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Correlation of change in phage type with pulsed field profile and 16S *rrn* profile in *Salmonella enteritidis* phage types 4, 7 and 9a

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SUMMARY

Using pulsed-field gel electrophoresis (PFGE) and 16S rRNA (*rrn*) analysis (ribotyping), the *in vivo* derivation of strains of *Salmonella enteritidis* PTs 9a and 7 from a strain of *S. enteritidis* PT 4 has been demonstrated. All strains were isolated from a single patient over a 6-week period. Further studies have demonstrated that in terms of pulsed-field profile and ribotype, the genotypes of the patient-derived strains differed from those of the reference strains of the respective phage types. It is concluded that when used in combination, these methods can provide evidence of phylogenetic relationships in apparently unrelated *S. enteritidis* phage types isolated during pathogenesis of disease.

INTRODUCTION

For epidemiological investigations, the method of choice for the primary differentiation of *Salmonella enteritidis* is phage typing. The scheme of Ward and colleagues [1] has defined 27 phage types (PTs) and a further 17 have subsequently been identified [2]. Change in phage type has been shown to be related to both plasmid acquisition and loss of the ability to express long chain lipopolysaccharide (LPS). For example, the acquisition of incompatibility group N drug resistance plasmids has been shown to result in the conversion of *S. enteritidis* PT 4 to *S. enteritidis* PT 24 [3]. Likewise the irreversible loss of the ability to express LPS has been shown to result in the conversion of *S. enteritidis* PT 4 to *S. enteritidis* PT 7 with a concomitant loss in virulence for BALB/c mice [4].

Pulsed-field gel electrophoresis (PFGE) provides a DNA fingerprint of the whole genome and may provide evidence of genetic heterogeneity which cannot be readily detected using other RFLP-based methods. For example, nine distinct *Xba* I-generated PFGE profile types within *S. enteritidis* PT 4 have recently been identified and it has been suggested that PFGE may provide a method of discriminating *S. enteritidis* PT 4 suitable for epidemiological investigations [5].

Ribotyping or 16S rRNA (*rrn*) gene analysis can also provide a molecular fingerprint based on the distribution of conserved multicopy gene sequences across the bacterial genome [6]. Although not as discriminatory as PFGE, this method has recently been applied to the analysis of faecal and blood isolates of *S. dublin*

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[7], and to the elucidation of phylogenetic relationships within *Salmonella* serogroup D1 [8]. However, as yet neither PFGE nor ribotyping have been applied to investigating changes in phage type within *S. enteritidis*. In this study we describe the combined use of PFGE and *rrn* gene analysis for elucidating genetic relationships and investigating the genotypic phylogeny of strains of *S. enteritidis* of three different phage types isolated from a single patient over a 6-week period.

MATERIALS AND METHODS

Bacterial strains

Strains of *S. enteritidis* used in this study are listed in Table 1. All strains had been phage typed by standard methods and assigned to phage types in accordance with the scheme of Ward and others [1]. Strains E2187, P130362 and P106583 are the reference strains of *S. enteritidis* phage types (PTs) 4, 7 and 9a respectively and were from the culture collection of the Laboratory of Enteric Pathogens (LEP). Strains P337433, P339184 and P339183 had been isolated from a single patient (patient A) over a 6-week period in 1993. P337433/A was a laboratory-derived mutant of PP337433, picked as a single colony from within the zone of lysis produced by typing phage 2 of the *S. enteritidis* typing scheme [1] during the phage typing of P337433. In the phage typing of *S. enteritidis*, strains belonging to *S. enteritidis* PT 4 are lysed by typing phage 2 at routine test dilution. In contrast, strains belonging to several other phage types, including *S. enteritidis* PT 9a are not lysed by this phage. Typing phage 2 was therefore chosen as a possible agent for the *in vitro* generation of spontaneous mutants of *S. enteritidis* PT 4 resistant to lysis by specific typing phages, including typing phage 2.

Phage typing, plasmid DNA extraction and salmonella plasmid virulence (spv) *gene homology*

Phage typing and the extraction and analysis of partially-purified plasmid DNA were by standard methods [1, 9]. Preparation of the *spv* gene probe (*spvBCDorfE*) and subsequent hybridization analysis was as described by Threlfall and colleagues [10].

Pulsed-field gel electrophoresis (PFGE)

The extraction of genomic DNA and the conditions for PFGE were as previously described [5] with the following modifications: intact chromosomal DNA was digested with *Spe* I (recognition sequence ACTAGT) and samples were then electrophoresed with Lambda ladder (48·5 kbp concatamers) through 1% agarose gels at 5·4 V/cm for 64 h. PFGE was performed using a CHEF DR II system (Biorad) and pulse times were ramped from 5 to 35 s during the run.

Preparation of genomic DNA and ribotyping

Genomic DNA for *rrn* gene probe analysis was prepared using the method of Ausubel and colleagues [11], digested separately with *Pvu* II and *Mlu* I according to the manufacturer's instructions (Boehringer Mannheim) and transferred to Hybond N membranes (Amersham) as previously described [7]. For the analysis of restriction site variation in and around the 16S rRNA (*rrn*) genes, membranes were hybridized with a 550 bp probe, generated by PCR employing the

Table 1. S. enteritidis *strains: source, plasmids, LPS, PFGE profiles and 16s* rrn *profiles (ribotypes)*

Strain no.	Phage type	Source	Year of isolation	Plasmids		LPS	PFP‡	16S *rrn* profile		Reference
				M_r (kbp)*	*spv*†		(*Spe* I)	(*Pvu* II)	(*Mlu* I)	
E2187	4§	Human	1967	57	+	+	1	D-RI	D-RI	9
P130362	7§	Environment	1988	57	+	−	2	D-RI	D-RI	This study
P106583	9a§	Human	1987	90	+	+	3	D-RI	D-RII	This study
P337433	4	Human‖	1993	57	+	+	4	D-RI	D-RIII	This study
P339184	7	Human‖	1993	57	+	−	5	D-RI	D-RIII	This study
P339183	9a	Human‖	1993	57	+	+	4	D-RI	D-RIV	This study
P337433/A	9a	Laboratory-derived	1994	57	+	+	4	D-RIII	D-RV	This study

* M_r, relative molecular mass.
† + indicates positive hybridization with *s*almonella *p*lasmid *v*irulence (*spv*) gene probe.
‡ PFP, Pulsed-field profile.
§ Type strain of phage type.
‖ Isolated from same patient.

primer sequences 5′GCAACGCGAAGAACCTTACC3′ and 5′GGTTACCTTGTTACGACTT3′ (nucleotides 966–985 and 1492–1510 of the *Escherichia coli rrn* B gene [12] as described by Chowdry and colleagues [7]. Detection was by chemiluminescence using standard protocols (Boehringer Mannheim Lumigen PPD) and ribotypes were designated in accordance with the scheme of Stanley and Baquar [13].

LPS analysis

LPS was prepared and examined by SDS–PAGE as described by Chart and colleagues [4].

RESULTS

General observations

S. enteritidis PT 4, strain P337433 was isolated from the faeces of patient A in November 1993. Four weeks later *S. enteritidis* PT 9a, strain P339183 was isolated from urine and after a further 2 weeks *S. enteritidis* PT 7, strain P339184 was isolated from the pubic ramus. *S. enteritidis* strain P337433/A was a laboratory-derived mutant of P337433, picked as a single colony from within the zone of lysis produced by typing phage 2 of the *S. enteritidis* typing scheme [1] during the phage typing of P337433. When grown in Nutrient Broth and subjected to full phage typing [1], the pattern of lysis of P337433/A was indistinguishable from that of P339183 (*S. enteritidis* PT 9a from patient A).

Plasmid content and spv *hybridization*

With the exception of P106583, the type strain of *S. enteritidis* PT 9a, the type strains of *S. enteritidis* PTs 4 and 7 and all strains of *S. enteritidis* of PTs 4, 7, and 9a from patient A were found to possess a single plasmid with a relative molecular mass (M_r) of 57 kbp which hybridized with the *spv* gene probe. In contrast P106583 possessed a single plasmid of approximately 90 kbp which also hybridized with *spv* probe.

Lipopolysaccharide

All strains of *S. enteritidis* of PTs 4 and 9a including the type strains and strains of these phage types from patient A produced long-chain LPS migrating with a typical 'ladder' pattern [4] on SDS–PAGE gels. Strains P130362 and P339184 (*S. enteritidis* PT 7) did not express long-chain LPS (results not shown).

PFGE

Digestion of genomic DNA with *Spe* I and analysis of the resulting fragments by PFGE revealed five unique Pulsed Field Profiles (PFPs) (Fig. 1), which have been designated *S. enteritidis* PFP (*Spe* I) 1 through to *S. enteritidis* PFP (*Spe* I) 5 (Table 1). Although all profiles shared at least 10 common fragments, strains of *S. enteritidis* PTs 4, 7 and 9a from patient A could be distinguished from the reference strains of their respective phage types by the presence of a fragment of approximately 320 kbp which was present in *Spe* I-generated PFPs of the patient isolates but not in those of the reference strains. As yet this fragment has not been detected in *Spe* I digests of over 30 other strains of *S. enteritidis* PT 4 studied by PFGE (N. G. Powell, unpublished observations).

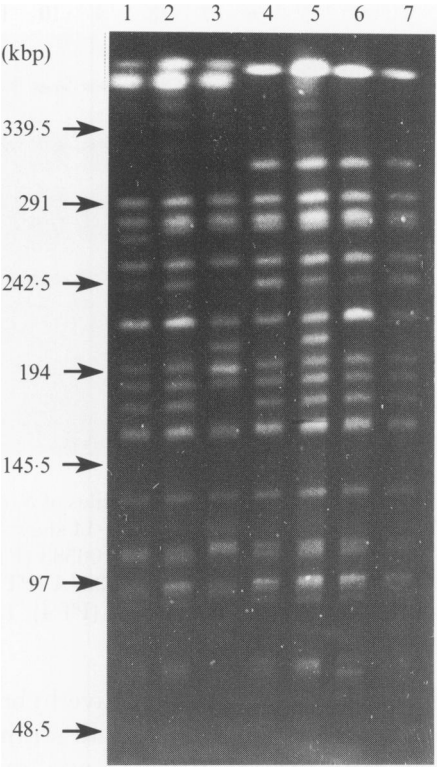

Fig. 1. PFGE profiles of *Spe* I-digested genomic DNA from *S. enteritidis* PTs 4, 7 and 9a. *Legend*: Tracks 1–7 contained: 1, E2187 (*S. enteritidis* PT 4); 2, P130362 (PT 7); 3, P106583 (PT 9a); 4, P337433 (PT 4); 5, P339184 (PT 7); 6, P339183 (PT 9a); 7, P337433/A (PT 9a).

The *Spe* I-generated PFGE profiles of the reference strains differed from each other by the presence of between two and five fragments. In particular P130362, the reference strain of *S. enteritidis* PT 7 could be distinguished from E2187, the reference strain of *S. enteritidis* PT 4 by the absence of a fragment of 270 kbp and the presence of one of 280 kbp. Although P106583, the reference strain of *S. enteritidis* PT 9 possessed the 280 kbp fragment, it lacked two fragments of 240 and 165 kbp and possessed an additional fragment of 200 kbp. P337433, P339184 and P339183, the strains of *S. enteritidis* of PTs 4, 7 and 9a from patient A all possessed the 280 kb fragment but not the fragment of 270 kbp identified in E2187. However, P339184 (PT 7) could be differentiated from P337433 and P339183 by the possession of an additional fragment of 200 kbp. In contrast the PFPs of PP337433, P339183 and P337433/A (the laboratory-derived strain of *S. enteritidis* PT 9a), were indistinguishable.

Identification of plasmid-associated fragments

Whereas E2187 and P130362 and the strains of *S. enteritidis* of PTs 4, 7 and 9a from patient A all possessed a *Spe* I-generated fragment of approximately 57 kbp (Fig. 1, lanes 1–2, 4–7), this fragment was not identified in P106583, the reference strain of PT 9a. In contrast, in P106583 a fragment of approximately 87 kbp was identified (Fig 1, lane 3) which was not present in all other strains tested. When

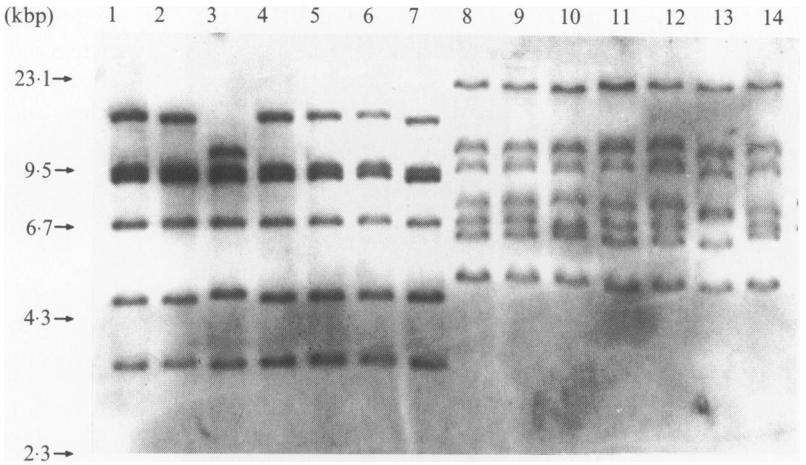

Fig. 2. *Pvu* II- and *Mlu* I-derived 16S rRNA gene profiles of *S. enteritidis* PTs 4, 7 and 9a. *Legend*: Tracks 1–7 show *Pvu* II digests; Tracks 8–14 show *Mlu* I digests. Track 1, E2187 (*S. enteritidis* PT 4); 2, P130362 (PT 7); 3, P106583 (PT 9a); 4, P337433 (PT 4); 5, P339184 (PT 7); 6, P339183 (PT 9a); 7, P337433/A (PT 9a); 8, E2187 (PT 4); 9, P130362 (PT 7); 10, P106583 (PT 9a); 11, P337433 (PT 4); 12, P339184 (PT 7); 13, P339183 (PT 9a); 14, P337433/A (PT 9a).

probed with the *spv* gene probe (see above), positive hybridization was observed with the 57 kbp band in E2187, P130362 and in all strains from patient A, and with the 87 kbp band in P106583. As P106583 was exceptional amongst the strains studied in possessing a *spv* plasmid of approximately 90 kbp (see above) it was therefore concluded that the band differences in the 57–90 kb region between the type strain of *S. enteritidis* PT 9a and the other strains studied were a function of *spv* plasmid carriage and not a result of heterogeneity in chromosomal structure in the different phage types.

Ribotype

Digestion of *S. enteritidis* DNA with *Pvu* II gave an apparent 16S *rrn* gene copy number of six, as opposed to *Mlu* I digests which, in all strains except P339183, indicated a copy number of seven (Fig. 2). This discrepancy may be explained either by two copies of the 16S gene being present on similarly-sized fragments not resolved by electrophoresis, or by the presence of two copies of the gene on a single *Pvu* II-generated fragment.

When *Pvu* II digests of genomic DNA were probed with the 16S rRNA probe, three distinct ribotypes were identified, which have been designated D-RI *Pvu* II through to D-RIII *Pvu* II. Of these, D-RI *Pvu* II was unique to P106583, the reference strain of *S. enteritidis* PT 9a and D-RIII *Pvu* II was exhibited by the laboratory-derived strain P337433/A. In particular, D-RIII *Pvu* II displayed *rrn*-carrying fragments of 14, 9·4 and 9·2 kbp as opposed to D-RI *Pvu* II, in which the three largest fragments were sized as 15, 9·5 and 9·3 kbp.

Ribotyping following digestion with *Mlu* I proved more discriminatory, revealing five distinct ribotypes (D-RI *Mlu* I–D-RV *Mlu* I). All reference strains shared two *Mlu* I fragments of 21·0 and 5·0 kbp not present in the patient isolates. In contrast the patient isolates all shared fragments of 23·0 and 4·8 kbp not

identified in the type strains. The reference strains of *S. enteritidis* PTs 4 and 7 shared identical *Mlu* I profiles (D-RI *Mlu* I), as did the patient-derived strains P337433 (PT 4) and P339184 (PT 7) (profile D-RIII *Mlu* I). The reference strain of *S. enteritidis* PT 9a could be clearly distinguished from those of *S. enteritidis* of PTs 4 and 7 by the loss of a band of 6·7 kbp and its replacement by a band of 6·5 kbp (D-RII *Mlu* I) and P339183, the strain of *S. enteritidis* PT 9a from patient A could be distinguished from the isolates of PTs 4 and 7 from patient A by differences in the size of several fragments and also by its apparent copy number of six. As with the *Pvu* II-generated ribotypes, the *Mlu* I-generated ribotype of P337433/A, the laboratory-derived strain of *S. enteritidis* PT 9a showed minor variation in band pattern; this ribotype has been designated D-RV *Mlu* I.

DISCUSSION

In terms of the interrelationships between strains of *S. enteritidis* belonging to PTs 4, 7 and 9a, loss of the ability to express long-chain LPS and the concomitant irreversible conversion of *S. enteritidis* PT 4 to *S. enteritidis* PT 7 is well-documented [4]. In contrast the possibility of conversion of strains of *S. enteritidis* of PTs 4 or 7 to *S. enteritidis* PT 9a has not been investigated either in relation to LPS-mediated interactions or to plasmid acquisition.

Previous studies [5] have demonstrated that in accordance with the definition of clonality for PFPs as described by Maslow and colleagues [14], PFPs obtained after digestion of genomic DNA from E2187, can be regarded as the archetypical PFPs for this phage type. The *Spe* I-generated PFP shown in Fig. 1, lane 1 and designated PFP (*Spe* I) may therefore be regarded as the archetypical *Spe* I PFP for *S. enteritidis* PT 4. PFP (*Spe* I) 2, the PFP of P130362 (PT 7 strain type) differs from PFP (*Spe* I) 1 by the presence of an additional fragment of 280 kbp and the loss of a fragment of 270 kbp. Such reorganization can result from a point mutation causing an isolated change in base sequence, thereby eliminating a restriction enzyme (RE) recognition site from the 270 kbp fragment in E2187. It is tempting to speculate that such a mutation also resulted in loss of the ability of P130362 to produce long-chain LPS. However, all other strains studied in this investigation possessed a fragment of 280 kbp and lacked a fragment of 270 kbp and of these, five were able to produce LPS. PFP (*Spe* I), the PFP of the type strain of *S. enteritidis* PT 9a could be differentiated from PFP (*Spe* I) 1 both by the presence of *spv* plasmid-associated bands of different M_r, and also by the lack of two chromosomal fragments totalling approximately 400 kbp, only partly compensated by the presence of an additional fragment of 200 kbp. The degree of dissimilarity between the reference strains of *S. enteritidis* PTs 4 and 7, and PT 9a is reinforced by the ribotyping results, both with *Pvu* II and *Mlu* I.

PFGE of *Spe* I-digested DNA from strains of *S. enteritidis* of PTs 4, 7 and 9a from patient A revealed genomic similarities; all strains characterized by the possession of a unique fragment of 320 kbp not observed in the reference strains of the respective phage types nor in 30 unrelated strains of *S. enteritidis* PT 4 which have been studied by PFGE. As there were no detectable differences in the profiles of the strains of *S. enteritidis* PTs 4 and 9a from patient A, it was concluded that P337433 (PT 4) was the probable progenitor of P339183 (PT 9a)

isolated from patient A 6 weeks after P337433. Although slight differences in ribotype were observed, these involved only minor variation in band position which were probably related to a point mutation resulting in change of phage type. In contrast, although P339184 possessed the 'unique' 320 kbp fragment common to all strains from patient A, this strain also possessed an additional fragment of approximately 200 kbp not observed in the strains of *S. enteritidis* PTs 4 and 9a from patient A. It is possible that the presence of this fragment is related to the inability of P339184 to produce LPS and this is currently being investigated. Nevertheless, the *Pvu* II- and *Mlu* I-generated ribotyping results, which provide evidence of *rrn*-carrying fragments conserved between P337433, P339184 and P339183 and not identified in the type strains of *S. enteritidis* PTs 4, 7 and 9a reinforce the hypothesis of the *in vivo* derivation of both P339184 and P339183 from P337433; the small differences in the *Mlu* I-generated ribotype of P339183 are currently being investigated.

In this study, equal discriminatory power was displayed by PFGE with *Spe* I and ribotyping with *Mlu* I. Each technique defined five sub-types with differences in ribotype being observed in strains with indistinguishable pulsed-field profiles and *vice versa*. In contrast ribotyping following digestion with *Pvu* II was less discriminatory, although confirming differences between the genomes of the reference strains of *S. enteritidis* PT 4 and *S. enteritidis* PT 9a.

As discussed above, it is highly probable that the *in vivo* loss of the ability to express LPS was responsible for the conversion of P337433 (PT 4) to P339184 (PT 7). However LPS-mediated interactions may be excluded from being involved in the conversion of P337433 to P339183 (PT 9a), since both these strains produced long-chain LPS. As loss of the ability to produce LPS is irreversible [4], this also eliminates the possibility of P339183 being derived from P339184. The possible involvement of plasmids may also be excluded, since both P337433 and P339183 had an identical plasmid content, both possessing single *spv* plasmids of 57 kbp.

The reactions of strains of *S. enteritidis* PTs 4, 7 and 9a with the *S. enteritidis* typing phages are such that it is very unlikely for a strain of *S. enteritidis* PT 7 to have been derived from a strain of *S. enteritidis* PT 9a [2]. In this study the conversion of *S. enteritidis* PT 4 to *S. enteritidis* PT 9a was achieved *in vitro* following spontaneous mutation of P337433 to resistance of one of the *S. enteritidis* typing phages. This demonstrated that *S. enteritidis* PT 9a can be derived from *S. enteritidis* PT 4 by a spontaneous mutation probably affecting phage receptor sites. The resultant strain, P337433/A, had a pulsed-field profile indistinguishable from that of P337433, and exhibited *Pvu* II- and *Mlu* I-generated ribotypes closely related to those of P337433 and P339183, with only minor differences in band position. We therefore conclude that in patient A, strains of *S. enteritidis* of PTs 7 and 9a were independently derived from a strain of *S. enteritidis* PT 4 during pathogenesis of disease either as the result of a mutation affecting the ability to express LPS (*S. enteritidis* PT 7), or affecting phage receptors (*S. enteritidis* PT 9a).

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