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Transduction of inositol-fermenting ability demonstrating phylogenetic relationships among strains of *Salmonella typhimurium*

By D. C. OLD, P. F. H. DAWES and RUTH M. BARKER
Bacteriology Department, University of Dundee Medical School, Ninewells Hospital, Dundee DD1 9SY, Scotland

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SUMMARY

The production of Inl$^+$ recombinants was readily demonstrated in transductional crosses from Inl$^+$ donor strains to Inl$^-$ recipient strains from different biotypes and biogroups. None of numerous crosses between different pairs of strains from biotypes 25 and 26 and the biogroup FIRN (biotypes 29, 30, 31 and 32) gave Inl$^+$ recombinants suggesting that the *inl* mutation was present at the same intragenic site in all of these strains. Strains of the FIRN biogroup (Fim$^-$Inl$^-$Rha$^-$Bxyl$^-$) are thought to have descended by successive *rha* and *fim* mutations from an ancestral bacterium of biotype 25 (Fim$^+$Inl$^-$Rha$^+$Bxyl$^-$). The sites of the *inl* mutations in other Inl$^-$ biotypes (9, 10, 27 and 25hi) were independent and each was different from that in strains from biotypes 25, 26 and FIRN.

1. INTRODUCTION

A new biotyping scheme for *Salmonella typhimurium*, developed by Duguid *et al.* (1975) from the original scheme of Kristensen, Bojlén & Faarup (1937), incorporated additional tests and distinguished more types. Thirty-two primary biotypes were recognisable by the possible combinations of positive and negative reactions in five primary tests with the substrates D-xylose, *meso*-inositol, L-rhamnose, *d*- and *meso*-tartrates. Subtypes were defined within the primary biotypes by ten secondary tests (Duguid *et al.* 1975). Full biotypes were designated by the primary biotype number and letters that indicated the results of the secondary tests. In a series of 2030 strains of international origin, 144 full biotypes were distinguished, including representatives of 19 of the 32 potential primary biotypes.

Duguid *et al.* (1975) showed that 1081 of these strains of *S. typhimurium*, i.e. those from primary biotypes 1, 2, 3, 4, 7, 17, 18, 19, 21 and 23 (Fig. 1), fermented *meso*-inositol in peptone water in 24 h at 37 °C, i.e. were Inl$^+$. Strains from primary biotypes 9, 25, 26, 27, 29, 30, 31 and 32 did not ferment inositol at 37 °C, i.e. were Inl$^-$. Although designated as non-fermenting on the basis of their mutant phenotype at 37 °C, the majority of strains of biotypes 29, 30, 31 and 32 (FIRN

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biogroup) and of biotypes 25 and 26 fermented inositol at 25 °C in 48 h, i.e. had a temperature-sensitive phenotype (Old, 1972; Duguid *et al.* 1975).

On the basis of their biotype findings, Duguid *et al.* (1975) constructed a hypothetical genealogical tree and a pattern of mutational (usually 'loss') pathways, whereby the known primary biotypes might have evolved from the presumed

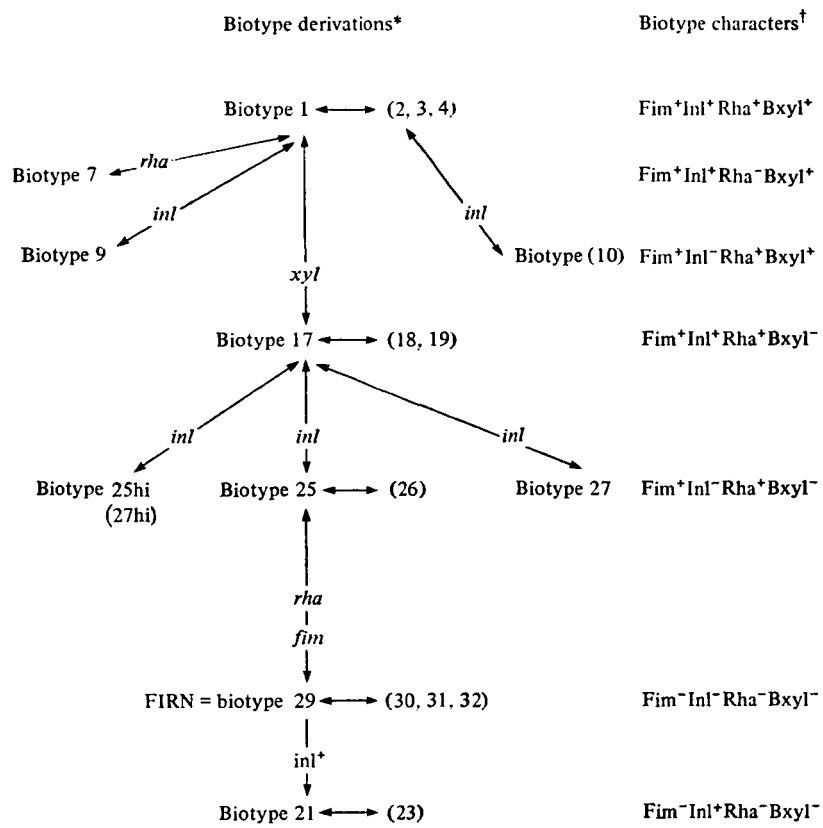

Fig. 1. Proposed relationships between the primary biotypes of *Salmonella typhimurium*. *In parentheses are the *d*-tartrate-negative (3, 19, 23, 27hi, 31), *meso*-tartrate-negative (2, 10, 18, 26, 30) and *d*-tartrate-negative, *meso*-tartrate-negative (4, 32), biotypes related to the major biotypes that are *d*-tartrate and *meso*-tartrate positive. † +/− Positive/negative with respect to the characters: Fim, production of type-1 fimbriae; Inl, fermentation of *meso*-inositol at 37 °C; Rha, fermentation of L-rhamnose; Bxyl, utilisation of D-xylose as sole carbon source.

archetypal biotype 1a, the commonest biotype and that in which all biotyping characters are positive. The relationships among the primary biotypes are indicated (Fig. 1, modified from Old & Duguid, 1979).

Considerable interest attaches to the question of whether the sites of a mutation in a phenotypically homogeneous group of strains isolated from natural sources are alike. If identical, the strains probably had a common origin and a common ancestor. If the sites are different, the strains might be unrelated or distantly

related because of their descent from different ancestral clones, that underwent independent mutations.

Thus, transduction studies have provided evidence that the sites of the mutations in *fim* are homologous in all *S. typhimurium* FIRN strains in biotypes 29, 30, 31 and 32 (Duguid, Old & Hume, 1962; Old, 1963; Old & Duguid, 1979). Those in *rha* are also homologous in FIRN strains (Duguid *et al.* 1962; Old, 1963; Morgenroth & Duguid, 1968). The likelihood, therefore, is that FIRN strains have had a single, common origin.

This paper describes transduction studies that suggest that the site of the *inl* mutation is the same in FIRN strains and in strains from biotypes 25 and 26, but different from those in strains from other Inl$^-$ biotypes. It provides evidence to substantiate the proposed relationships of biotypes presented in Fig. 1, in particular the origin of FIRN from biotype 25.

2. MATERIALS AND METHODS

(i) *Bacteria.* We tested 153 of the naturally occurring strains of *S. typhimurium* biotyped by Duguid *et al.* (1975), and strain S911 of the Inl$^-$ biotype 10 described by Barker, Old & Sharp (1980). The definitive phage types (Anderson *et al.*, 1977) were determined at the Division of Enteric Pathogens, Colindale Avenue, London. Table 1 indicates the biotype (BT), phage type (PT), source, date and place of isolation of these strains. The Inl$^+$ donor strains were S844 (BT1a/PT1), S2317 (BT2a/PT89) and S375 (BT3a/PT1). The majority of the strains in the Inl$^-$ biogroups were tested both as donors and recipients in the transduction experiments.

(ii) *Culture media.* Nutrient broth was Nutrient Broth No. 2 (Oxoid). Inositol peptone water (IPW) and inositol minimal-salts agar (IMA) were prepared as described previously (Duguid *et al.* 1975).

(iii) *Biotyping.* The biotyping media and tests have been described (Duguid *et al.* 1975).

(iv) *Donor phages.* The propagation of phage P22 on donor strains and the titration of lysates followed the methods of Old & Duguid (1971). Most lysates contained $5–50 \times 10^9$ phages/ml; from occasional strains, e.g. some from biotype 26, lysates of only moderate titre ($5–40 \times 10^8$/ml) were obtained.

(v) *Transduction experiments.* An overnight nutrient broth culture of each strain to be tested as recipient was used to inoculate (with *c* 10^6 bacteria) a series of IPW which were incubated, usually for 3–4 h, until just turbid (10^8 bacteria/ml). Donor phages were mixed with the recipient bacteria in IPW at a multiplicity of 1–5. Most donor–recipient pairs were tested in duplicate; for some, additional tests were performed. In each experiment, the recipient bacteria were also tested in 'no-phage' control cultures to confirm that they did not give spontaneous Inl$^+$ mutants. 'Homologous phage' tests confirmed that self-crosses of the recipient strains did not generate Inl$^+$ progeny. Control mixtures of the recipient bacteria and donor phage from one of the three Inl$^+$ strains (Table 1) were

Table 1. *Biotype, phage type, source and date and place of isolation of strains of* Salmonella typhimurium *used in transduction experiments*

Biogroup or biotype	Full biotype	Phage type	No. of strains	Source*	Date	Place†
Inl+	1a	1	1	H	1960	Asia
	2a	89	1	B	1963	UK
	3a	1	1	U	1956	U
BT25	25a	2, 3, 5, 16, 46, 58, 70, 83, 126, 135, 140, 153, 158, 173	19	A, B, E, H	1953–66	Africa, Aus Eur, UK
	25b	10, 66	2	H	1958, 69	Eur, UK
	25d	16, 88	3	A, H	1962, 63	UK
	25gz	16	1	H	1947	UK
	25x	6, 49, 76, 135	7	A, H, M	1956	Aus, Eur, UK
BT26	26a	2, 6, 16, 29, 44, 49, 57, 114, 135, 185 200, 204, 207	20	B, En, H, Monkey	1958–75	Aus, Eur, NA UK
	26d	29	1	B	1965	UK
	26f	44	1	H	1955	Asia
	26i	29, 44	4	H, Guinea-pig	1959–64	Aus, UK
	26z	132	1	H	1959	UK
FIRN	29b	13, 89, 129, 161, 166	7	A, En, H, O	1958–70	Africa, NA, Eur, UK
	29bd	56	1	H	1958	UK
	29bf	13, 40, 89	4	En, H	1952–63	Africa, Asia, NA
	30by	80	6	A, B, En	1962–64	UK
	31b	1, 13, 14, 25, 29, 40, 41, 85, 92, 93, 120, 123, 126, 195	24	A, E, En, H	1955–71	Eur, NA, UK
	31bd	141	2	A, B	1965, 72	UK
	31bg	58, 71	2	A, Guinea-pig	1959, 62	Eur, UK
	31bi	14, 86	2	A, B	1963, 69	NZ, UK
	32b	1, 14, 81, 161	4	A, H	1958–67	Eur, UK
	32bf	80	1	A	1958	UK
	32bef	146	1	H	1962	NA
	32bi	40	2	E, H	1963	UK
BT27	27i	165	1	H	1966	UK
	27ei	94	1	H	1963	SA
	27dfgi	2	1	A	1962	NA
'BT'25hi‡	25hi	99	7	A, En, H, P	1958–65	UK
	25hix	99	1	M	1965	UK
	25bhi	99	1	E	1962	NA
	25ghi	99	1	A	1969	Eur
	25fghi	99	1	A	1969	Eur
	27hi	99	6	A, C, H, M	1958–64	NA, UK
	27fhi	99	1	A	1969	CA
	27hiz	99	2	P	1969	Eur
BT9	9a	18	1	H	1952	UK
	9b	22	1	H	1956	SA
	9f	49, 68, 141	3	H	1967–72	UK
	9i	1, 20a, 59, 94, 106	7	A, E, En, H	1956–63	SA, UK
BT10	10a	104b	1	H	1974	UK

included in all experiments. The mixtures were cultured for 72 h; the temperature of incubation was 37 °C throughout.

(vi) *Characterization of Inl⁺ transductants.* Each IPW culture that showed a colour change from purple to yellow, indicating acid production, was plated on IMA and incubated for 48–72 h. Purified colonies were biotyped by the primary tests of Duguid *et al.* (1975).

3. RESULTS

The aggregated results of the transduction and 'no-phage' control tests with the recipient strains of *S. typhimurium* of different biogroups or biotypes are given in Table 2. The proportion of tests, donor strains, recipient strains and combinations of strains that gave Inl⁺ recombinants is shown for each class of biogroup cross. That the Inl⁺ bacteria were recombinants is clear from the finding that there were no Inl⁺ bacteria in any of the 'no-phage' and 'homologous' control cultures of the inositol non-fermenting (Inl⁻) recipients.

(i) *Transduction from Inl⁺ donors to Inl⁻ recipients*

The results show that phage propagated on Inl⁺ donor strains (from biotypes 1a,2a and 3a) produced Inl⁺ recombinants from 136 of the 137 naturally occurring Inl⁻ recipient strains. The exceptional strain S2804 (BT31b/PT120) had been shown previously to be poorly susceptible as a recipient for transduction of *fim* (Old & Duguid, 1979). The three Inl⁺ strains were equally competent donors, and together they gave positive results in 617/689 tests. The Inl⁻ recipients, regardless of biogroup, did not differ markedly in their susceptibility to transduction of *inl.* Although we did not determine the proportion of Inl⁺ transductants originally produced, the frequency of transduction may have been low, for test mixtures plated on IMA sometimes gave negative results with competent donor-recipient pairs that had shown a high frequency of transduction when selection of transductants was in IPW for 72 h.

(ii) *Transduction from Inl⁻ donors to Inl⁻ recipients*

Table 2 shows that Inl⁺ recombinants were obtained in many crosses among naturally occurring strains of different Inl⁻ biogroups. The Inl⁻ strains from some biogroups, e.g. biotypes 9, 10 or 25hi (see footnote, Table 1) were nearly as effective as the Inl⁺ donors in crosses with recipients from other Inl⁻ biogroups.

(*a*) *Biotypes 25, 26 and FIRN.* Inl⁺ recombinants were not obtained in any of 1550 tests in which 581 different combinations of FIRN strains were tested as

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* Isolated from a source that was: A, avian; B, bovine; C, canine; E, equine; H, human; M, murine; O, ovine; P, porcine; En, environmental; U, unspecified.

† Place of isolation was: Aus, Australia; CA, Central America; Eur, Europe (other than UK); NA, North America; NZ, New Zealand; SA, South America; UK, United Kingdom; U, Unspecified.

‡ The nine strains of phage type 99 and of biotype 27hi differ from the strains of biotype 25hi in being *d*-tartrate non-fermenting. For ease of reference, this group is called 'BT' 25hi.

donor–recipient pairs; in any of 678 tests of 211 pairs of biotype 25 strains, nor in any of 450 tests of 184 pairs of biotype 26 strains. Furthermore, Inl$^+$ recombinants were not detected in 988 tests in reciprocal crosses of the classes BT25 × BT26, BT25 × FIRN and BT26 × FIRN. Because we used strains from these biotypes

Table 2. *Transduction of ability to ferment* meso-*inositol* (*at* 37 °*C*) *between donor and recipient strains of different biogroups of* Salmonella typhimurium

Donor strain biogroup or biotype	Results given for	Number of tests, donors, recipients or pairs* giving Inl$^+$ bacteria/number tested in crosses with Inl$^-$ recipient strains of biogroup or biotype:					
		BT25	BT26	FIRN	BT27	'BT'25hi	BT9
Inl$^+$	Tests	99/103	74/80	283/336	23/26	81/84	57/60
	Donors	3/3	3/3	3/3	3/3	2/2	2/2
	Recipients	28/28	22/22	54/55	3/3	20/20	9/9
	Pairs	41/41	28/28	102/103	7/7	27/27	16/16
BT25	Tests	0/678	0/76	0/182	3/18	14/50	26/36
	Donors	0/20	0/12	0/7	2/7	6/8	7/7
	Recipients	0/28	0/9	0/23	2/2	4/5	4/4
	Pairs	0/211	0/22	0/41	4/6	8/10	8/10
BT26	Tests	0/116	0/450	0/188	9/23	3/46	18/32
	Donors	0/8	0/13	0/7	3/3	3/7	6/6
	Recipients	0/13	0/22	0/23	1/1	3/4	4/4
	Pairs	0/46	0/184	0/38	3/3	4/7	7/8
FIRN	Tests	0/238	0/188	0/1550	35/68	80/156	121/130
	Donors	0/23	0/23	0/32	18/22	21/22	22/22
	Recipients	0/10	0/7	0/55	2/2	4/4	4/4
	Pairs	0/79	0/64	0/581	13/16	28/36	43/50
BT27	Tests	15/24	12/20	34/64	0/32	8/30	13/20
	Donors	2/2	2/2	2/2	0/2	2/2	2/2
	Recipients	8/10	5/7	16/22	0/3	4/6	4/4
	Pairs	5/7	6/6	13/18	0/6	5/9	3/4
'BT'25hi	Tests	49/60	25/38	86/124	19/26	0/676	17/28
	Donors	4/4	4/4	3/3	3/4	0/18	3/3
	Recipients	10/11	8/8	21/22	3/3	0/20	4/4
	Pairs	18/18	12/12	29/32	4/4	0/313	7/7
BT9	Tests	30/36	27/28	82/116	10/12	18/32	71/178
	Donors	3/3	3/3	3/3	3/3	3/3	9/10
	Recipients	8/8	5/5	22/22	2/2	4/4	9/9
	Pairs	10/11	10/10	24/24	3/4	7/8	31/59
BT10	Tests	2/2	2/4	4/4	2/2	4/4	4/4
	Donors	1/1	1/1	1/1	1/1	1/1	1/1
	Recipients	1/1	2/2	2/2	1/1	2/2	2/2
	Pairs	1/1	2/2	2/2	1/1	2/2	2/2
None (no phage control)	Tests	0/80	0/58	0/212	0/19	0/66	0/42
	Recipients	0/28	0/22	0/55	0/3	0/20	0/9

* The number of 'pairs' of strains crossed was the number of combinations of different strains, regardless of which was donor or recipient; thus, transductions (A × B) and (B × A) were counted as for a single 'pair' cross.

already known to be effective recipients of *inl*, the absence of Inl$^+$ recombinants in these latter inter-biotype crosses was most significant. On the other hand, although we made fewer tests and assessed fewer combinations of pairs, Inl$^+$ recombinants were produced at high frequency in reciprocal crosses between strains from biotypes 25, 26 and FIRN and those from Inl$^-$ biogroups 25hi, 27, 9 and 10.

(*b*) *Biotype 27*. The three Inl$^-$ strains of this biogroup did not give Inl$^+$ recombinants in crosses with one another, but did so both as donors and recipients with strains from all other Inl$^-$ biogroups.

(*c*) *Biotype 25hi*. Among 20 strains of phage type 99 from this biogroup, we performed 676 tests representing 313 donor–recipient pair crosses, but did not detect Inl$^+$ recombinants. We did so, however, when these same strains were crossed with strains from other Inl$^-$ biogroups (Table 2).

(*d*) *Biotype 9*. Reciprocal crosses between strains of biotype 9 and those of other Inl$^-$ biogroups were usually fertile (Table 2). So too were crosses among different strains of biotype 9: in 71 of 178 tests and in 31 of 59 donor–recipient pairs tested. This was the only Inl$^-$ biogroup, the members of which were fertile giving Inl$^+$ recombinants in intrabiotype crosses.

(*e*) *Biotype 10*. The single representative of biotype 10 was tested only as a donor and gave Inl$^+$ recombinants in crosses with the other six biogroups (Table 2). It was not tested as a recipient because it gave spontaneous Inl$^+$ mutants in 48–72 h.

(iii) *Characterization of Inl$^+$ transductants*

The isolated clones of Inl$^+$ transductants retained the other biotype characters of the recipient strain. Thus, the Inl$^+$ transductants from recipients of FIRN biotypes had the phenotype, Fim$^-$Inl$^+$Rha$^-$Bxyl$^-$, a rare combination in wild type strains found only in the two FIRN-like members from biotypes 21 and 23 (see Fig. 1). The biotypes of the Inl$^+$ recombinants isolated from recipient strains of biotypes 9, 10, 25, 26 and 27 were, respectively, 1, 2, 17, 18 and 19.

4. DISCUSSION

Strains of the FIRN biogroup (Fim$^-$Inl$^-$Rha$^-$Bxyl$^-$), constituting all members of biotypes 29, 30, 31 and 32, comprised 13% of the 2030 naturally occurring cultures biotyped by Duguid *et al.* (1975). Previous transductional studies suggested that the site of the *fim* mutation and that of the *rha* mutation were in each case identical, or less probably overlapping, in all FIRN strains (Duguid *et al.* 1962; Old, 1963; Morgenroth & Duguid, 1968; Old & Duguid, 1979). Further evidence that FIRN strains have a common evolutionary origin emerges from the present study which suggested that they are also homologous in the site of their *inl* mutation. These findings support the hypothesis that all FIRN strains have been derived from a single FIRN ancestral clone that diversified in secondary biotype characters and phage types (Duguid *et al.* 1975; Anderson *et al.* 1978; Old & Duguid, 1979).

The suggestion by Morgenroth & Duguid (1968) that FIRN strains in turn had descended from a strain of biotype 25 (Fim$^+$Inl$^-$ Rha$^+$Bxyl$^-$) by successive mutations at two sites in the *rha* region and one or more in the *fim* region (see Fig. 1) received confirmation from the finding that the majority of strains from the FIRN biogroup and those from biotypes 25 and 26 were phenotypically alike, i.e. they had a temperature-sensitive inositol-fermenting character (Old, 1972).

We included in our study representative strains from these biotypes (see Table 1) that had the secondary biotype character '*i*' (i.e. inability to ferment inositol at 25 °C); they did not behave differently from the majority of strains from biotypes 25, 26 and FIRN in which the temperature-sensitive inositol function had been conserved. Other experiments have suggested that strains of biotypes 25, 26 and FIRN with the secondary biotype character '*i*' have the original mutation responsible for the inositol temperature-sensitive phenotype in the majority of strains and a further masking mutation in *inl* (D. C. Old & Ruth M. Barker, unpublished results). Thus, both biotyping and genetic data are consistent with the idea that the *inl* mutations in strains from biotypes 25, 26 and FIRN are identical and that FIRN strains have descended from an ancestral strain of biotype 25 as outlined in Fig. 1.

Although Inl$^+$ recombinants had not been found in crosses among Inl$^-$ strains from biotypes 25, 26 and FIRN, they were found in crosses between these strains and those from other Inl$^-$, but unrelated, biogroups (namely 9, 10, 25hi and 27) suggesting that the site of the *inl* mutation in the former differed from those in the latter biogroups. Although the phenotypic primary characters, Fim$^+$, Inl$^-$, Rha$^+$, and Bxyl$^-$, are common to strains of biotypes (i) 25 and 26, (ii) 25hi and (iii) 27, the sites of the *inl* mutations in these three groups must be non-identical for crosses between members of any of the three groups were fertile (Table 2). We have provisionally suggested that each of these three Inl$^-$ lines was derived independently from an ancestral clone such as biotype 17 (Fim$^+$Inl$^+$Rha$^+$ Bxyl$^-$) as shown in Fig. 1.

Among the 2030 strains of *S. typhimurium*, there were only three members of biotype 27 (excluding those of BT27hi/PT99), and these apparently did not establish themselves as epidemic strains. On the other hand, cultures of phage type 99, i.e. members of the biogroup 25hi or 27hi which are characteristically associated with avian hosts, are representatives of a clone that has established itself on a world-wide basis (Anderson *et al.* 1978). Our finding that they did not yield Inl$^+$ recombinants in the many intrabiogroup crosses performed supports the suggestion that they are probably members of a single strain that during its establishment has diversified in biotype characters.

The strains of biotype 9 (Fim$^+$Inl$^-$Rha$^+$Bxyl$^+$) are thought to be distantly related in phylogenetic terms to other Inl$^-$ biogroups (Duguid *et al.* 1975), and so it was not unexpected to find that their *inl* mutations were different from those in the Inl$^-$Bxyl$^-$ groups 25(26), 25hi, 27 and FIRN. The finding that Inl$^+$ recombinants were produced in many crosses between different naturally occurring strains of biotype 9 was evidence that the intragenic sites of the *inl* mutations

were different in the different members that were fertile in intrabiotype 9 crosses. We have not determined how many different independent *inl* mutations there are among the biotype 9 strains, particularly because, unlike the nine strains that we tested as recipients, some other biotype 9 strains were unstable in their *inl* mutation and gave spontaneous Inl$^+$ mutants in 48–72 h. Since many of the biotype 9 strains differed in phage type, biotype and source (Duguid *et al.* 1975; Anderson *et al.* 1978) they probably arose independently by *inl* mutations from different biotype 1 ancestors (see, for example, the origin of the clone BT9f/PT141 from BT1f/PT141; Barker & Old, 1979).

The phenotypic characters of the Inl$^-$ biotypes 9 and 10 (Fim$^+$Inl$^-$Rha$^+$Bxyl$^+$) are similar but it is clear that their *inl* mutations are independent (Table 2). Epidemiological evidence (Barker *et al.* 1980) suggested the derivation of the biotype 10 from biotype 2 and this has been indicated in Fig. 1.

The most likely derivations of strains of biotypes 25 (or 26), 25 (or 27) hi and 27 would have been from a strain of biotype 17 (Fim$^+$ Inl$^+$Rha$^+$Bxyl$^-$). Genetic evidence for such a relationship is not yet available, but characterization of a series of strains of *S. typhimurium* for their D-xylose enzyme activities (Mortlock & Old, 1979), showed that strains from 11 different Bxyl$^-$ biotypes behaved similarly (but differently from Bxyl$^+$ strains) and these findings are in agreement with the hypothesis of the derivation of all Bxyl$^-$ biotypes from a biotype 17 ancestral clone (Old & Mortlock, 1979).

The transduction method, therefore, has been useful in tracing and clarifying further parts of the genealogy of the biotypes of naturally occurring strains of *S. typhimurium*.

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