.6- and 4.1-kilobase pair (kb) HindIII 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 HindIII 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 *E. coli* plasmids.

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 non-pathogenic 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-kr 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 TnI-mediated conduction (30). This method allowed us to construct TnI 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 plasmids of *Shigella* species 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-MK2 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).

**TnI insertion mutagenesis.** The strategy of TnI insertion mutagenesis was described previously as the TnI-mediated conduction system (30). This method is based on the facts that TnI-related ampicillin transposons transpose onto the plasmid DNA more readily than onto the cellular chromosome (13) and that a cointegrate between TnI donor and recipient is formed as an intermediate of TnI-mediated transposition (25). Plasmid pTH10 (8), used as the TnI donor, is a derivative of RP4 that is temperature sensitive for maintenance and confers upon its host resistance to kanamycin, tetracycline, and ampicillin (Km, Tc, and Ap', 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, *Corresponding author.*
pTH10 was cultured in the medium containing kanamycin and ampicillin at nonpermissive temperature (42°C) for maintenance of plasmid pTH10 to select co-integrates 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 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 (provided by T. Hashimoto) was digested with HindIII 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 [32P]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 [35S]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 15% (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).

### TABLE 1. Bacterial strains and plasmids used

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristics</th>
<th>Invasiveness* in LLC-MK2 cells</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>S. sonnei</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HW383</td>
<td>nad, pSS120, cryptic plasmids</td>
<td>+ 30</td>
<td></td>
</tr>
<tr>
<td>HW436</td>
<td>HW383(pTH10)</td>
<td>+ 30</td>
<td></td>
</tr>
<tr>
<td>S. dysenteriae</td>
<td>Serotype 1, a large plasmid, cryptic plasmids</td>
<td>+ 30</td>
<td></td>
</tr>
<tr>
<td>HW257</td>
<td>Serotype 1, a large plasmid</td>
<td>+ N. Okamura</td>
<td></td>
</tr>
<tr>
<td>S. boydii</td>
<td>Serotype 2a, nad, pSF140, cryptic plasmids</td>
<td>+ 30</td>
<td></td>
</tr>
<tr>
<td>S. flexneri</td>
<td>Serotype 1b, no large plasmid, cryptic plasmids</td>
<td>- 30</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>E. coli</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HW906</td>
<td>O124, a large plasmid, cryptic plasmids</td>
<td>+ M. Ohashi</td>
<td></td>
</tr>
<tr>
<td>HW907</td>
<td>O136, a large plasmid</td>
<td>+ M. Ohashi</td>
<td></td>
</tr>
<tr>
<td>HB101</td>
<td>ara-14 leu proA2 lacY1 glnV44 galK2 recA13 rpsL20 xyl-5 mtl thi hsdS20</td>
<td>- 2</td>
<td></td>
</tr>
<tr>
<td>P678-54</td>
<td>thr leu lacY minA minB gal str thi</td>
<td>- 1</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pSS120</td>
<td>Virulence plasmid from <strong>S. sonnei</strong> HW383, nonconjugative, I antigen</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>pSF140</td>
<td>Virulence plasmid from <strong>S. flexneri</strong> 2a HW283, nonconjugative</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>PTH10</td>
<td>Temperature-sensitive derivative of RP4, conjugative, Ap' (TnI) Km' Te'</td>
<td>8</td>
<td>T. Iino</td>
</tr>
<tr>
<td>pACYC184</td>
<td>Cloning vector, Cm' Te' 3</td>
<td>T. Hashimoto</td>
<td></td>
</tr>
<tr>
<td>pHSG415</td>
<td>Cloning vector, Ap' Km' Cm', temperature-resistant derivative of pHSG415</td>
<td>3</td>
<td>T. Hashimoto</td>
</tr>
<tr>
<td>pSC101::Tnl</td>
<td>Tnl probe, Te' Ap' (Tnl)</td>
<td>pTH10-derived Tnl insert of pSC101</td>
<td></td>
</tr>
</tbody>
</table>

* Abbreviations and nomenclature

+ Invasive; –, noninvasive.
RESULTS

Insertion mutagenesis of pSS120 and identification of Tn1 insertion sites of noninvasive mutants. To identify regions of plasmid pSS120 which were required for cell invasion, we constructed independent Tn1 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-MK2 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::Tn1. These results strongly suggested that the ampicillin resistance transposon Tn1 of plasmid pTH10 inserted onto the 120-MDa form I plasmid, pSS120. Resulting pSS120::Tn1 plasmids of seven noninvasive strains are referred to as pHW502, pH503, pH504, pHW505, pH506, pH510, and pH511.

Tn1 insertion sites of these plasmids were determined by hybridization with a Tn1 probe, pSC101::Tn1, after cleavage of plasmid DNAs with HindIII restriction enzyme, which does not cut Tn1 (10). The Tn1 probe hybridized with HindIII fragments of four different sizes: the 15.0-kb HindIII fragments of pHW502, pH503, and pH504; the 9.1-kb fragment of pHW505; the 8.8-kb fragment of pHW506; and the 7.6-kb fragments of pH510 and pH511. These HindIII fragments were cloned into the HindIII 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 Tn1 demonstrated that four HindIII fragments of pSS120 were different from each other. Four distinct HindIII fragments of pSS120 seemed to be necessary for cell invasion.

Isolation of DNA sequence of pSS120 corresponding to the pH605 and pH613 DNAs. For a mutation of the invasiveness gene to occur by Tn1 insertion, it is necessary to clone DNA fragments which rescue the mutation. We further analyzed pH605 and pH613, which were pACYC184-ligated recombinants of the 9.1-kb HindIII fragment of pHW505 and the 7.6-kb HindIII fragment of pH510, respectively (Fig. 1). We tried to identify parental HindIII fragments corresponding to the Tn1-containing HindIII fragments of pH605 and pH613. A part of the DNA sequence of pH605 or pH613 shown in Fig. 1 was used as a DNA probe to isolate the corresponding sequences. Plasmid pSS120 was partially digested with HindIII restriction enzyme and cloned into the HindIII site of a vector plasmid, pHSG415. A total of 300 recombinants were screened for homology with the DNA probe by colony hybridization (6). We isolated six recombinants, pHW625, pH626, pH627, pH629, pH635, and pH641, which showed homology with the DNA probe of pH605. HindIII-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 HindIII fragment, which hybridized with the DNA probe from pH605 (Fig. 2 and 3). With the DNA probe of pH613, three recombinants, pH626, pH641, and pH647, were isolated. The cloned inserts all contained a common core of 2.6-kb HindIII fragment (Fig. 3), which hybridized with the DNA probe from pH613 (data not shown). Plasmids pHW626 and pH641 shared the 2.6- and 4.1-kb HindIII fragments (Fig. 3). We constructed a restriction cleavage map of a part of the plasmid pSS120 with those inserts of recombinants (Fig. 3).
The parental 4.1- and 2.6-kb HindIII fragments corresponding to the TnI-containing HindIII 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 (Cm²), and chloramphenicol-resistant transformants were examined for cell invasion. The invasiveness phenotype of transformants is summarized in Table 2. None of the parental HindIII fragments were capable of restoring invasiveness to E. coli without mutant plasmids. All recombinants containing the 4.1-kb HindIII fragment (e.g., pHW626 and pHW629) restored invasiveness to E. coli HB101(pHW505). A recombinant containing only the 2.6-kb HindIII fragment (e.g., pHW647) could not restore the mutation(s) of pHW510 and pHW511. To restore the mutation(s) of pHW510 and pHW511, one contiguous molecule of 2.6- and 4.1-kb HindIII fragments (e.g., pHW626) was required. These results indicated that the 6.7-kb DNA sequence containing 2.6- and 4.1-kb HindIII 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 TnI 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 TnI-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 HindIII fragment (e.g., pHW626 and pHW629) were unstable when purified without chloramphenicol; approximately 30% of the cells lost chloramphenicol resistance. Plasmids pHS415 and pH647, the latter a recombinant clone containing the 2.6-kb HindIII 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 HindIII 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 HindIII 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 HindIII fragment (e.g., pHW647) or the vector plasmid pHS415 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 TnI-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 HindIII fragment was able to complement the mutation of plasmid pHW505 (Table 2). We then cloned the 4.1-kb HindIII fragment of pHW505 into the HindIII 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 TnI 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 HindIII 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 HindIII fragment could not restore the mutation of pHW510 and pHW511 (Table 2). A plasmid containing 2.6- plus 4.1-kb HindIII 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 HindIII 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. 2.** HindIII digestions of recombinant plasmids and hybridization with a 32P-labeled DNA probe of pHW605. Lanes: 1, HindIII-digested phage λ DNA; 2, HindIII- and PstI-digested pHW605; 3 to 10, HindIII-digested pHS415, pHW628, pHW629, 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 32P-labeled DNA probe of pHW605.
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 EcoRI fragment (Fig. 3) within the 4.1-kb HindIII 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 peptides of 38, 41, 47, and 80 kDa (Fig. 4, lane 8). The 1.6-kb EcoRI 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 HindIII fragments of pSS120, from 300 recombinants of partially HindIII-digested pSF140 with the vector plasmid pHSG415². Plasmids pHW628, pHW636, and pHW644, which hybridized with a probe of pHW605, had a common core of 4.1-kb HindIII 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 HindIII 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 HindIII 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 HindIII fragments as those of pSS120.

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 32P-labeled 4.1- or 2.6-kb HindIII 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
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 Tnl-mediated conduction system that we developed proved to be useful and convenient for constructing a series of Tnl insertions in the nonconjugative plasmid, as well as for transferring the plasmid into another strain.

Using the Tnl-mediated conduction system and Tnl insertion mutagenesis, we found that Tnl inserted into four different HindIII fragments of pSS120 in mutants losing cell invasiveness. One such HindIII fragment, the 4.1-kb HindIII fragment of pSS120, was able to restore invasiveness to strain HB101(pHW505), a Tnl 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 Tnl-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 HindIII fragment of the same size of pSF140 was also able to restore invasiveness to strain HB101(pHW505). The 4.1-kb HindIII 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 HindIII 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 HindIII fragments were necessary for the expression of 41-, 47-, and 80-kDa polypeptides. Deletion of the 1.6-kb EcoRI fragment within the 4.1-kb HindIII 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 EcoRI fragment contains DNA sequences important for the expression of all four polypeptides, i.e., a promoter 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 Tnl insertions in 2.6- and 4.1-kb HindIII fragments and analyzing the products of insertions.

Maurerli 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. 4.** Polypeptides expressed in minicells by recombinant clones. Polypeptides were labeled with [35S]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 (vector); 7, pHW626; 8, pHW651. Molecular weight standards were phosphorylase b (92,500), bovine serum albumin (66,200), ovalbumin (45,000), carboxic 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 Tnl or might be a result of Tnl insertion. The polypeptide indicated by a′ seemed to be a truncated product of a.

**FIG. 5.** Plasmids of Shigella species and enteroinvasive E. coli and hybridization with a 32P-labeled 4.1-kb DNA fragment. Lanes: 1, S. boydii pp343; 2, S. sonnei HW583; 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 32P-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 pHSW26 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.

ACKNOWLEDGMENTS

We thank A. Wake for his interest in our work and T. Hashimoto, T. Iino, N. Okamura, and M. Ohashi for providing us with their plasmids and strains. We also thank T. L. Hale for helpful suggestions and Y. Sakakibara for critical reading of this manuscript.

This work was supported by a grant from the Ministry of Education of Japan.

LITERATURE CITED