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Phage conversion in *Salmonella enterica* serotype Enteritidis: implications for epidemiology

S. RANKIN[1] AND D. J. PLATT[2]*

[1] *Scottish Salmonella Reference Laboratory, Stobhill NHS Trust, 133 Balornock Road, Glasgow G21 3UW*

[2] *University Department of Bacteriology, Glasgow Royal Infirmary, Castle Street, Glasgow G4 0SF*

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SUMMARY

A model system for the study of phage conversion of *Salmonella enterica* serotype Enteritidis is reported. Temperate phages 1,2,3 and 6 from the phage typing scheme were used to convert several individually recognized phage types into others. Phage type 4 was converted to PT8, PT6a to PT4, PT6a to PT7, PT13 to PT13a and PT15 to PT11; some new phage lysis patterns were also detected.

This model was used to examine the relationships between phage types within a previously defined clonal lineage, SECLIII, to establish whether or not Enteritidis like *Salmonella enterica* serotype Typhi and *Salmonella enterica* serotype Paratyphi B possessed type determining phages. We were able to convert PT1 to PT20, and PT15 to PT11.

INTRODUCTION

In the 1940s Craigie observed that many strains of *Salmonella enterica* serotype Typhi carried temperate phages [1, 2]. He believed that carriage of these phages made their host strains resistant to the Vi-typing phages. He lysogenized type A with a temperate phage isolated from type D1 and found that the newly lysogenized culture reacted as type D1. It was later shown [3,4] that type specificity in Typhi is partly controlled by the carriage of temperate phages, which have been called 'type determining' phages. Around the same time Boyd and colleages [5], showed that 'symbiotic' bacteriophages were present in a large percentage of strains of *Salmonella enterica* serotype Typhimurium, and showed that the identification of these phages in cultures of Typhimurium could therefore be used for tracing the origin and following the spread of an outbreak of infection, as cultures for related cases and carriers all contained the same type.

Enteritidis is the most frequently isolated serotype from cases of human food poisoning, and World Health Organization surveillance data on human salmonellosis for 1979–87 indicate that this infection increased in both Europe and

* Corresponding author.

the Americas [6]. It has, however, become increasingly apparent that the distribution of phage types in Europe and the USA is very different. Rodrigue and colleagues [7] reported that PT8 and PT13a accounted for 83% of the Enteritidis subtypes in their study and stated that this was similar to the distribution of these phage types (79%) in Canada [8]. This is in contrast to the prevalence of PT4 and PT8 (85%) among Enteritidis isolated in England and Wales [9]. These data indicate that phage typing alone provides little epidemiological discrimination among strains that are currently predominant. Compared with Typhimurium there have been few studies that used phage typing combined with other typing methods to increase Enteritidis strain discrimination. Threlfall and colleagues [10] attempted to subdivide Enteritidis phage types by plasmid profile typing and concluded that plasmid profile typing was not as discriminatory as phage typing for the subdivision of Enteritidis.

Rodrigue and colleagues [7] further included antimicrobial resistance patterns and concluded that phage typing and plasmid profile typing complemented each other. These anomalies have led to increased confusion as both studies reached different conclusions. Recently, Stanley and colleagues [11] identified evolutionary lines among Enteritidis phage types based on the distribution of the insertion sequence IS200. Three clonal lineages were described, SECLI, SECLII and SECLIII, which contained 13, 8 and 5 phage types respectively. SECLIII contained the phage types 11, 14, 15, 18 and 20. Phage types 11 and 15 harbour plasmids of 80 kb (pOG691) and 54 kb (pOG701) respectively, which we believe are molecular variants of the Enteritidis serotype-specific plasmid (SSP) [12]. Phage type 9a which is absent from Stanley's study has also consistently been shown to harbour the 80 kb plasmid seen in PT11 strains.

The Enteritidis phage-typing scheme uses 10 phages from 3 sources. Four of the final phages were obtained by direct isolation from lysogenic strains of Enteritidis (1, 2, 3 and 6). Five were phage adaptations (5 from 3; 8 from 6; 9 from 4; 10 from 8, and 4 from a phage of unknown origin) also, one phage (7) was isolated from sewage. The current investigation focused on those phages which were isolated directly from lysogenic strains and not adapted in any way. It addressed the hypothesis that Enteritidis strains are often lysogenic for one or more phages, and that a temperate phage isolated from a particular 'phage type' is able to convert a different 'type' into either the phage type from which the original phage was isolated, another recognized phage type, a strain that does not conform to any previously recognized type (RDNC) or a strain which shows negative reactions with all the typing phages (untypable). In the Enteritidis phage-typing scheme, as in that of Paratyphi B [13], we question whether some phages carry out the dual function of phage typing and type determination.

The specific aims of the present study were (*a*) to develop a model system for the study of phage conversion among strains of Enteritidis using those temperate phages of the typing scheme which were obtained directly from Enteritidis; (*b*) to test this model within a clonal lineage, SECLIII, based on a genotypic parameter proposed by Stanley and colleagues [11], to determine whether phage conversion occurred between phage types solely within or outwith the lineage to establish whether the genotypic relationship defined by the IS200 lineage was concordant with phenotypic characterization by phage type.

METHODS

Bacterial strains

Strains of Enteritidis described in this study were obtained from the collection held by the Scottish Salmonella Reference Laboratory. Details of their source and plasmid content are listed in Table 1. Phage typing was as described by Ward and colleagues [9] and with the exception of one strain (PT6a) only antibiotic-sensitive strains were included.

Plasmid profile (PPA) and restriction endonuclease fragmentation pattern (REFP) analysis.

Plasmid DNA was examined in crude lysates by a modification of the method of Platt and Sommerville [14]. The equivalent of 12–20 colonies of an overnight culture on nutrient agar (Oxoid, CM3) was suspended in 300 μl of electrophoresis buffer (EB) (89 mM Tris, 89 mM boric acid, 1·25 mM EDTA, pH 8·2) in 1·5 ml Eppendorf tubes. Two hundred microlitres of sodium dodecyl sulphate (SDS) (10% w/v in EB) were added and the tubes mixed gently by inversion. After incubation at 50 °C for 5 min the crude lysates were centrifuged for 15 min (9980 g) in a micro-centrifuge. One hundred microlitres of the supernatant fraction were loaded onto a vertical agarose gel (0·7%), sigma, type II) together with 5 μl of tracking dye (25% sucrose, 8 mM sodium acetate, 3·5 mM SDS and 0·7 mM bromophenol blue). Electrophoresis was carried out for 1 h at 100 V followed by 4 h at 200 V (constant voltage). Gels were stained with ethidium bromide (6 μg/ml) for 15 min, viewed using a UV transilluminator (365 nm) and photographed on type 665 film (Polaroid). The molecular weight of plasmids was determined by reference to plasmids of known size (kb); Rts 1 (180), RA-1 (127), R1 (93), R702 (69) and RP4 (54). Supercoiled ladder (Life Technologies, Paisley, UK) was used for the molecular weight estimation of small plasmids (< 16 kb).

Restriction enzyme fingerprinting was performed as previously described in detail by Platt and colleagues [15]. Plasmid DNA was extracted and purified by a modification of the method of Birnbom and Doly [16]. Restriction enzymes were supplied by Life Technologies, Paisley, UK and used according to the manufacturer's instructions.

Phage conversion

Using typing phages at routine test dilution (RTD)

Phages were supplied by LEP, Colindale. Indicator organisms were grown in 3 ml double strength nutrient broth (Difco) that contained 0·85% sodium chloride (phage broth) at 37 °C for 2 h with shaking. This was used to flood phage agar plates (double strength nutrient broth that contained 0·85% NaCl + 1·3% agar (Difco). The seeded plates were allowed to dry and spotted with 2–3 drops of the typing phage under examination. The plates were incubated at 38·5 °C overnight and the following morning a discrete plaque, together with a small amount of the surrounding culture, was cut from the agar. Each plaque was transferred to 20 ml phage both in a screw-capped bottle and incubated at 38·5 °C overnight. A 10 μl loop was used to streak a nutrient agar plate (Oxoid) and after overnight incubation at 38·5 °C single colonies were selected for phage typing.

Table 1. Salmonella enterica *serotype Enteritidis strains used in the study*

Strain designation	Phage type	Plasmid profile (kb)	Source
SE1	1	54	Typing strain
SR901977	1	54: 3·6	Human
SR924098	2	54	Human
GRI 16485	4	54	PT4 Control
SR923224	6a	54: 45	Human, Amp®
SR901610	7	54: 3·0	Poultry
K/1896	8	54	Kasauli 1979
GRI 10688	9a	80	PT9a Control
SR88190	11	80	PT11 Control
SR872651	13	54	Human
SR881508	15	54	PT15 Control
SR922218	23	54	Human
GRI 7389	8	ND	Plasmid free control, PT8
SR901179	4	ND	Human

Propagation of phage from Enteritidis phage types 9a, 11 and 15

Individual strains were examined for the presence of temperate phages by induction with mitomycin C. The organisms were grown in 20 ml phage broth for 4 h followed by the addition of 1 ml of a mitomycin C solution (10 mg/ml). The broths were incubated overnight at 38·5 °C, then centrifuged at 3000 r.p.m. for 15 min. The supernatant fluids were carefully removed into glass universals and treated with 0·14% toluene to kill any residual bacteria. The lysates were tested on a lawn of logarithmically grown Enteritidis PT1 (SR901977). The following morning discrete plaques were removed to 20 ml phage broth and incubated for 4 h. These were centrifuged at 3000 r.p.m. for 15 min. The supernatant fluid was removed to a sterile universal and heated at 57 °C for 40 min to kill residual bacteria.

A further centrifugation step was involved to remove dead bacteria, after which the lysate was ready for use. Indicator organisms were spotted with lysate, as before, and after incubation plaques removed to 20 ml phage both. A nutrient agar plate was inoculated from each broth and 5–10 colonies were selected for phage typing.

Sterility controls

Before use all lysates were filter sterilized using a 0·2 μm filter (Arcodisc, Gelman Sciences); nutrient agar plates were also inoculated from this filtrate to test sterility. The strains used were chosen as they were readily distinguishable from each other during individual studies using PPA or REFP's. We used these different REFP's to confirm distinction between phage convertants and the control strains.

RESULTS

Enteritidis phage types 1, 2, 4, 6a, 8, 9a, 13 and 15 were treated individually with 10 μl of a routine test dilution of one or more typing phages 1, 2, 3 and 6 and single colonies were subsequently phage typed (Table 1). In each case 5 colonies

Table 2. *Phage conversion in* Salmonella enterica *serotype Enteritidis*

Original phage type	Treated with phage no.*	Observed phage type(s)
1	1	1
1	2	1
1	3	1
1	3	Untypable
1	6	1
2	1	RDNC
4	2	8
6a	6	7
6a	6	4
6a	6	6a
7	2	7
8	2	8
8	6	8
13	6	13a
13	6	RDNC
15	6	11
15	6	15

* Phage number in the typing scheme.

were phage typed, unless stated otherwise. Phage type 1 remained as PT1 when treated with phages 1, 2, 3, and 6. Phage type 2 converted to RDNC when treated with phage 1 and 2 distinct lysis pattern were observed (results not shown). When PT4 was treated with phage 2 the lawn of cells showed almost complete lysis on all 5 plates. In all cases, however, there were discrete colonies of which 10 were subsequently re-phage typed. Two of the 10 were untypable and the remaining 8 typed as PT8. Phage type 6a when treated with phage 6 showed one PT6a, 2 convertants to PT4 and 2 convertants to PT7. Phage type 8 was treated with phages 2 and 6. In both cases 10 colonies were phage typed and showed no conversion from PT8 to any other phage type. All 5 phage type 9a colonies tested remained as PT9a when treated with phage 6. Phage type 13 converted to PT13a, in 4 of 5 colonies, when treated with phage 6, the other became RDNC. Phage type 15 converted to PT11 when treated with phage 6, in 3 of 5 cases, the other 2 resultant organisms typed as RDNC (Table 2).

Fig. 1 shows the REFP's (*Pst*1) of phage type convertants 6a to 4; 6a to 7; 15 to 11 and 13 to 13a. The convertants harboured the plasmid(s) present in the parent strain and not those typical of the phage type to which they had converted. The conversion of PT6a to PT4 resulted in the loss of the 45 kb plasmid present in the PT6a strain before conversion.

Phage types 1 and 15 treated with a PT11 lysate

Enteritidis SE1 was the PT1 strain used for conversion. Ten colonies were phage typed, 8 of which converted to PT20, 1 became rough and untypable and 1 showed no change. When fingerprinted using *Pst*1 all of these colonies harboured the 54 kb SSP only (Fig. 2).

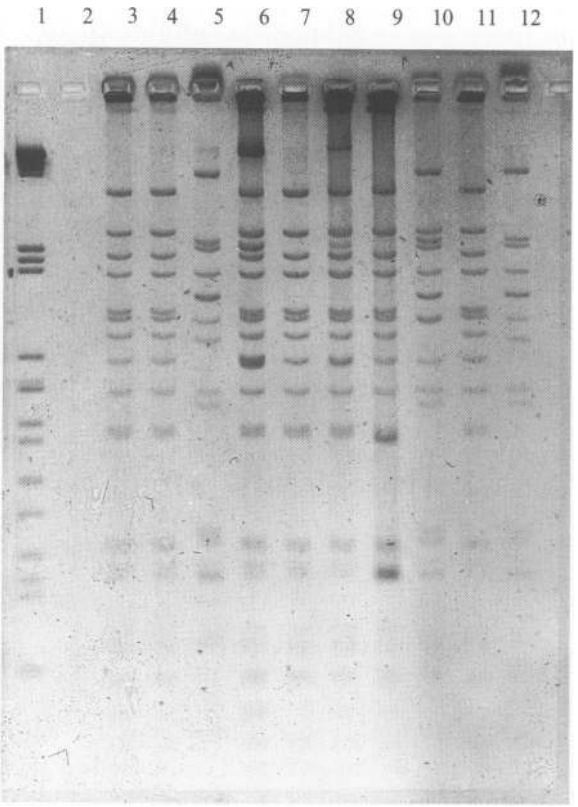

Fig. 1. REFP's using *Pst*1 of Enteritidis phage convertants. Lanes: 1, *Pst*1/*Kpn*1 digested lambda DNA; 2, Blank; 3, PT6a converted to PT4; 4, PT13 to PT13a; 5, PT15 to PT11; 6, PT6a to PT7; 7, GRI16485; 8, 903224; 9, 901610; 10, 881190; 11, 872651; 12, 881508. Each of the convertants still harboured the plasmid which was present in the parent strain.

Phage types 1 and 15 treated with a lysate from PT9a

Enteritidis SE1 was the PT1 strain used. Five colonies were examined; of these, 4 had converted to PT20, the other had all the PT1 reactions but was negative with phage 6. The *Pst*1 fingerprint, however, showed that the PT20 colonies harboured 2 plasmids of 54 and 3·6 kb. This indicated that contamination had occurred in the PT9a lysate and the lysogenized stain was not SE1 but SR901977, which was the PT1 strain on which the PT9a lysate was propagated. Five colonies of PT15 were examined. One remained as PT15, 2 had converted to PT1, 1 to PT20 and 1 to PT11. *Pst*1 fingerprint analysis showed that both the PT11 and PT15 isolates harboured a 54 kb plasmid (pOG701). The remaining 3 isolates harboured 2 plasmids of 54 and 3·6 kb. We were thus able to exclude the conversion of PT15 to PT1 or PT20 using a lysate from PT9a, as clearly contamination has again occurred from the PT9a lysate. However, the strain which had converted to PT11 was shown, by REFP analysis, to have originated from the PT15 strain by phage conversion.

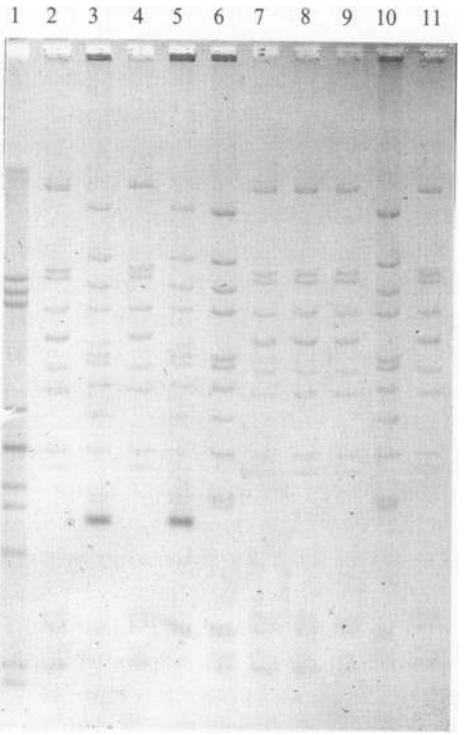

Fig. 2. REFP's using *Pst*1 of Enteritidis phage convertants from phage types 1 and 15. Lanes: 1, *Pst*1 digested lambda DNA; 2, PT15 converted to PT11; 3, PT1 to PT20; 4, PT15 to PT11; 5, PT1 to PT20; 6, PT1 (SE1) to PT20; 7, 8 and 9; PT15 to PT11; 10, GRI 16485; 11, 881508; Each of the convertants still harboured the plasmid present in the original parent strain.

DISCUSSION

Phage typing of Enteritidis has become the preferred method for the primary subdivision of this serotype in epidemiological investigations [17, 18]. An interesting observation which emerged was that of the conversion of PT4 to PT8 by lysogenization with phage 2 of the typing scheme. An analogy to this phenomenon has been documented for the Typhi Vi-phage types F1 and F2 [3]. F2 always carries a phage to which F1 is highly susceptible. The phage can produce lysis on F1 and can also transform it into F2; both processes lead to the disappearance of F1. Enteritidis phage types 4 and 8 thus merit further investigation. Clearly, if they are readily interconvertible, there is nothing to be gained from their recognition as separate types. If, however, as we presume, they are relatively stable under field conditions we can continue to derive useful information from their separate identification. In the UK and Europe PT4 predominates and a similar situation exists in the USA and Canada where PT8 is the predominant type [8, 18]. Attempts have been made to further characterize phage types using molecular techniques e.g. plasmid profile analysis (PPA) [10], restriction endonuclease fingerprint analysis (REFP) [19, 20] and IS200 analysis [11, 17]. Each of these methods has offered its own conclusions but overall they have contributed little to epidemiology.

Phage conversion has been shown to occur in Enteritidis, both with representative phages from the panel of 10 used in the typing scheme and also with lysates prepared from mitomycin C induced organisms. This phenomenon is not new; among the best-known converting phages is P22 which lysogenizes salmonellae of serogroup B with the appearance of factor 1 [21] and phage 14 which lysogenizes salmonellae of serogroup C, with the appearance of factor 14 [22]. Lysogeny, however, is not always accompanied by detectable changes in serological specificity. There were no detectable serotypic changes found in any of the phage type convertants in this study (results not shown).

Some lysogenic strains liberate phage, during growth, in numbers approximately proportional to the number of bacteria: this occurs simply by cell death [23]. Therefore, in a mixed population phage conversion may be expected to occur. Double-type infections may therefore be found with salmonellae where there is no reason to suspect that more than one type was initially involved. In such instances it is usually found that the lytic patterns of the 2, or more, types, present a partial similarity [13]. This phenomenon has been documented, albeit unknowingly, for Enteritidis [24]. In this study by Hickman-Brenner and colleagues, one strain was initially typed as PT23 and subsequently typed as PT8, another originally typed as PT14b was later found to be a mixture of PT14b and PT8. Their observation 'that a few strains appeared to revert to more stable phage types could pose a problem' is profound given the results presented here. It is possible that due to the isolation and characterization procedures used in routine diagnostic laboratories, which rely primarily on single colony isolation and sub-culture, this phenomenon passes largely unrecognized. There have been cases in this laboratory (unpublished observations) in which apparent family outbreaks yield members from which infecting organisms have different phage types. This is currently taken to imply that (*a*) the organisms are unrelated or (*b*) more than one organism was present in the initial infecting source. The latter conclusion prevails in salmonella epidemiology, and we argue that the results presented provide some clarification of these anomalies.

That relationships exist between the phage types of Enteritidis has until very recently been unrecognized. Frost and colleagues [25] demonstrated conversion between PT4 and PT24 based on the acquisition of a plasmid. Chart and colleagues [26] showed that PT4 converted to PT7 by loss of lipopolysaccharide and became rough and further suggested [27] that strains belonging to PTs 23 and 30 might have originated from strains belonging to PT4, 6, 8, 13a or 24 and Threlfall and colleagues [28] have subsequently shown interrelationships between strains of several phage types, based on the loss/acquisition of an *Inc*N plasmid. The phage conversion data presented here extend the range of mechanisms by which phage types can change.

The advent of strain discrimination methods based on genotypic analysis provides the potential for a phylogenetic framework; the distribution of IS200, among Enteritidis [11] suggested three clonal lineages within each of which a range of phage types was associated albeit on the basis of a single representative of each phage type. The phage type conversions described here, notably PT1 (SECLI) into PT20 (SECLIII), PT6a (SECLI) into PT7 (SECLII) and PT4 (SECLI) into PT8 (SECLII), strongly suggest that phage types are polyphyletic.

In conclusion we have shown that 'phage type' is not an 'independent' characteristic of strains of Enteritidis and as a consequence it should be recognized that whereas phage typing may provide good discrimination and remain a useful epidemiological tool it is not a coherent system of subtyping. We are in agreement with Harvey and colleagues [29] in that we believe phage typing will remain a useful tool which should, however, be complemented with the newer molecular methods and interpreted with considerable caution.

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