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Molecular characterization of plasmids in *Salmonella enteritidis* phage types

D. J. BROWN*, E. J. THRELFALL†, M. D. HAMPTON AND B. ROWE

Laboratory of Enteric Pathogens, Central Public Health Laboratory, 61, Colindale Avenue, London NW9 5HT

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SUMMARY

Plasmids in selected type strains of 26 of the *Salmonella enteritidis* phage types have been characterized by restriction enzyme fingerprinting and by DNA–DNA hybridization with oligonucleotide probes for *S*almonella *p*lasmid *v*irulence (*Spv*) genes. With one exception, the fingerprints of the 38 MDa plasmids studied were homogeneous but there was heterogeneity in the fingerprints of 59 MDa plasmids found in 4 of the type strains. However all 38 MDa and 59 MDa plasmids were related as was a 45 MDa plasmid identified in the type strain of phage type 19. A 3·5 kb fragment homologous to *SpvC* was conserved in *Hin*d III digests of all 38 MDa and 59 MDa plasmids, and in the related 45 MDa plasmid. In contrast a 65 MDa plasmid found in the type strain of phage type 10 was not related to these three plasmid molecular weight groups and did not carry the *SpvC* gene.

INTRODUCTION

Since 1988 *Salmonella enteritidis* has been the most common salmonella serovar isolated from cases of food-poisoning in England and Wales [1]. Phage typing has provided a rapid method of discrimination for this serotype and 27 phage types (PTs) have been described [2]. A minority of strains of *S. enteritidis* may also be differentiated by plasmid profile typing. However, although 25 of the 27 type strains carried plasmids, 18 of the plasmid-carrying strains were found to possess a plasmid of 38 megadaltons (MDa) [3]. This plasmid corresponds to that previously designated the *S. enteritidis* 'serotype-specific' plasmid [4]. However 6 of the phage type strains carried a plasmid of approximately 59 MDa and of these, 1 strain carried additional plasmids of 4·0 and 3·8 MDa (PT6a) and 1, an additional plasmid of 4·0 MDa which coded for ampicillin resistance (PT19). The type strain of PT10 was characterized by a single plasmid of 65 MDa and two strains which carried plasmids of 38 MDa also carried plasmids of 70 MDa [3].

Numerous DNA–DNA hybridization studies have been carried out to identify plasmids involved in the virulence of salmonellas for BALB/c mice [5–7]. Such studies have also demonstrated considerable homology in the 'serotype-specific'

* Present address: Department of Veterinary Microbiology, Royal Veterinary and Agricultural University, 13, Bülowsvej, DK-1870 Frederiksberg C., Denmark.

† Author for correspondence.

plasmids of *S. typhimurium*, *S. enteritidis*, *S. dublin* and *S. choleraesuis*. The regions responsible for virulence in the *S. typhimurium*, *S. dublin* and *S. choleraesuis* plasmids have also been sequenced [8–10].

The purpose of this study was to characterize 38, 45, 59 and 65 MDa plasmids in the type strains of the *S. enteritidis* phage types on the basis of their restriction endonuclease fingerprints and the presence and distribution of *m*ouse *k*illing *f*actor (*mkf*) (now termed *S*almonella *p*lasmid *v*irulence (*Spv*) genes). The latter property was investigated by using oligonucleotide gene probes prepared against a region of the *SpvC* gene of the *S. typhimurium* serotype-specific, mouse virulence plasmid [8]. The results are presented.

METHODS

Bacteria

The *S. enteritidis* phage type strains in which plasmid DNA was characterized are listed in Table 1. P102936, the type strain of PT13a, which was not characterized by Threlfall and colleagues [3], but has subsequently been shown to carry a single plasmid of 38 MDa was also included. The type strain of PT6 used in this investigation, P99327, was not that studied previously and has been found to carry four plasmids of 65, 38, 2·6 and 1·0 MDa. Additional strains of PT15 are also listed in Table 1. For *Hin*d III restriction enzyme fingerprinting and DNA–DNA hybridization tests. *S. typhimurium* strain P100836 (PT49) was included as a control. Previous studies (E. J. Threlfall, unpublished) have demonstrated that this strain possesses a 60 MDa plasmid carrying the *SpvC* gene.

Plasmid extraction and characterization

Plasmid DNA was extracted by a modification of the method of Kado and Liu [7] as described by Threlfall and colleagues [8] and visualized after electrophoresis at 150 V for 2·5 h on vertical 0·7% (w/v) Tris–borate agarose gels (Sigma, Type II, Medium EFO). Plasmids were sized in relation to plasmids of known molecular weights carried in strains 39R861 [12] and V517 [13].

Restriction enzyme fingerprinting

For restriction enzyme fingerprinting, plasmid DNA was extracted by the method of Olsen [14] and digested with the restriction endonucleases *Pst* I and *Hin*d III according to the manufacturer's instructions (Gibco/BRL, UK). Endonuclease digests of plasmid DNA were visualized after electrophoresis for 16 h at 20 V on horizontal 0·8% (w/v) Tris–acetate agarose gels, using a BRL H5 Horizontal Gel apparatus.

Construction of oligonucleotide gene probes

Two oligonucleotide sequence probes were synthesized from the published amino acid sequence of the *SpvC* gene of the 60 MDa *S. typhimurium* virulence plasmid [8]. The sequences selected were:

(a) *SpvC*-3 (from 334–358):
5′-(C-G-A-G-A-A-T-C-A-C-C-T-C-A-G-T-C-T-C-A-G-G-G-C)-3′

(b) *SpvC*-4 (from 712–733):
5′-(C-G-A-G-T-G-G-G-C-G-T-G-A-T-G-G-T-G-G-C-G)-3′

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Table 1. *Strains of* S. enteritidis*: phage type and plasmid content*

Strain no.	PT*	Plasmids (MDa†)					
P66040‡	3		59				
E2187‡	4			38			
P99327‡	6	65		38		2·6	1·0
E2468‡	8			38			
E3945‡	10	65					
E2109‡	11		59				
P102936‡	13a			38			
E2387‡	14		59				
E2042‡	15			38			
P121901	15			38			
P116792	15		59				
P130088	15		59				
E1949‡	19			45	4·0¶		
P68147‡	20		59				

* PT, phage type.
† MDa, megadaltons.
‡ indicates type strain.
¶ ampicillin resistance plasmid.

Factors governing the choice of oligosequence were: (i) about 20–30 base pairs (bp) in length; (ii) GC-rich ends, especially 3′; (iii) no hairpins or internal repeats. The temperatures of dissociation (Td) were high, being 76 °C and 72 °C respectively. The oligonucleotide probes were synthesized using an Applied Biosystems DNA Synthesizer and the single-stranded DNA was stored in water at −20 °C.

Labelling with digoxigenin-11-dUTP

For the labelling reactions, 200 ng of oligonucleotide DNA was mixed together with 2·5 μl tailing buffer (1·4 mM sodium cacodylate, 300 mM Tris pH 7·2, 1 mM dithiothreitol), 3·6 μl $CoCl_2$ (7 mM), 2·5 digoxigenin-11-dUTP (100 mM) (Boehringer Mannheim), 25 units of calf thymus terminal transferase (Boehringer Mannheim) and the same volume made up to 25 μl with sterile distilled water. The mixture was incubated at 37 °C for 1·5 h. No further treatment was carried out and the labelled probes were stored at −20 °C.

Blotting and hybridization

Following electrophoresis, gels containing linear DNA fragments were blotted onto Hybond nylon hybridization membranes (Amersham UK) using an LKB Vacuoblot Apparatus (LKB-Produkter, Sweden). Vacuoblotted membranes were then prewashed with 10 ml/50 cm² hybridization solution (5 × SSC, 0·5% blocking reagent (BCL), 0·1% N-lauryl sarcosine, 0·02% SDS) at 60 °C for 1 h. The membranes were pre-hybridized with 10 ml/50 cm² hybridization solution at 2–3 h at 60 °C. The membranes were then incubated overnight at 65 °C with 1 ml of hybridization solution for every 50 cm² membrane, and probe equivalent to 25 ng/ml each of both *SpvC*-3 and *SpvC*-4. All steps were performed in heat-sealed plastic bags with gentle shaking in a G24 Environmental Incubator Shaker (New Brunswick, USA).

Finally, the membranes were washed twice with 5 × SSPG/0·2% SDS at 65 °C for 10 min and once with 2 × SSPE/0·2% SDS for 5 min. Membranes were air-dried and stored, or hybridization was detected immediately as described by BCL.

RESULTS

Restriction enzyme fingerprinting

When digested with *Hin*d III, 38 MDa plasmids in the type strains of *S. enteritidis* PTs 1, 2, 4, 5, 8, 9, 12, 13, 13a, 15, 17, 18 and 23 had identical fingerprints. Those of the 38 MDa plasmids in E2187 and E2042, the type strains of PT4 and PT15, and of P121901 (PT15) are shown in Fig. 1*a*. Because of the presence of additional plasmids, some of which were cleaved by *Hin*d III, comparison of the fingerprints of the 38 MDa plasmids in the type strains of PTs 4a, 6, 21, 22 and 25 with those of the 38 MDa plasmid in strains carrying only this plasmid group could only be subjective. Nevertheless, the seven bands characteristic of *Hin*d III digests of the 38 MDa plasmid in E2187 were observed in *Hin*d III digests of plasmid DNA in the type strains of PTs 4a, 6, 21, 22 and 25 and it seems highly probable that the 38 MDa plasmids in these strains are closely related if not identical to the 38 MDa plasmid in E2187.

Similarly, when digested with *Pst* I, with the exception of E2042 (PT15) the fingerprints of the 38 MDa plasmids in the type strains of PTs 1, 2, 4, 5, 8, 9, 12, 13, 13a, 17, 18 and 23 were also identical. The *Pst* I digests of the 38 MDa plasmids in E2042 (PT15), E2187 (PT4), E2468 (PT8), P121901 (PT15) and P102936 (PT13a) are shown in Fig. 2.

Previous studies [3] had demonstrated that the type strains of PTs 3, 11, 14 and 20 carried plasmids of approximately 59 MDa. When digested with both *Hin*d III and *Pst* I, the fingerprints of the 59 MDa plasmids in the type strains of these phage types were found to be similar but not identical. The *Hin*d III digests of the 59 MDa plasmids in P66040 (PT3), E2387 (PT14) and E2109 (PT11) are shown in Fig. 2*a* and the *Pst* I fingerprints of the 59 MDa plasmids in P66040 and E2109 in Fig. 2. Although none of these 59 MDa plasmids was identical, they all appeared closely related to the 38 MDa plasmid in E2187 (PT4). For example, these plasmids had 5 of 7 fragments in common with the 38 MDa plasmid in *Hin*d III digests (Fig. 1*a*). However, in *Pst* I digests the degree of similarity was not quite as evident, with only 5 of 14 fragments in common for digests of the 38 MDa plasmid in E2187 and the 59 MDa plasmid in P66040, and 6 of 14 fragments in common with the 38 MDa E2187 plasmid and the 59 MDa plasmid in E2109 (Fig. 2).

The 65 MDa plasmid in the type strain of PT10, E3945, had a distinctive fingerprint when digested with both *Hin*d III (Fig. 1*a*) and *Pst* I (data not shown). In *Hin*d III digests, there was only one band in common with digests of the 59 MDa plasmids and none with those of the 38 MDa plasmids (Fig. 1*a*).

The subculture of E1949 (PT19) used in this study, which had been stored on Dorset's Egg medium at 18 °C, was found to carry two plasmids with MWs of 45 and 4·0 MDa, the latter plasmid coding for resistance to ampicillin. In contrast the subculture used in an earlier study carried plasmids of 59 and 4·0 MDa [3]. Subsequent re-examination of the original subculture, which had been maintained at 4 °C, showed that this strain still carried plasmids of 59 and 4·0 MDa. It would

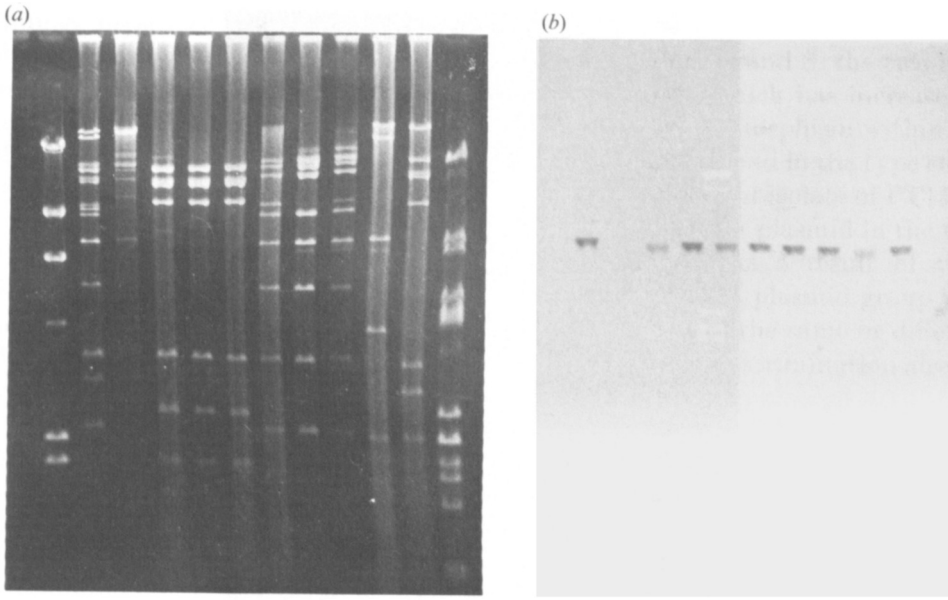

Fig. 1. *Hind* III restriction enzyme fingerprints of plasmids in *Salmonella enteritidis* phage types and hybridization with digoxygenin-labelled oligonucleotide probes for the *SpvC* gene. (*a*) Track 1: λ/*Hind* III; Track 2: P100836 (*S. typhimurium*); Track 3: P99327 (PT6); Track 4: E2187 (PT4); Track 5: E2042 (PT15); Track 6: P121901 (PT15); Track 7: P66040 (PT3); Track 8: E2387 (PT14); Track 9: E2109 (PT11); Track 10: E3945 (PT10); Track 11: E1949 (PT19); Track 12: λ/*Pst* I. (*b*) As with (*a*). Digoxygenin-labelled *SpvC* oligonucleotide hybridization with a 3·5 kb *Hind* III fragment in Tracks 2, 3, 4, 5, 6, 7, 8, 9 and 11.

therefore appear that spontaneous loss of DNA from the 59 MDa plasmid in E1949 had occurred following storage of the subculture at 18 °C, resulting in the formation of a plasmid of 45 MDa. When this 45 MDa plasmid was digested with both *Hind* III (Fig. 1*a*) and *Pst* I (Fig. 2), a close relationship with the 38 MDa plasmid group was apparent. Five of 7 bands were in common in *Hind* III digests and there were at least 12 of 14 common fragments in *Pst* I digests. The 4·0 MDa ampicillin resistance plasmid did not digest with *Pst* I and can be clearly seen in its supercoiled form (Fig. 2).

Plasmids in S. enteritidis *PT15*

When digested with *Pst* I, fragment length polymorphism was observed in the 38 MDa plasmid in E2042, the type strain of PT15 (Fig. 2). *S. enteritidis* PT15 is only rarely isolated in the UK and only three strains of this phage type were isolated between 1981 and 1989 [2]. These three isolates were therefore screened for plasmid DNA and the plasmid fingerprints compared.

One of these isolates of PT15, P121901, carried a 38 MDa plasmid, and two, P116792 and P130088, plasmids of 59 MDa. The *Hind* III and *Pst* I fingerprints of the 38 MDa plasmid in P121901 were identical with those of E2187 (PT4) and the fragment length polymorphism observed in the *Pst* I digest of the 38 MDa plasmid in E2042 was not evident (Figs 1*a* and 2). The 59 MDa plasmids in P116792 and P130088 had *Hind* III and *Pst* I fingerprints indistinguishable from those of the 59 MDa plasmid in P66040 (PT3, data not shown).

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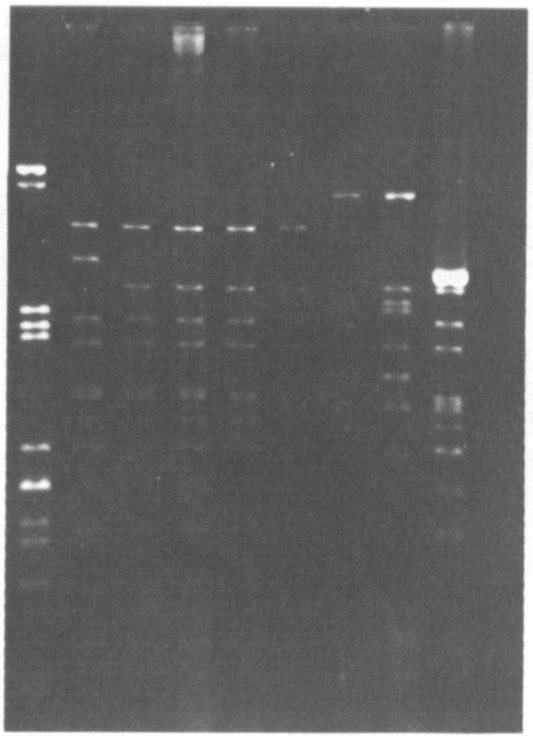

Fig. 2. ***Pst*** **I restriction enzyme fingerprints of plasmids in** ***Salmonella enteritidis*** **phage types. Track 1: λ/*Pst* I; Track 2: E2042 (PT15); Track 3: E2187 (PT4); Track 4: 2468 (PT8); Track 5: P121901 (PT15); Track 6: P102936 (PT13a); Track 7: P66040 (PT3); Track 8: E2109 (PT11); Track 9: E1949 (PT19).**

Homology with SpvC *probe*

Following digestion with *Hin*d III, linear DNA fragments of selected plasmids were transferred by vacuoblotting onto nylon membranes and tested for homology with oligonucleotide probes for *SpvC*.

A 3·5 kb fragment from *Hin*d III digests of all the 38 MDa and 59 MDa plasmids in the type strains and in additional strains of PT15, including the variant plasmid in E2042, was found to hybridize with the *SpvC* probes. Hybridization was also exhibited with an identical fragment from *Hin*d III digests of the 45 MDa plasmid of E1949 (PT19) and the 60 MDa serotype-specific plasmid carried by the control strain of *S. typhimurium* strain P100836 (Fig. 1*b*).

In the two strains containing 65 MDa plasmids, a 3·5 kb fragment from a *Hin*d III digest of plasmid DNA in P99327 (PT6) hybridized with the probes. However, as this strain also carried a 38 MDa plasmid (Table 1), this positive result is likely to have resulted from hybridization with a 3·5 kb *Hin*d III fragment from the 38 MDa plasmid. In contrast there was no hybridization with any fragments resulting from *Hin*d III digests of the 65 MDa plasmid carried by E3945 (PT10).

DISCUSSION

Previous studies have suggested that a 'serotype-specific' plasmid of approximately 38 MDa is common to the majority of strains of *S. enteritidis* [4]. The

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findings presented here demonstrate that such a plasmid is not common to all phage types of *S. enteritidis*, although it is present in PTs 4 and 8, the two most common phage types in the UK [2] and also to PT13a, which has increased in incidence in recent years. Restriction fragment length polymorphism within this plasmid was observed in only one instance, in a 38 MDa plasmid in the type strain of PT15. However a second 38 MDa plasmid from a subsequent isolate of PT15 did not show this heterogeneity, which suggests that the 38 MDa plasmid in the type strain of PT15 may be extremely rare if not unique. As a result of these observations, we conclude that fingerprinting of the 38 MDa plasmid group with the enzymes *Hin*d III and *Pst* I in strains of *S. enteritidis* of the same or different phage types is unlikely to significantly extend the degree of discrimination already achieved by phage typing and plasmid profile typing.

With respect to all 38 MDa plasmids examined, DNA–DNA hybridization demonstrated that virulence for BALB/c mice was encoded by genes carried on a 3·5 kb *Hin*d III fragment. This *Hin*d III fragment was conserved in 59 MDa and 45 MDa plasmids found in some of the type strains, which were related to the 38 MDa plasmid on the basis of restriction enzyme fingerprints. The fragment was also common to the 60 MDa serotype-specific plasmid carried by the control strain of *S. typhimurium*, P100836.

The 59 MDa plasmid group found in some phage types of *S. enteritidis* was heterogeneous, although the pattern exemplified by the 59 MDa plasmid in the type strain of PT11 was also exhibited by 59 MDa plasmids carried by two of four strains of PT15 examined (data not shown). However, on the basis of their restriction enzyme fingerprints all plasmids of this group were related to each other and to the 38 MDa plasmid group. All plasmids of the 59 MDa MW group carried the *SpvC* genes located on a 3·5 kb *Hin*d III digest fragment. Analysis of the digest fingerprints of the 45 MDa plasmid in the type strain of PT19 demonstrated that this plasmid was also closely related to the 38 and 59 MDa plasmid groups and likewise carried the *SpvC* genes on a 3·5 kb *Hin*d III fragment. In contrast, the 65 MDa plasmid in the type strain of PT10 did not appear to be related to these three groups and did not exhibit *SpvC* homology.

These findings demonstrate that although the majority of phage types of *S. enteritidis* carry a 38 MDa plasmid which cannot be differentiated by restriction enzyme fingerprinting, there is considerable molecular variation in plasmids of 45, 59 and 65 MDa, carried by a minority of the phage type strains. Nevertheless, the *SpvC* region appears to have been conserved on all plasmids of these molecular weight groups other than that of 65 MDa identified in the type strain of PT10. Further studies are necessary to understand the evolutionary development of these virulence plasmids in different *S. enteritidis* phage types and to elucidate their relationship with similar plasmids in other serotypes of epidemiological importance in humans and food animals.

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