Plasmid-mediated Serum Resistance and Alterations in the Composition of Lipopolysaccharides in *Salmonella dublin*

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(Received 30 December 1987; revised 11 March 1988)

Survival rates of *Salmonella dublin* in rabbit serum after culture for 1 h at 37 °C were compared between a wild-type strain (5240) carrying a 50 MDa plasmid, a plasmid-cured strain (C524), and a cured strain containing the 50 MDa plasmid tagged with Tn1 (5241). Strain C524 was more susceptible to the bactericidal activity of normal serum than its parent strain 5240 (percentage survival <1% and $52.5 \pm 9.2\%$, respectively). On the other hand, the percentage survival of strain 5241 was significantly increased ($90.4 \pm 4.0\%$), indicating that the reintroduction of the plasmid into the cured strain restored the serum resistance. Moreover, this change in the serum resistance properties correlated with changes in the neutral sugar composition of the lipopolysaccharides (LPS) of these strains, suggesting that the 50 MDa plasmid is necessary for O-side chain expression in the LPS of *S. dublin*.

INTRODUCTION

Salmonella dublin is primarily associated with cattle; it causes acute diarrhoea, septicaemia and abortion in adult cattle and a chronic disease in calves (Walton, 1972). Milk-borne outbreaks of infection due to *S. dublin* have also been reported in man (Small & Sharp, 1979; Fierer, 1983). In both cattle and man, the organism is highly invasive (Walton, 1972; Fierer, 1983).

In a previous report (Terakado *et al.*, 1983), we demonstrated that *S. dublin* strains from cattle harboured a characteristic 50 MDa plasmid and suggested that this plasmid plays an important role in the virulence of *S. dublin*, since the loss of the plasmid resulted in the loss of virulence for mice. Several workers (Baird *et al.*, 1985; Chikami *et al.*, 1985; Heffernan *et al.*, 1987; Helmuth *et al.*, 1985; Manning *et al.*, 1986) have also reported that a similar plasmid was associated with the virulence of *S. dublin*. Although their precise function(s) have not been elucidated, other virulence-associated plasmids have been reported in several serotypes of *Salmonella* such as *S. typhimurium* (Baird *et al.*, 1985; Hackett *et al.*, 1987; Helmuth *et al.*, 1985; Jones *et al.*, 1982; Michiels *et al.*, 1987; Pardon *et al.*, 1986), *S. enteritidis* (Helmuth *et al.*, 1985; Nakamura *et al.*, 1985), *S. choleraesuis* (Helmuth *et al.*, 1985; Kawahara *et al.*, 1988) and *S. gallinarum* (Barrow *et al.*, 1987).

During our investigations on the role of the 50 MDa plasmid of *S. dublin* in pathogenicity, we observed that resistance to rabbit and guinea-pig serum in *S. dublin* was mediated by the plasmid. In Gram-negative pathogens serum resistance is considered to be an important virulence factor conferred either by lipopolysaccharides (LPS) or by protein components of the outer membrane (Buchanan & Pearce, 1971). This prompted us to analyse the composition of the LPS in *S. dublin*. This paper deals with the serum resistance, and the changes in the composition of *S. dublin* LPS, associated with the presence of the 50 MDa plasmid.

Table 1. *Bacterial strains and plasmids used*

Strain/plasmid	Relevant characteristics	Source/reference
<i>S. dublin</i>		
5240	Wild-type, pTE800	Terakado <i>et al.</i> (1983)
C524	pTE800-cured	This work
5241	C542(pTE8001)	This work
<i>E. coli</i>		
TH471	pTH10	Harayama <i>et al.</i> (1980)
Plasmids		
pTE800	50 MDa plasmid from <i>S. dublin</i> 5240, non-conjugative	Terakado <i>et al.</i> (1983)
pTE8001	50 MDa::TnI, non-conjugative	This work

METHODS

Bacterial strains and plasmids. These are listed in Table 1. The virulent wild-type strain 5240 was isolated from a field outbreak of bovine salmonellosis (Terakado *et al.*, 1983). The derivative strain C524, cured of its plasmid (pTE800), was obtained from strain 5240 after treatment with ethidium bromide (700 µg ml⁻¹ in L broth). Strain 5241 was constructed by transformation of C524 with pTE8001 (a plasmid derived from pTE800 by insertion of TnI). Antigenic analysis of these three strains showed that C524 and 5241 possessed O (9, 12) and H (g, p:-) antigens identical to those of the parental strain 5240.

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

Plasmid analysis. Small-scale isolation of plasmid DNA was done by the method of Birnboim & Doly (1979). Large-scale preparation of plasmid DNA was done by CsCl/ethidium bromide density-gradient ultracentrifugation at 140000 g for 15 h at 20 °C in a Beckman VTi 65 rotor. Horizontal electrophoresis was done in 0.8% (w/v) agarose gels using Tris/borate buffer (89 mM-Tris, 2.5 mM-EDTA, 8.0 mM-boric acid, pH 8.4). DNA was usually electrophoresed for 5 h at a constant voltage of 100 V. The restriction enzymes *Hind*III and *Eco*RI were obtained from Takara Shujo Co. Plasmid DNA was digested as recommended by the supplier.

Tagging of *S. dublin* plasmid with TnI. The method for labelling the plasmid with TnI was essentially as previously reported (Danbara *et al.*, 1987). For the tagging of the 50 MDa plasmid with TnI as a selective marker, a thermosensitive mutant of RP4, pTH10 (Harayama *et al.*, 1980), into which a 3.3 MDa ampicillin-resistance transposon TnI had been inserted, was conjugatively introduced into strain 5240 and the transconjugants were grown at 42 °C in the presence of ampicillin. The transconjugants resistant to ampicillin but susceptible to kanamycin and tetracycline were selected after 42 °C incubation, because pTH10 contains genes encoding resistance to ampicillin, kanamycin and tetracycline. The transposition of TnI into the 50 MDa plasmid was confirmed by an increase in the size of the original plasmid by 3.3 MDa.

Reintroduction of the plasmid into the cured strain. The plasmid tagged with TnI was reintroduced into C524 by transformation, because the plasmid was not conjugative. Competent cells were prepared by the rubidium chloride method (Kushner, 1978). A 200 µl volume of the competent cells was mixed with 2 µg plasmid DNA in 20 µl TE buffer (10 mM-Tris/HCl, 1 mM-EDTA, pH 8.0) and the mixture was agitated for 3 min at 42 °C after cooling on ice for 30 min. It was then added to 2.5 ml L broth, incubated for 6 h at 35 °C with gentle shaking, and then spread on L agar containing ampicillin (25 µg ml⁻¹). Colonies that grew at 37 °C were isolated.

Serum resistance. Sera from New Zealand White rabbits, whose complement components are genetically determined (Komatsu *et al.*, 1985), were used for the assay of bactericidal activity. Two types of sera, with the following characteristics, were used: (a) the levels of the third (C3) and eighth (C8) components of complement in the serum were normal (C3, 1 mg ml⁻¹; C8, 80 µg ml⁻¹); and (b) the level of C3 was normal but that of C8 was deficient (0 µg ml⁻¹). These sera were given by Dr M. Komatsu and were kept at -80 °C until use. Bacteria from an early-exponential-phase broth culture were washed twice with phosphate-buffered saline (0.15 M-NaCl, 10 mM-sodium phosphate buffer, pH 7.2; PBS) and diluted 1000-fold in PBS, in a multiwell tissue-culture plate (Sumitomo Chemical Laboratories). Samples (10 µl) of this dilution (approximately 10⁴ organisms) were added to 90 µl of serum and incubated at 37 °C. Percentage survival was calculated as the ratio of the bacterial count after 1 h to the count at zero time, multiplied by 100. The experiments were repeated three times and the results were expressed as geometric means ± standard deviations.

Analysis of neutral sugars in LPS. LPS was extracted from 500 ml of 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 into trimethylsilyl derivatives (Sweeley *et al.*, 1963). GLC was performed on a Hewlett-Packard model 5880A gas chromatograph fitted with a flame

Table 2. Molar ratios of neutral sugars in LPS of *S. dublin* strains

Symbols for sugars: Rha, rhamnose; Man, mannose; Gal, galactose; Glu, glucose; Tyv, tyvelose; Hep, heptose. Monosaccharides were estimated by GLC. (see Methods).

Strain	Plasmid	Rha	Man	Gal	Glu	Tyv	Hep
5240	pTE800	9.1	8.5	11.5	4.9	4.5	1.0
C524	Cured	2.0	2.2	5.8	4.0	0.6	1.0
5241	pTE8001	7.9	8.5	11.0	3.3	4.2	1.0

ionization detector. The separations were carried out on a flexible fused capillary column coated with methylsilicone SP2100 (Hewlett Packard). The column temperature was programmed at 2.5 °C min⁻¹, from 195 °C to 250 °C. The amounts of neutral sugars in the LPS were calculated as molar ratios relative to heptose.

RESULTS

Reintroduction of the 50 MDa plasmid tagged with TnI into the cured strain. The 50 MDa plasmid from *S. dublin* strain 5240, designated pTE800, was cleaved by *Hind*III into 9 fragments, and by *Eco*RI into 10 fragments. In the *TnI* tagging experiments, several kinds of 50 MDa plasmids into which *TnI* had been inserted were obtained. One of them, designated pTE8001, into which *TnI* was inserted in *Hind*III cleavage fragment 3, was selected and reintroduced into the cured strain C524 by transformation. One of the purified transformants obtained was designated as 5241 and used for further experiments.

Serum resistance. In C3-normal but C8-deficient serum, there was no significant difference in viable numbers between the wild-type strain 5240, the plasmid-cured strain C524 and the plasmid-reintroduced strain 5241. All of them showed an increase in viable numbers after culture for 1 h at 37 °C, with percentage survival values of 153.4 ± 15.7 for strain 5240, 138.0 ± 20.0 for strain C524 and 192.5 ± 7.6 for strain 5241 (means of three experiments $\pm$ SD). In contrast, there was a marked difference in the C3-normal and C8-normal serum. In this serum, strain C524 showed a marked decrease after culture for 1 h at 37 °C, and viable numbers were less than 10² organisms per 100 μ l of mixed culture (less than 1% survival). A slight decrease in viable numbers was also observed in both plasmid-positive strains 5240 and 5241 (percentage survival 52.5 ± 9.2 and 90.4 ± 4.0 , respectively), but reduction rates were much lower than that of strain C524, indicating that the reintroduction of pTE8001 into the cured strain restored serum resistance.

Composition of neutral sugars in LPS. The composition of the neutral sugars in LPS preparations obtained from the three *S. dublin* strains was determined by GLC (Table 2). In the plasmid-cured strain C524, the molar ratios of almost all the sugars were decreased compared with the parent strain 5240, the greatest reduction (by more than 80%) being recorded for tyvelose. In contrast, the molar ratios in the plasmid-reintroduced strain 5241 were close to those of the wild-type, except for glucose.

DISCUSSION

Serum resistance seems to be an important virulence factor in invasive serotypes of *Salmonella*, since it promotes survival of the organisms. Plasmids associated with serum resistance have been reported in several bacteria such as *Escherichia coli* (Binns *et al.*, 1982; Moll *et al.*, 1980) and *S. typhimurium* (Hackett *et al.*, 1986). More recently, Hackett *et al.* (1987) have cloned the gene responsible and reported that serum resistance in *S. typhimurium* is mediated by an 11 kDa polypeptide encoded by a 2.1 kb fragment. Helmuth *et al.* (1985) reported that only plasmid-positive strains of *S. dublin* were resistant to the bactericidal activity of guinea-pig serum, although they were not able to reintroduce the plasmid into strains lacking the plasmid.

The present study demonstrated that curing strain 5240 of *S. dublin* of the 50 MDa plasmid resulted in high susceptibility to C3-normal rabbit serum compared with that of the wild-type parent strain. In contrast, the survival rate of the plasmid-reintroduced strain was significantly

increased in such serum, indicating that serum resistance in *S. dublin* is associated with the presence of the 50 MDa plasmid. Moreover, a similar correlation was observed in our previous experiments on the virulence of the strain for mice (data not shown). When the C8-deficient serum was used for the assay of bactericidal activity, however, no such difference in viable numbers was observed among *S. dublin* strains examined. This finding seems to be reasonable, because C8 plays an important role in the cell membrane attack mechanism and complement-mediated cell lysis (Stolfi, 1968).

Alterations in the sugar composition of LPS have been reported in *S. typhimurium* infected with a derepressed ColIb mutant plasmid or an R64-11 plasmid, also derepressed in their transfer (Derylo *et al.*, 1975). Bacteria carrying such plasmids showed a marked decrease in amounts of neutral sugars in the LPS, while bacteria carrying the wild-type ColIb, or the revertant of a *drd* mutant to the wild-type, showed increased amounts of sugar. However, the non-colicinogenic strain resulting from the elimination of such a *drd* mutant plasmid showed no changes in the sugar composition of the LPS (Derylo *et al.*, 1975). These results suggest that the mechanism of changes in the sugar composition of the LPS mediated by such *drd* mutant plasmids might be different from that mediated by the 50 MDa plasmid of *S. dublin*. As to genetic properties, the 50 MDa plasmid of *S. dublin* exhibited fertility inhibition and belonged to incompatibility group FII (unpublished data).

Although the mechanism of changes in sugar composition mediated by the 50 MDa plasmid is unknown, it is interesting that, in our experiments, such changes were correlated with changes in survival rates of *S. dublin* strains in C3-normal serum. Recently, Grossman *et al.* (1987) reported that the size and distribution of LPS determine serum resistance in *S. montevideo*. On the other hand, Hackett *et al.* (1987) reported that a plasmid-cured *S. typhimurium* strain did not express normal LPS, but introduction of the gene for an 11 kDa protein into the strain rendered it serum resistant without restoration of normal LPS synthesis. Heffernan *et al.* (1987) have reported that the biological function of the plasmid of *S. dublin* appears to be to act primarily at the level of the reticuloendothelial system and possibly to destroy tissue macrophages. To elucidate the precise functions and gene products of the 50 MDa plasmid of *S. dublin* will require the cloning of the gene(s) determining serum resistance. This is now in progress.

This study was supported by grant A12601 provided by the Ministry of Education, Science, and Culture of the Japanese government.

We thank Professor M. Yoshikawa, Institute of Medical Science, University of Tokyo, and Dr M. Komatsu, National Institute of Animal Husbandry, for supplying *E. coli* TH471 and rabbit sera. We also thank Mr Y. Mukohara, Mr K. Koiwa and Mr T. Nakano for technical assistance.

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