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Possible Relationship of a 36-Megadalton *Salmonella enteritidis* Plasmid to Virulence in Mice

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All of the *Salmonella enteritidis* strains isolated from diseased animals (61 strains) and from beef (2 strains) in Japan and in West Germany (1 strain), except for 2 strains isolated from ducks, harbored either a 36-megadalton (Md) plasmid alone or in combination with several other plasmids of different sizes. It is likely that these 36-Md plasmids from various *S. enteritidis* strains were derived from the same origin because their plasmid DNAs showed the same cleavage patterns obtained with *Eco*RI, *Hin*dIII, and *Bam*HI. We also suggested that this plasmid is native to *S. enteritidis*. Tests carried out on two strains isolated from ducks which naturally lacked this plasmid and one strain whose plasmid was artificially cured showed that the strains without the 36-Md plasmid showed less virulence compared to a wild-type strain harboring the 36-Md plasmid, suggesting that this 36-Md plasmid might be associated with virulence for mice.

In our epidemiological investigation of plasmids in *Salmonella* strains isolated from diseased animals, we found that a 36-megadalton (Md) plasmid was present either alone or in combination with other plasmids in most *Salmonella enteritidis* strains. Recently, virulence-associated plasmids in *Salmonella* spp. (4, 8), *Shigella* spp. (2, 7), and *Yersinia* spp. (1, 9) have been reported by many workers. In *Salmonella* spp., a 60-Md plasmid of *S. typhimurium* (4) and 50-Md plasmid of *S. dublin* (8) were reported to be associated with the virulence for mice. Therefore, we investigated the possible association of this 36-Md plasmid with virulence for mice.

In this experiment, as shown in Table 1, 63 strains isolated from diseased animals (61 strains) and from beef (2 strains) in Japan between 1972 and 1982 and 1 strain isolated from West Germany (supplied by R. Sakazaki, National Institute of Health) were checked. Many of these strains were samples obtained during outbreaks, and some strains were isolated consistently at the same farm over a long period of time. Some strains were isolated from various organs of the same animals.

Plasmid was detected by a modified rapid method described by Kado and Liu (5). Bacterial cells were grown overnight in 10 ml of L broth at 37°C, harvested by centrifugation, and suspended in 1 ml of E buffer (40 mM Tris acetate, 2 mM EDTA [pH 7.9]). The cells were then lysed by addition of 2 ml of freshly prepared lysing solution (3 g of sodium dodecyl sulfate, 0.6 g of Tris, 6.4 ml of 2 N NaOH in 100 ml of distilled water), incubated for 1 h at 55°C, and followed by extraction with 6 ml of phenol-chloroform (1:1 [vol/vol]). After centrifugation, the supernatant was subjected to agarose gel electrophoresis for detection and sizing of plasmid DNA. The molecular weight standards were R27 (112 Md), R40a (96 Md), R16 (69 Md), RP4 (36 Md), Rs-a (23 Md), and small size plasmid of *Escherichia coli* V517 (6). For restriction analysis, the supernatant was further extracted with phenol-chloroform and centrifuged. The aqueous phase was dialyzed against 25% polyethylene glycol 6000 for 3 h and then against 0.1× SSC (1× SSC is 0.015 M sodium citrate plus 0.15 M NaCl) for 18 h at 4°C. The restriction endonucleases *Hin*dIII, *Bam*HI, and *Eco*RI were used under condition recommended by the supplier (Takara-Shuzo, Kyoto, Japan).

For tagging of *S. enteritidis* plasmid by transposition, the donor *E. coli* TH471 carries a temperature-sensitive mutant of RP4, pTH10, into which ampicillin (Ap) resistance transposon Tn*1* had been inserted (3). This pTH10 plasmid, which contains genes resistant to Ap, kanamycin, and tetracycline was transferred into an *S. enteritidis* strain by conjugation in HI broth for 3 h at 37°C. Selection was performed in glucose minimal medium containing Ap (50 μg/ml). The transconjugant showing resistance to Ap, kanamycin, and tetracycline was incubated in HI broth at 42°C overnight, and derivatives that were resistant to Ap but sensitive to kanamycin and tetracycline were selected. The size of plasmids contained in these bacteria was determined to make sure that the Tn*1* transposon was inserted into the plasmid and not into the chromosome. For curing of the plasmid, Ap-resistant derivatives harboring the plasmid with Tn*1* were subcultured daily in HI broth containing 1 mg of ethidium bromide per ml at 42°C and plated on drug-free HI plates each time a subculture was made. The colonies were screened for the loss of Ap resistance by replica plating.

As listed in Table 1, all strains of *S. enteritidis*, except for two strains isolated from ducks, were found to harbor the 36-Md plasmid either alone or with other plasmids of different sizes. In the test of drug resistance, about one-third of the *S. enteritidis* strains showed resistance to various antibiotics examined; however, all strains that harbored only 36-Md plasmid were sensitive to drugs. Conjugative R plasmids, including thermosensitive ones, were detected in 17 strains; the sizes of these R plasmids transferred to *E. coli* ML1410 (Recipient strain) varied, but none was 36 Md. These results clearly indicate that the 36-Md plasmid thus far detected in *S. enteritidis* was not associated with drug resistance.

In restriction enzyme analysis of 36-Md plasmid DNAs obtained from four strains of *S. enteritidis* isolated from four geographical sources, we found that these plasmid DNAs shared almost all fragments when digested by *Eco*RI,

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TABLE 1. Distribution of plasmid DNA in *S. enteritidis*

Source	Mass of plasmid (Md)	No. of strains
Chicken	36	1
Duck	58	2
Pig	36	1
Cattle	120, 36, 1.4	1
	105, 36,	3
	103, 36	3
	96, 36	2
	96, 36, 1.4	4
	85, 36	5
	36	28
	36, 32	1
	36, 5.6, 3.7	9
	36, 1.5	1
Beef	36, 16.5, 2.0	1
	36	1
West Germany	36	1

*Hin*dIII, and *Bam*HI, suggesting that these 36-Md plasmids were identical (Fig. 1).

For tagging of plasmids with transposon Tn*1*, we used *S. enteritidis* L-119 isolated from the spleen of a dairy cow as a representative strain. This strain was designated as strain AL1190, and the 36-Md plasmid of this strain was pNL2001. The derivative of AL1190 with a Ap-resistant transposon; Tn*1*, inserted in pNL2001 to give pNL2002 was designated as AL1191, and the plasmid-cured derivative was designated as AL1192. This plasmid-cured strain AL1192 was obtained after the fifth passage in HI broth containing ethidium bromide. These three strains, AL1190, AL1191, and AL1192, showed smooth phase in colony formation on HI agar and were not agglutinated by the acriflavin test. Antigenic analysis showed that all of the strains possessed identical O(1,9,12) and H(g,m:−) antigens. By 20 basic biochemical properties and motility, these strains were indistinguishable.

The virulence of two strains (AL1190 and L-34), isolated from cattle, carrying only the 36-Md plasmid was compared to that of two other strains (L-73 and L-74), isolated from ducks, carrying 58-Md plasmid. The 50% lethal dose (LD_{50}) of the two strains carrying 36-Md plasmid was 10^4 bacteria

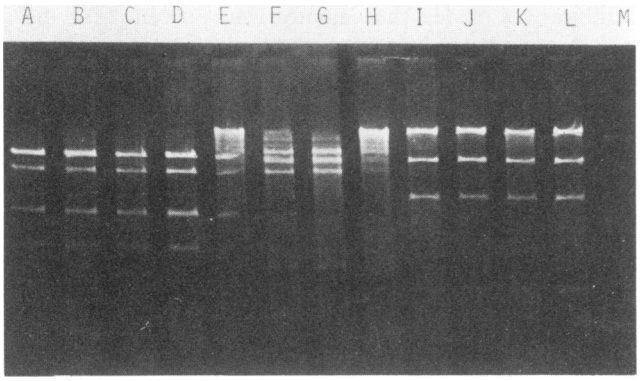

FIG. 1. Restriction analysis of 36-Md plasmid DNAs from *S. enteritidis*. Gel electrophoresis was on 1% agarose. Lanes: A through D, *Eco*RI (L-22, L-119, L-34, L-596, respectively); E through H, *Hin*dIII (L-22, L-119, L-34, L-596, respectively); I through L, *Bam*HI (L-22, L-119, L-34, L-596, respectively); M, *Eco*RI (lambda).

TABLE 2. LD_{50} for mice subcutaneously administered *S. enteritidis*

Strain	Plasmid size (Md)	LD_{50}
L-34	36	$10^{4.34}$
AL1190	36	$10^{4.83}$
L-73	58	$10^{7.96}$
L-74	58	$10^{6.68}$

on subcutaneous administration (Table 2). In contrast, strains L-73 and L-74, which naturally lacked this plasmid, exhibited less virulence. Next, the virulence of AL1190, AL1191, and AL1192 was compared. With oral administration, the LD_{50}s of strains AL1190 and AL1191 were both approximately 10^6 bacteria (Table 3), whereas strain AL1192, which did not carry the 36-Md plasmid, did not cause death. By subcutaneous administration, the LD_{50}s of these two strains harboring the plasmid were approximately $10^{4.5}$ bacteria, but that of strain AL1192 was $>10^{7.9}$. On the contrary, in chick virulence tests, in which 1-day-old chicks were inoculated orally with 10 to 30 *S. enteritidis* organisms and these chicks were reared with six other uninoculated chicks of the same age for 1 week, strains L-73 and L-74 isolated from ducks were virulent; however, two strains harboring the 36-Md plasmid isolated from a cow and beef and strains AL1190 and AL1192 were less virulent for chicks (Table 4).

The data presented in this paper showed that every strain of *S. enteritidis* harbored at least one plasmid and that except for the two strains isolated from ducks, they harbored the 36-Md plasmid either alone or with several other plasmids of different sizes, including some conjugative R plasmids. As for the 36-Md plasmid, it was suggested that these 36-Md plasmids from various *S. enteritidis* strains were derived from the same origin because their plasmid DNAs showed the same cleavage patterns obtained with *Eco*RI, *Hin*dIII, and *Bam*HI. We also suggested that this plasmid is native to *S. enteritidis*.

In all strains of *Salmonella dublin*, a 50-Md plasmid which might be associated with virulence for mice was detected, and their plasmid DNAs also had identical cleavage patterns with various restriction enzymes (8). Neither the 36-Md plasmid of *S. enteritidis* nor the 50-Md plasmid of *S. dublin* was associated with basic detectable biochemical properties and drug resistance, although conjugative R plasmids coexisted with the 36-Md plasmid in *S. enteritidis*. It is of interesting that *S. enteritidis* and *S. dublin*, both belonging to D group in *Salmonella* spp., harbored such plasmids without detectable properties even though their sizes are different.

To investigate the role of the 36-Md plasmid in virulence, several experiments were carried out. At first, we compared

TABLE 3. *S. enteritidis* virulence in mice

Route of administration and strain	Plasmid	LD_{50}
Oral		
AL1190	pNL2001	$10^{5.71}$
AL1191	pNL2002	$10^{6.24}$
AL1192		$>10^8$
Subcutaneous		
AL1190	pNL2001	$10^{4.51}$
AL1191	pNL2002	$10^{4.44}$
AL1192		$10^{7.85}$

TABLE 4. Orally administered *S. enteritidis* virulence in chicks

Strain no.	Source	Plasmid size (Md)	Mortality		Recovery from spleen
			Inoculated	Uninoculated	
L-73	Duck	58	2/2	6/6	8/8
L-74	Duck	58	2/2	6/6	8/8
L-34	Beef	36	1/2	0/6	8/8
L-120	Cattle	36	1/2	2/6	8/8
AL1190	Cattle	36	0/2	0/6	8/8
AL1192	Cattle		0/2	0/6	2/8

the virulence for mice between the strains that carried this plasmid and those that carried a different plasmid. The former exhibited higher virulence for mice than the latter, which did not naturally contain this plasmid. When the 36-Md plasmid was eliminated from wild-type *S. enteritidis*, it resulted in a significant decrease in virulence for mice. With oral administration, the plasmid-cured *S. enteritidis* strain was not lethal to mice even though the parent strain was fully virulent, and this strain was also 1,000-fold less virulent when mice were infected subcutaneously than was the parent strain that harbored the 36-Md plasmid.

These results suggest that this 36-Md plasmid in *S. enteritidis* may be associated with the virulence for mice, although this plasmid does not seems to be associated with virulence for chicks because of the results obtained from chick virulence test.

In *Salmonella typhimurium*, virulence of a strain lacking a 60-Md plasmid which was associated with virulence for mice was increased by transferring this plasmid to it (4). In addition to comparing the virulence between parent and cured strains of *S. enteritidis*, reintroduction of the 36-Md plasmid into the cured strain should be carried out. Reintroduction is presently under investigation to show that other changes induced during the curing procedure did not occur.

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