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Large Plasmids Associated with Virulence in *Shigella* Species Have a Common Function Necessary for Epithelial Cell Penetration

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Large plasmids (120 to 140 megadaltons) associated with virulence of *Shigella sonnei*, *S. flexneri* 2a and *S. dysenteriae* 1 were transferred from each strain into *Escherichia coli* K-12 and avirulent *S. flexneri* 1b strains by ampicillin transposon (Tn*1*)-mediated conduction. Strains with the virulence plasmid could penetrate tissue culture cells irrespective of the original host of the plasmid.

Virulence of dysentery-producing bacterial pathogens is associated with the presence of a 120-megadalton (Mdal) form I plasmid in *Shigella sonnei* (12), a 140-Mdal plasmid in *Shigella flexneri* (13), a 140-Mdal plasmid in enteroinvasive *Escherichia coli* (11), a 140-Mdal plasmid in *Shigella dysenteriae* (10), and a 6-Mdal plasmid in *Shigella dysenteriae* 1 (16, 17). Non-self-conjugative 120- to 140-Mdal plasmids of *Shigella* species and enteroinvasive *E. coli* encode determinants necessary for invasion of epithelial cells in vitro (11–13). Hale et al. (2) have shown that 120-Mdal plasmid of *S. sonnei* shares many homologous sequences with the 140-Mdal plasmid and that the polypeptides expressed by all of the plasmids are similar among *S. sonnei*, *S. flexneri*, and invasive *E. coli*. It would be expected that the *S. sonnei* 120-Mdal plasmid would carry the same invasion determinants as *S. flexneri*. However, Sansonetti et al. (11) demonstrated that invasiveness of enteroinvasive *E. coli* that has lost this plasmid can be restored by an *S. flexneri* but not an *S. sonnei* virulence plasmid. The result impressed on us some differences in extrachromosomal function between *S. flexneri* and *S. sonnei*. In this paper, however, we report that a 120-Mdal plasmid of *S. sonnei* also has the same function necessary for cell penetration as those of *S. flexneri* and *S. dysenteriae*.

To compare the function of plasmid on invasiveness among *Shigella* species, it is necessary to transfer a nonconjugative 120- to 140-Mdal plasmid from one strain to another and examine the restoration of invasiveness in the strain which receives the plasmid. We utilized the system of transposon-mediated conduction (1) to efficiently transfer a nonconjugative plasmid into a recipient by a conjugative plasmid. Plasmid pTH10 (3) (provided by T. Iino), which was used as the conjugative plasmid, is a derivative of RP4 that is temperature sensitive for maintenance, belongs to the P incompatibility group, and confers on its host resistance to kanamycin, tetracycline, and ampicillin (Tn*1*). Tn*3*-related ampicillin transposons Tn*3*, Tn*1*, and Tn*801* transpose onto the plasmid DNA more readily than onto the chromosome DNA (7, 8). When a strain bearing both pTH10 and a nonconjugative plasmid with no drug resistance markers is cultured in the medium containing kanamycin and ampicillin at the nonpermissive temperature (42°C) for maintenance of plasmid pTH10, a cointegrate between pTH10 and the nonconjugative plasmid which is formed as an intermediate of Tn*1*-mediated transposition (15) will be selected.

Based on this assumption, we carried out the following experiments. *S. sonnei* HW383 as a nonconjugative 120-Mdal form I plasmid (Fig. 1, lane b), designated pSS120 that is essential for cell penetration and form I antigen formation (6, 12). *S. sonnei* HW383 bearing plasmid pTH10 (Fig. 1, lane c) was grown at 42°C on Penassay agar (Penassay broth plus 1.5% agar) containing kanamycin (25 μg/ml) and ampicillin (100 μg/ml). The resultant colonies were suspended in 1 ml of drug-free Penassay broth at a concentration of 2×10^8 cells per ml. The suspension was mixed with 5 ml of *E. coli* K-12 HW80 culture, a nalidixic acid-resistant derivative of WA921 [*thr leu met thi lac hsdS*(r_K^- m_K^-)] (18) (provided by K. Mise) at 39°C for 4 h. The mixture was spread on the Penassay agar plates containing nalidixic acid (50 μg/ml), kanamycin (25 μg/ml), and ampicillin (100 μg/ml), and the plates were incubated at 42°C for 48 h. The resultant transconjugants were obtained at a frequency of 1×10^{-5} to 3×10^{-5} per donor cell. Of 10 transconjugants, 9 agglutinated in the antiserum (Denka-seiken Co., Tokyo, Japan) specific for *S. sonnei* form I antigen and carried the two plasmids similar in size to plasmids pTH10 and pSS120, respectively (Fig. 1, lane e). Plasmid pTH10 was cured from the transconjugant by culture in drug-free medium at 42°C for about 20 generations. The pTH10-cured strain (Fig. 1, lane f) still retained ampicillin resistance and form I antigen. As a 120-Mdal form I plasmid of *S. sonnei* is physically unstable (6), we could easily obtain form I-negative mutants from the pTH10-cured, ampicillin-resistant, form I-positive strain. The mutants always lost ampicillin resistance and the plasmid similar in size to plasmid pSS120, designated pSS120::Tn*1*. These results strongly suggested that ampicillin transposon Tn*1* of plasmid pTH10 inserted onto the 120-Mdal form I plasmid pSS120. Plasmid pSS120 seems to have been transferred by the cointegrate between plasmid pSS120 and plasmid pTH10, which was formed as an intermediate of Tn*1*-mediated transposition. Plasmid pSS120::Tn*1* was transferred from strain HW80(pTH10 + pSS120::Tn*1*) into *E. coli* K-12 X1037 (*met hsdR* Rif^r; rifampicin resistance) provided by M. Inoue) at the frequency of 5×10^{-3} to 8×10^{-3} per donor cell, and further from strain X1037(pTH10 + pSS120::Tn*1*) into avirulent *S. flexneri* 1b HW1002 (Fig. 1, lanes g and h), which was a nalidixic acid-resistant derivative of avirulent *S. flexneri* 1b HW1001, losing a 140-Mdal plasmid essential for cell penetration (10) at a frequency of

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0.5×10^{-6} to 2×10^{-6} per donor cell in a way similar to that described above. A 140-Mdal plasmid, designated pSF140, of *S. flexneri* 2a HW283 and a 140-Mdal plasmid, designated pSD140, of *S. dysenteriae* 1 HW257 were also transferred from each strain into *E. coli* K-12 HW80 and *S. flexneri* 1b HW1002, based on the same hypothesis as described above (data not shown).

The virulence of each strain was examined by the ability of strains to invade tissue culture cells and produce keratoconjunctivitis (Serény test) in a guinea pig (14) as described previously (17). We used rhesus monkey kidney epithelial cells (LLC-MK_2) (4) as tissue culture cells instead of HeLa cells because invasive bacteria could penetrate LLC-MK_2 cells more efficiently than HeLa cells (unpublished data). Table 1 shows that the strains of *S. flexneri* 1b HW1002 which had large plasmids associated with virulence could penetrate LLC-MK_2 cells and produce keratoconjunctivitis in guinea pigs irrespective of the original hosts of the plasmids. Plasmids pSS120, pSF140, and pSD140 were sufficient to allow *E. coli* K-12 to invade culture cells in vitro but not to provoke keratoconjunctivitis in guinea pigs. Chromosomal loci from *Shigella* species are required for full expression of virulence of *E. coli* K-12 in animal models as demonstrated by Sansonetti et al. (9).

Our results clearly demonstrated that plasmid pSS120 of *S. sonnei* encodes determinants necessary for invasion of culture cells and that they function in *E. coli*, *S. sonnei*, and *S. flexneri*. Sansonetti et al. (11) reported that a 120-Mdal form I plasmid of *S. sonnei* could not restore virulence in avirulent *E. coli* strains. But they did not show the retension of virulence determinants of the 120-Mdal form I plasmid by retransferring the plasmid from *E. coli* to an avirulent *S. sonnei* strain. It is possible that some deletions had occurred in the virulence determinants of the form I plasmid. Indeed, we found some deletions of plasmid pSS120 in *E. coli* K-12 HW80 after transferring it from *S. sonnei* HW383 (unpublished data). The system of transposon-mediated conduction described in this paper should facilitate the production of Tn*1*-tagged mutants specific for nonconjugative virulence plasmids of *Shigella* species and the analyses of the mutations losing virulence in *E. coli* K-12.

TABLE 1. Virulence properties of various strains bearing large plasmids necessary for cell penetration

Host strain	Plasmid	O-antigen phenotype	Virulence in assays[a]	
			LLC-MK_2 (% of infected cells)	Serény
S. sonnei HW383	pSS120	Form I	95.0	+
S. flexneri HW283	pSF140	2a	95.0	+
S. dysenteriae HW257	pSD140	1	95.0	+
E. coli K-12 HW80	None	R[b]	0.5	−
	pTH10	R	0.5	−
	pTH10 + pSS120::Tn*1*	Form I	90.0	−
	pSS120::Tn*1*	Form I	91.0	−
	pSF140::Tn*1*	R	95.0	−
	pSD140::Tn*1*	R	94.5	−
S. flexneri HW1002	No large plasmids	1b	0.5	−
	pTH10	1b	0.5	−
	pTH10 + pSS120::Tn*1*	1b + form I	95.0	+
	pTH10 + pSF140::Tn*1*	1b	96.0	+
	pTH10 + pSD140::Tn*1*	1b	96.0	+

[a] Assays for virulence were done with cultures grown at 37°C as described previously (17). The percent is that of infected LLC-MK_2 cells containing five or more bacteria in 200 cells selected at random. The Serény test was done as described previously (17). Strains which produced keratoconjunctivitis within 3 days were considered virulent (+).

[b] R, Rough.

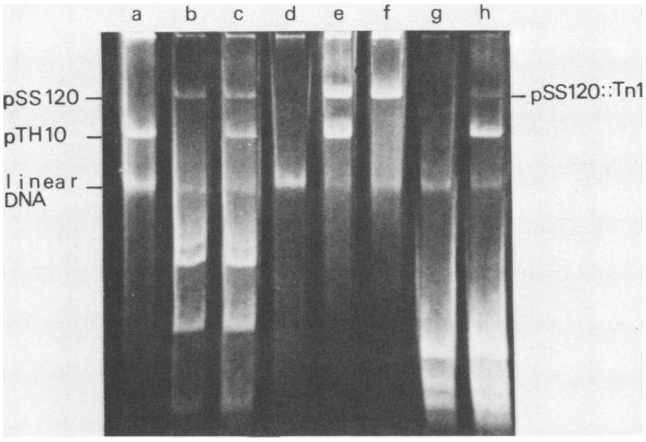

FIG. 1. Analysis of plasmid DNA from *S. sonnei*, *E. coli* K-12, and *S. flexneri* 1b with plasmid pSS120 and its Tn*1*-tagged derivative, pSS120::Tn*1*. Bacterial cells were cultured at 30°C and plasmid DNAs were prepared by the method of Kado and Liu (5). Plasmid DNA was fractionated by electrophoresis through a 0.8% vertical agarose gel in Tris-borate buffer (2.5 mm disodium EDTA, 89 mM boric acid, 89 mM Tris base) at 100 V for 4 h. Shown is an ethidium bromide-stained agarose gel containing plasmid DNA from the following strains (lanes): a, *E. coli* K-12 HW80(pTH10); b, *S. sonnei* HW383; c, *S. sonnei* HW383(pTH10); d, *E. coli* K-12 HW80; e, *E. coli* K-12 HW80(pSS120::Tn*1* + pTH10); f, *E. coli* K-12 HW80(pSS120::Tn*1*); g, *S. flexneri* 1b HW1002; h, *S. flexneri* 1b HW1002(pSS120::Tn*1* + pTH10)

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