Transposon insertion mutagenesis of a genetic region encoding serum resistance in an 80 kb plasmid of *Salmonella dublin*

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Using transposon insertion mutagenesis with TnI or Tn5, we obtained *Salmonella dublin* mutant strains that showed either diminished serum resistance (five mutants) or diminished mouse lethality (two mutants). Detailed restriction cleavage analysis to determine the single sites of transposon insertion in an 80 kb plasmid (pTE800) indicated that a region for serum resistance was located within a 3.0 kb region of the *Sa*I cleavage fragment 5 and the *Hind*III fragment 2, while the region for mouse lethality was within a 6.0 kb region of the *Sa*I fragment 2 and the *Hind*III fragment 1. When the TnI-containing *Sa*I fragment 5 was reconverted, by homologous recombination, to the original *Sa*I fragment 5 (9.6 kb), serum resistance was recovered to the same level as that of a parent strain 52401. Moreover, the change in the serum resistance correlated with changes in the neutral sugar composition of the LPS. The mutation in the plasmid in strain TE4-55 that gave diminished mouse lethality was also reversed by recombination with the cloned *Sa*I fragment 2 (15.0 kb), with concomitant recovery of mouse lethality. These results indicate that the genetic region for serum resistance is different from that for mouse lethality, and that the gene for serum resistance is closely involved with the expression of the neutral sugar composition of the LPS of *S. dublin*.

Introduction

Salmonellosis is a zoonosis of worldwide economic importance. Infection of animals with various serovars of *Salmonella* may result in serious disease and constitutes a vast reservoir for the disease in man. Although several bacterial structures and products have been suggested to play a role in the virulence of *Salmonella* (Stocker & Mäkelä, 1986), many details of its virulence, such as its invasion from the intestinal tract and its role in systemic infection, are not understood.

In the last few years, plasmids associated with virulence have been reported in several serovars of *Salmonella*, such as *S. typhimurium* (Baird *et al.*, 1985; Gulig & Curtiss, 1987; Hackett *et al.*, 1986; Helmuth *et al.*, 1985; Jones *et al.*, 1982; Pardon *et al.*, 1986), *S. dublin* (Baird *et al.*, 1985; Chikami *et al.*, 1985; Helmuth *et al.*, 1985; Manning *et al.*, 1986; Terakado *et al.*, 1983), *S. enteritidis* (Helmuth *et al.*, 1985; Hovi *et al.*, 1988; Nakamura *et al.*, 1985), *S. choleraesuis* (Helmuth *et al.*,

1985; Kawahara *et al.*, 1988), *S. gallinarum* (Barrow *et al.*, 1987) and *S. pullorum* (Barrow & Lovell, 1988). Several workers have succeeded in identifying genetic regions for mouse lethality on an 80 kb plasmid of *S. dublin* (Beninger *et al.*, 1988; Baird *et al.*, 1985; Williamson *et al.*, 1988b); and on a 100 kb plasmid of *S. typhimurium* (Gulig & Curtiss, 1988; Michiels *et al.*, 1987). A gene for serum resistance has also been cloned from the 100 kb plasmid of *S. typhimurium* (Hackett *et al.*, 1987).

We have shown previously that the 80 kb virulence plasmid, pTE800, of *S. dublin* is associated with virulence phenotypes such as mouse lethality and serum resistance (Terakado *et al.*, 1983, 1988). Moreover, we found that the serum resistance properties of *S. dublin* correlated with changes in the neutral sugar composition of the LPS, suggesting that part of the LPS biosynthesis of this organism might be mediated by the 80 kb plasmid (Terakado *et al.*, 1988). Little information is available, however, as to whether the genetic regions mediating serum resistance and mouse lethality are the same. In this communication, we show that the location of a genetic

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locus for serum resistance on the 80 kb plasmid is different from that for mouse lethality, and that the gene(s) for serum resistance is involved in O-side-chain expression of the LPS of *S. dublin*.

Methods

Bacterial strains and plasmids. These are listed in Table 1. *S. dublin* 52401 is a spontaneous nalidixic-acid-resistant mutant obtained from the virulent wild-type strain 5240 (Terakado *et al.*, 1988).

Media. Nutrient broth (Difco) and L broth [10 g tryptone (Difco), 5 g yeast extract (Difco), 1 g glucose and 5 g NaCl l⁻¹; pH 7.4] were used as liquid media. Mueller-Hinton agar (Difco) and L agar [15 g Bacto-agar (Difco) per litre of L broth] were used as solid media.

Plasmid analysis. Isolation of plasmid DNA, agarose gel electrophoresis and restriction enzyme cleavage were done as described previously (Terakado *et al.*, 1988).

Preparation of mutants. Transposon insertion mutants of *S. dublin* 52401 were prepared by random insertion of either TnI or Tn5. For the insertion of TnI, a thermosensitive mutant of RP4 (pTH10, Harayama *et al.*, 1980), was introduced by conjugation into strain 52401 at 30 °C, and transconjugants were grown at 42 °C in L broth containing ampicillin (50 µg ml⁻¹). Transconjugants resistant to ampicillin, but susceptible to kanamycin and tetracycline, were identified by replica plating. For Tn5 insertion, a thermosensitive mutant of R388::Tn5 (Sasakawa & Yoshikawa, 1987) was introduced by conjugation into strain 52401 at 30 °C, and transconjugants obtained were grown at 42 °C in L broth containing kanamycin (50 µg ml⁻¹). Transconjugants

resistant to kanamycin but susceptible to trimethoprim and sulfamethoxine were selected by replica plating. The transposition of TnI and Tn5 into the 80 kb plasmid pTE800 was confirmed by restriction digestion analysis.

Cloning and transformation. A given plasmid and the vector plasmid (pACYC184; Chang & Cohen, 1978) were independently digested with an appropriate restriction endonuclease. Following incubation of the cloning vector with alkaline phosphatase, vector DNA and plasmid fragments were mixed and incubated in ligation buffer with T4 DNA ligase at 16 °C for 18 h. The ligated DNA was used to transform *Escherichia coli* or *S. dublin*, rendered competent by the rubidium chloride method (Kushner, 1978). To determine the sites of transposon insertion, plasmid fragments contained TnI or Tn5 were cloned into the vector, and transformed *E. coli* were selected on agar containing both chloramphenicol and ampicillin (for TnI) or chloramphenicol and kanamycin (for Tn5). Identification of the clones was confirmed by restriction digestion and electrophoresis.

Homologous recombination between pTE800::TnI or Tn5 plasmids and cloned SalI fragments. To confirm the locations of genetic regions for serum resistance and mouse lethality on pTE800, recombinant plasmids containing cloned *SalI* fragments 5 (pTE1002) or 2 (pTE1003) were introduced separately into the mutant strains TE24 and TE4-55 by transformation. Transformants of these mutants were selected on L agars containing nalidixic acid (25 µg ml⁻¹) and chloramphenicol (25 µg ml⁻¹), and either ampicillin (25 µg ml⁻¹, for TE24) or kanamycin (25 µg ml⁻¹, for TE4-55). Transformants obtained in this way were subcultured three times at 37 °C in L broth without antibiotics. Clones resistant to nalidixic acid, but susceptible to ampicillin (for TE24) or kanamycin (for TE4-55), were selected by replica plating. The

Table 1. *Bacterial strains and plasmids used*

Strain/plasmid	Relevant characteristics	Source/reference
<i>S. dublin</i>		
52401	Nalidixic-acid-resistant mutant of 5240 (pTE800)	Terakado <i>et al.</i> (1988)
TE24	52401 derivative showing diminished serum resistance (pTE8002)	This work
TE241	TE24 derivative (pTE80021)	This work
TE4-55	52401 derivative showing diminished mouse lethality (pTE8003)	This work
TE4-551	TE4-55 derivative (pTE80031)	This work
<i>E. coli</i>		
MC1061	<i>hsr lac leu</i>	Casadaban & Cohen (1980)
TH471	pTH10[RP4(<i>Ts</i>):TnI]	Harayama <i>et al.</i> (1980)
YSH10	R388(<i>Ts</i>):Tn5]	Sasakawa & Yoshikawa (1987)
Plasmids		
pTE800	80 kb plasmid from <i>S. dublin</i> 5240	Terakado <i>et al.</i> (1988)
pTE8002	pTE800 derivative in which TnI has been inserted into the <i>SalI</i> fragment 5	This work
pTE8003	pTE800 derivative in which Tn5 has been inserted into the <i>SalI</i> fragment 2	This work
pTE80021	Recombinant of pTE8002 and pTE1002	This work
pTE80031	Recombinant of pTE8003 and pTE1003	This work
pACYC184	Vector plasmid	Chang & Cohen (1978)
pTE1002	pACYC184 derivative carrying a cloned <i>SalI</i> fragment 5 of pTE800	This work
pTE1003	pACYC184 derivative carrying a cloned <i>SalI</i> fragment 2 of pTE800	This work

identities of clones were confirmed by plasmid profiling and restriction endonuclease digestion.

Serum resistance. Sera from New Zealand White rabbits, whose complement is genetically determined (Komatsu *et al.*, 1985), were used to assay bactericidal activity. Bacteria from an early-exponential-phase L broth culture were washed twice with phosphate-buffered saline (0.15 M-NaCl, 10 mM-sodium phosphate buffer, pH 7.2) and diluted with it 1000-fold. Samples (10 µl) of this dilution (approximately 10^5 organisms) were added to 90 µl of normal rabbit serum and incubated at 37 °C. Percentage bacterial survival was calculated as the ratio of the bacterial count after 90 min to the count at time zero, multiplied by 100. The experiments were repeated three times and the results were expressed as geometric means $\pm$ standard deviations.

Analysis of neutral sugars in LPS. LPS was extracted from the cells of a 500 ml overnight L broth culture and purified by the method of Westphal & Jann (1965). The neutral sugars in the LPS were estimated by gas-liquid chromatography (GLC), after hydrolysis for 4 h at 100 °C with 1 M-HCl and conversion to trimethylsilyl derivatives (Sweeley *et al.*, 1963).

Infection of mice. Mouse virulence of each strain was examined by means of intraperitoneal injection of female ICR mice (4-week-old, Shizuoka Animal Co.). Preliminary results indicated that the LD₅₀ of the parent strain 52401 was usually $10^{5.3}$. Overnight cultures were diluted 1000 fold into L broth and incubated for a further 5 h at 37 °C with shaking. After harvesting by centrifugation, bacteria were washed twice in 0.9% (w/v) saline. For each strain, 0.5 ml volumes of 1000-fold dilutions in saline ($1.5\text{--}2.2 \times 10^6$ organisms) were inoculated intraperitoneally into 20 mice (five mice were housed per cage). Deaths were monitored for up to 7 d following inoculation.

Results

Mutants showing diminished serum resistance and mouse lethality

Using transposon insertion mutagenesis with TnI or Tn5, we obtained seven mutants of *S. dublin* 52401 that showed either diminished serum resistance or diminished mouse lethality. All insertions were located in the

80 kb plasmid pTE800. Fig. 1 shows the restriction map of the part of pTE800 containing the *Hind*III and *Sal*I cleavage fragments within which the TnI and Tn5 insertions have been located. The TnI-insertion sites in the five mutants (TE13, 14, 16, 24, 40) that showed susceptibility to rabbit serum were localized within a 3.0 kb region on *Sal*I cleavage fragment 5 and *Hind*III fragment 2 (Fig. 1). The Tn5 insertion sites of two mutants (TE4-55, 6-61) that showed diminished mouse lethality were within a 6.0 kb region of the *Sal*I fragment 2 and *Hind*III fragment 1. Virulence phenotypes of two representative mutants (TE24 and TE4-55) and their parent strain 52401 are shown in Table 2. The mutant strain TE24, carrying pTE8002, in which TnI had been inserted into the *Sal*I fragment 5, showed diminished serum resistance, but the ability of this mutant to kill mice was similar to that of the parent strain 52401. In contrast, strain TE4-55, carrying pTE8003, in which Tn5 had been inserted into the *Sal*I fragment 2, showed normal resistance to serum but a marked decrease in mouse lethality. No significant differences were observed between the colony morphologies, qualitative agglutination reactions and biochemical properties of these mutants and those of the parent strain 52401.

Changes of virulence phenotype in mutants with cloned fragments

The *Sal*I fragment 5 of pTE800 was introduced into TE24, on the recombinant plasmid pTE1002, by transformation. When transformants were subcultured in L broth without antibiotic selection, several derivatives that retained resistance to nalidixic acid but which had lost resistance to ampicillin were identified. Restriction endonuclease cleavage analysis revealed that plasmid pTE80021, obtained from one representative clone

Table 2. Virulence phenotypes of various mutants obtained from *S. dublin* 52401

Strain	Drug resistance phenotype*	Serum resistance (% survival)†	Mouse lethality (No. of dead mice/total no.)‡
TE24	Amp ^R , Nal ^R	0.05 $\pm$ 0.01	18/20
TE241	Nal ^R	120.0 $\pm$ 26.4	19/20
TE4-55	Kan ^R , Nal ^R	89.6 $\pm$ 16.1	1/20
TE4-551	Nal ^R	99.5 $\pm$ 18.8	19/20
52401	Nal ^R	96.0 $\pm$ 17.5	20/20

* Amp^R, Nal^R, Kan^R; ampicillin, nalidixic acid, kanamycin resistance.

† Percentage survival was calculated as the ratio of the bacterial count after 90 min to the count at zero time, multiplied by 100 (see Methods).

‡ The inoculum was 1.5×10^6 for 52401 and TE24 and 1.7×10^6 for TE241, TE4-55 and TE4-551. The number of mice dying within 7 d was recorded.

Fig. 1. Sites of TnI and Tn5 insertion in pTE800. The size of pTE800 is 80 kb (estimated from the sum of the sizes of restriction-enzyme-generated fragments). ○, Location of TnI-inserts that showed diminished serum resistance; ●, TnI-inserts that failed to diminish serum resistance; □, Tn5-inserts that showed diminished mouse lethality; ■, Tn5-inserts that failed to diminish mouse lethality. Restriction endonuclease sites: S, *SalI*; H, *HindIII*.

Fig. 2. Agarose gel (0.8%) electrophoretic patterns of the restriction endonuclease *SalI* fragments of plasmids obtained from various mutants of *S. dublin* 52401. Lane 1, a *HindIII* λ digest (size reference standard). Lanes 2, 3, 4, 5 and 6 are digests of plasmids (pTE800, pTE8002, pTE80021, pTE8003, pTE80031) obtained from 52401, TE24, TE241, TE4-55 and TE4-551, respectively.

(TE241), gave the same fragment pattern after restriction with *SalI* as did pTE800 (Fig. 2), suggesting that the TnI-containing *SalI* fragment 5 of pTE8002 in TE24 had been replaced, probably by homologous recombination, with the cloned *SalI* fragment 5 from pTE800. Moreover, TE241 had almost the same level of serum resistance as 52401 (Table 2). In a second series of experiments, another mutant, TE4-551, originating from TE4-55, in which the Tn5-containing *SalI* fragment 2 of pTE8003 in TE4-55 was replaced with the cloned *SalI* fragment 2 from pTE800, recovered full virulence for

Table 3. Molar ratios of neutral sugars in LPS of mutants obtained from *S. dublin* 52401

Rha, rhamnose; Man, mannose; Gal, galactose; Glu, glucose; Tyv, tyvelose; Hep, heptose. The monosaccharides were estimated by GLC (see Methods).

Strain	Rha	Man	Gla	Glu	Tyv	Hep
TE24	3.4	4.0	5.0	4.0	0.5	1.0
TE241	6.0	7.0	6.5	3.0	1.7	1.0
52401	6.0	6.8	6.2	2.8	1.6	1.0

mice (Table 2). The derivative plasmid pTE80031, obtained from TE4-551, was also indistinguishable from pTE800 (Fig. 2).

Changes of neutral sugar composition in LPS

The molar ratios of the neutral sugars in the LPS preparations obtained from various strains of *S. dublin* are shown in Table 3. In the TnI-insertion mutant, TE24, the molar ratios of almost all sugars, except for glucose, were lower than those of the parent strain 52401. In contrast, the molar ratios in TE241 were higher than those in TE24, and similar to those of 52401. No differences were observed between TE4-55 and 52401 (data not shown).

Discussion

In Gram-negative pathogens, serum resistance is considered to be an important virulence factor, conferred (at least in part) by either LPS or protein components of the outer membrane (Buchanan & Pearce, 1971). Plasmids associated with serum resistance have been reported in

several bacteria such as *E. coli* (Binns *et al.*, 1982; Moll *et al.*, 1980) and *S. typhimurium* (Hackett *et al.*, 1986). Hackett *et al.* (1987) cloned a gene expressing an 11 kDa serum-resistance protein encoded by the cryptic plasmid of *S. typhimurium*. Introduction of the cloned 2.1 kb fragment carrying the gene into a plasmid-cured strain restored serum resistance without restoring either LPS synthesis or mouse lethality. Rhen & Sukupolvi (1988) reported that the *traT* gene of the *S. typhimurium* virulence plasmid is responsible for plasmid-mediated serum resistance and that the *traT* gene region lies outside the previously defined virulence determinant region.

In a previous report (Terakado *et al.*, 1988), we demonstrated that serum resistance of *S. dublin* is associated with the presence of an 80 kb virulence plasmid, but the genetic region for serum resistance was not identified at that time. In the present study, the cloned *SalI* fragment 5 of this plasmid restored serum resistance to the mutant strain TE24, indicating that at least one genetic region for serum resistance is located within a 3.0 kb region of *SalI* fragment 5. In contrast, the loss of mouse virulence resulting from the mutation in the plasmid in TE4-55 was recovered by recombination between the mutant plasmid and the *SalI* fragment 2 from pTE800. These results indicate that the locations of these two virulence genes are separate. It is unclear whether the genetic region for serum resistance located on pTE800 (obtained from *S. dublin*) is related to the serum resistance locus of the virulence plasmid of *S. typhimurium* reported by other workers (Hackett *et al.*, 1987; Rhen & Sukupolvi, 1988) because no detailed restriction maps have been reported for that plasmid. Using dot hybridization experiments, however, no homology was detected between the cloned *SalI* fragment 5 of pTE800 and the virulence plasmid of *S. typhimurium* LT2, suggesting that the virulence plasmids of these *Salmonella* serovars mediate serum resistance by different mechanisms (data not shown). On the other hand, the location of the genetic region for mouse virulence in pTE800 agreed well with those determined for other *S. dublin* plasmids (Baird *et al.*, 1985; Beninger *et al.*, 1988), suggesting close homology among these virulence determinants. Moreover, it was reported recently that a common genetic region for mouse virulence is present in plasmids of other *Salmonella* serovars (Beninger *et al.*, 1988; Williamson *et al.*, 1988a; Woodward *et al.*, 1989).

LPS is one of the major constituents of the outer membrane of Gram-negative bacteria, and its biosynthesis is known to be mediated by several chromosomal gene clusters (Mäkelä & Stocker, 1984). However, recent studies have revealed that the LPS structures of *Shigella sonnei* (Kopecko *et al.*, 1980), *Shigella dysenteriae*

(Watanabe & Timmis, 1984), *E. coli* (Riley *et al.*, 1987), and *Salmonella* (Popoff & Minor, 1985) are also influenced by plasmids. Therefore, plasmid-mediated alteration of LPS could be a common phenomenon among enteric bacterial pathogens.

The present study demonstrated that the neutral sugar composition of LPS in the mutant strain TE24 can be changed by introduction of the cloned *SalI* fragment 5 from the 80 kb virulence plasmid, pTE800, indicating that LPS biosynthesis of *S. dublin* is influenced by functions encoded on that fragment. Moreover, the change in the neutral sugar composition of the LPS was found to correlate with changes in serum resistance of *S. dublin*, as reported previously (Terakado *et al.*, 1988). In recent studies, however, correlation between LPS and serum resistance properties was observed only in *S. dublin*, and not in other *Salmonella* serovars such as *S. typhimurium*, *S. enteritidis* and *S. choleraesuis* (Kawahara *et al.*, 1989). In order to understand the relationship of these two functions in *S. dublin*, a detailed analysis of the gene products mediated by the cloned *SalI* fragment 5 is now in progress.

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