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Demonstration of different mutational sites controlling rhamnose fermentation in FIRN and non-FIRN rha⁻ strains of *Salmonella typhimurium*: an essay in bacterial archaeology

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1. INTRODUCTION

Strains of *Salmonella typhimurium* isolated from natural sources have been differentiated into a number of fermentation types, or 'biotypes', according to their ability to ferment rhamnose, inositol, xylose, glycerol, *d*-tartrate, *i*-tartrate, *l*-tartrate and citrate (Kristensen, Bojlén & Faarup, 1937; Harhoff, 1948; Kauffmann, 1954; Kallings & Laurell, 1957). Another differential character is fimbriation, i.e. the ability to form the non-flagellar filamentous appendages, fimbriae, described by Houwink & Van Iterson (1950), Brinton, Buzzell & Lauffer (1954) and Duguid, Smith, Dempster & Edmunds (1955).

In a series of 775 wild-type strains of *S. typhimurium*, Duguid, Anderson & Campbell (1966) found a correlation between the characters of fimbriation and rhamnose fermentation. Out of 669 strains that were rha⁺, i.e. fermented rhamnose in peptone water within 24 h at 37 °C, 663 were also fim⁺, i.e. genotypically fimbriate; 335 of the rha⁺ strains, including four that were fim⁻, were also inl⁺, i.e. fermented inositol. On the other hand, 101 out of 106 rha⁻ strains were fim⁻; 100 of these were fim⁻ inl⁻ rha⁻, and such strains are designated 'FIRN' (fimbriation, inositol and rhamnose negative). Although they are thus correlated in their occurrence, the fim⁺ and rha⁺ characters are transferred independently in phage transductions (Duguid, Old & Hume, 1962; Old, 1963) and col-factor-mediated conjugations (Subbaiah & Stocker, 1962; Stocker, Smith & Subbaiah, 1963, cited from Sanderson & Demerec, 1965) and map in different positions on the chromosome.

Considerable epidemiological interest attaches to the question of whether the sites of a mutation in a phenotypically homogeneous group of strains isolated from natural sources are identical or different. If they are identical, the strains probably have a common origin and have descended from a single ancestral bacterium in which the mutation took place. If the sites are not identical, the strains probably have descended from different ancestral bacteria that underwent independent mutations, and so are unlikely to be closely related to one another. Evidence indicating whether the sites of a mutation in two phenotypically similar strains are different or identical may be obtained by the observation of whether or not the

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strains recombine to yield the wild type when they are crossed by generalized transduction with phage. Demerec *et al.* (1954) and Hartman (1956), in studies of galactose-negative or auxotrophic mutants of *S. typhimurium* strains LT2 and LT7, found that most pairs of strains with mutations in the same gene locus yielded galactose-positive or prototrophic recombinants when crossed by transduction. Their results showed that independently produced mutations generally give rise to non-identical alleles and that non-identical alleles are generally distinguishable by their ability to recombine in transductions.

The transduction method was first used to study the relationships between strains with mutational deficiencies acquired in the wild state, by Stocker & Edgar (1959). All of fifteen wild-type nicotinamide-requiring (nic$^-$) strains of *S. typhimurium* phage-type 2, isolated in Britain, Australia, Italy and South Africa, were found by these workers to produce nic$^+$ recombinants when transduced with phage A3 grown on a nic$^-$ laboratory mutant of *S. typhimurium* strain LT2; none of the fifteen wild-type strains produced nic$^+$ recombinants when transduced with phage propagated on any of seven of these fifteen strains. The results suggested that the 15 nic$^-$ strains had mutations at identical sites and therefore that they had descended either from a common ancestral nic$^-$ mutant bacterium or from different nic$^-$ bacteria produced by independent mutations occurring at a single site specially liable to mutation ('hot spot').

Later, Duguid *et al.* (1962) and Old (1963) used the transduction method in a study of wild-type fim$^-$ and rha$^-$ strains of *S. typhimurium*. They obtained fim$^+$ recombinants in crosses between FIRN and non-FIRN fim$^-$ strains, but not in any cross between different FIRN strains. They concluded that the site of the fim$^-$ mutation was probably identical in all strains of the FIRN group. Old obtained similar findings for the site of the rha$^-$ mutation in the same series of FIRN strains. The combined results suggested that all FIRN strains have descended from a common ancestral FIRN bacterium.

The present paper describes the results of attempts to obtain rha$^+$ recombinants by transduction in 23 FIRN (fim$^-$ inl$^-$ rha$^-$) and 14 non-FIRN rha$^-$ (fim$^+$ inl$^+$ rha$^-$) wild-type strains of *S. typhimurium*. Most of the strains, twenty-one FIRN and nine non-FIRN rha$^-$, were different from those examined by Duguid *et al.* and Old. The purpose of the work was to determine whether the non-FIRN rha$^-$ group of strains resembles the FIRN group in being homogeneous in respect of the sites of their rha$^-$ mutations, and thus whether the non-FIRN rha$^-$ strains, which were isolated in Britain, America and Australia, are likely to have descended from a common ancestor. Observations were also made on the patterns of rha$^+$ mutation in the two groups.

2. MATERIALS AND METHODS

(i) *Strains*

We studied twenty-three FIRN and fourteen non-FIRN rha$^-$ strains of *Salmonella typhimurium* isolated from different sources. They were selected from a series under examination for biotype and phage-type by one of us (J.P.D.) and

Dr E. S. Anderson. Their particulars are shown in Table 1. Boyd's (1956) strain Q 1 (our SL 375) was used both as phage indicator and as wild-type rha$^+$ donor strain. We also examined three weakly rhamnose-fermenting (rha^w) mutant strains derived in different experiments from FIRN strain 2306.

Table 1. *Strains of* Salmonella typhimurium *used as donors and recipients in the transduction experiments*

Group of strains	Strain no.	Source	Place of origin	Date of isolation	Phage-type	Biotype
Non-FIRN rha$^-$ (fim$^+$ inl$^+$ rha$^-$)	1179	Man	Australia	1960	U 128	19Xd
	1180	Man	Australia	1960	27	19Xd
	1541	Man	U.S.A.	1961	26	19Xd
	1992	Man	Australia	1963	26	19Xd
	2002	Man	Australia	1963	26	19Xd
	2037	Man	Australia	1963	26	19Xd
	2212	Man	England	1962	26	19Xd
	2213	Man	England	1962	26	19Xd
	2241	Pig	Wales	1962	25	19Xd
	2323	Man	England	1963	23	19Xd
	2368	Mutton	Australia	1964	26	19Xd
	2382	Foodstuff	England	1964	U 38	19Xd
	6768	Man	England	1958	31	19Xd
	6899	Man	England	1958	U 58	19Xd
FIRN (fim$^-$ inl$^-$ rha$^-$)	706	Guinea-pig	Sweden	1959	U 45	18
	735	Unknown	Unknown	.	1	17
	1289	Man	England	1962	U 41	17
	1294	Man	England	1962	14a	17
	1723	Man	England	1963	14	17
	1804	Unknown	Netherlands	1960	26	17
	1877	Man	England	1964	NT	17
	1879	Man	England	1964	14	17
	1881	Man	England	1964	13	17
	2025	Man	Australia	1963	Un	15
	2205	Fowl	England	1962	14c	18
	2235	Man	England	1962	14b	17
	2306	Horse	England	1963	U 86	16
	2309	Canary	England	1963	29	17
	2321	Coypu	England	1963	26	17
	2322	Turkey	England	1963	U 86	17
	2333	Sheep	England	1963	U 86	17
	2540	Calf	Scotland	1965	U 128	17
	2555	Fowl	England	1965	U 131	17
	2565	Man	England	1965	U 84	16
	2571	Guinea-pig	England	1961	U 18	16
	2578	Man	England	1963	U 97	17
	2581	Bird	England	1964	U 151	17
Rhamnose-positive (fim$^+$ inl$^+$ rha$^+$)	SL 375	(Boyd's strain Q 1)		.	1	6
Rhamnose-weak (fim$^-$ inl$^-$ rha^w)	2306 rha^w-1	(Spontaneous mutants		.	.	.
	2306 rha^w-2	derived from FIRN		.	.	.
	2306 rha^w-3	strain 2306)		.	.	.

Phage-type NT = new type; Un = untypable. Biotype 19Xd is a new biotype; see text.

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The phage-types of the strains were determined at the Enteric Reference Laboratory, Colindale, London. They included twelve of the types 1–31 described by Callow (1959) and eleven types (U 18–U 151) more recently distinguished in the ERL.

The biotypes were determined according to Harhoff's scheme as described by Kallings & Laurell (1957). The FIRN strains belonged to the established biotypes 15, 16, 17 and 18. These four types differ from the others in not fermenting either inositol or rhamnose, and they differ from one another in their reactions with *d*-tartrate, *i*-tartrate, citrate and glycerol. The non-FIRN rha$^-$ strains all belonged to a single biotype that is different from the twenty-one established ones. This new biotype is provisionally designated '19Xd', because it resembles biotype 19 in all characters except that it gives a positive Bitter xylose (X) and a negative *d*-tartrate (d) reaction. The Bitter fermentation test is done by culture in a liquid salts–ammonia medium with a very small content of peptone; a positive reaction is given by strains that ferment the sugar strongly and rapidly, and a negative reaction by weakly fermenting and non-fermenting strains. All the strains and biotypes used were prototrophic and capable of growth on an ammonia–salts minimal medium containing a utilizable source of carbon and energy.

The *transducing phage* was salmonella phage P22 (PLT22, Zinder & Lederberg, 1952), which is known to be a general transducing phage.

(ii) *Culture media*

Nutrient broth was Bacto heart infusion broth (Difco) adjusted to pH 7·2. Nutrient agar was this broth solidified with 1·5% Oxoid agar. In semi-solid agar the concentration of agar was 0·3%.

Rhamnose minimal medium. The basic minimal medium (Davis & Mingioli, 1950) contained: K_2HPO_4, 7 g; KH_2PO_4, 3 g; $(NH_4)_2SO_4$, 1 g; $MgSO_4.7H_2O$, 0·1 g; Oxoid Ionagar No. 2, 15 g; de-ionized water, 1000 ml. A separately sterilized solution of L(+)-rhamnose (British Drug Houses) was added to the basic medium to give a final concentration of 3 g rhamnose per 1000 ml medium.

Enriched rhamnose minimal medium was rhamnose minimal medium plus dehydrated nutrient broth, 0·1 g per 1000 ml.

Rhamnose peptone water used for fermentation tests was Oxoid peptone water plus L(+)-rhamnose, 10 g per 1000 ml., and bromocresol purple as indicator of pH (range 6·8–5·2). The rhamnose was sterilized separately and added to the sterile peptone water. The medium (pH 7·2) was dispensed in 3 ml volumes in 6 ml screw-capped bottles.

(iii) *Culture methods*

Incubation was aerobic and at 37 °C. Plates were plastic Petri dishes 9 cm in diameter containing 20 ml agar medium. Viable counts were done by spreading measured volumes of diluted culture on plates of nutrient agar. The diluting solution was 0·85% (w/v) NaCl to which 3% (v/v) of nutrient broth was added. Overnight (*c.* 18 h) broth cultures for experiments were inoculated from stock

cultures on Dorset egg slopes. Transduction and mutation plates were inoculated with 0·2 ml of phage-treated or untreated broth culture, which was spread as uniformly as possible over the whole surface of the plate and allowed to dry in before the start of incubation.

(iv) *Preparation of donor phages*

Donor phages were propagated by the soft agar layer method of Swanstrom & Adams (1951). About 10^6 phages and 3×10^8 bacteria of the donor strain were mixed in 20 ml melted semi-solid agar at *c.* 46 °C and poured on to five well-dried nutrient agar plates. The plates were incubated at 37 °C and as soon as semi-confluent lysis was visible the five soft agar layers were scraped off and mixed together in 15 ml nutrient broth. The phage was freed from bacteria and agar debris by repeated centrifugation and then sterilized by the addition of 0·4 ml chloroform per 10 ml; the chloroform was removed after 15 min. To avoid errors arising from the persistence of small numbers of unadsorbed wild-type phage virions in the preparations of 'donor phage', the phage was passed a second time through the chosen experimental donor strain of bacteria and only this second lysate was used in transductions as donor phage.

The donor-phage preparations were titrated on lawns of the indicator bacterium SL 375 and each was diluted to a concentration of 2×10^{10} plaque-forming units per ml. Tests for sterility were made at the time of preparation and every 6 weeks thereafter by inoculation into broth and on to enriched rhamnose minimal medium. At the end of the series of experiments the titre of each phage preparation was found to be nearly at its original value.

(v) *Transduction experiments*

An overnight broth culture of the bacterial strain to be tested as recipient was diluted 1 in 50 in fresh broth and then incubated for $3\frac{1}{2}$ h at 37 °C, so that it yielded about $3{\cdot}5 \times 10^8$ log.-phase bacteria per ml. One ml of this recipient culture was mixed with 0·1 ml of the donor phage preparation and the mixture was held for 12 min at 37 °C in a water bath to allow adsorption of the phage. The input ratio of phage virions to bacteria was about 6 to 1. Two 0·2 ml samples of the mixture, which together contained about $1{\cdot}3 \times 10^8$ phage-infected bacteria, were then spread over two plates of enriched rhamnose minimal medium (20 ml/plate). Similar samples of the recipient bacteria, grown in the same way but without the addition of phage, were inoculated on two other plates as controls, to demonstrate the frequency of mutation to rha$^+$. The plates were incubated for 65 h at 37 °C and then scored for colonies of rha$^+$ recombinant or mutant bacteria. The long period of incubation was required because rha$^+$ bacteria grow relatively slowly on enriched rhamnose minimal medium and take about 48 h to form moderate-sized colonies.

Confirmation of the identity of the rha$^+$ bacteria. About 10 % of all rha$^+$ colonies were subcultured on rhamnose minimal medium to confirm the genetic stability

of their acquired rha$^+$ character. A smaller proportion were tested for fermentation of inositol and agglutination with salmonella O 4 serum. Except for a few of them, whose appearance suggested that they were airborne contaminants, all showed the serological character of *S. typhimurium* and the inositol reaction of the recipient strain under test.

Expression of results. The average number of rha$^+$ colonies obtained per two plates in several repeated experiments with the same pair of donor and recipient strains is recorded as the result for the cross of that pair of strains. The value represents the number of rha$^+$ recombinant (or mutant) bacteria obtained per $1{\cdot}3 \times 10^8$ bacteria plated, i.e. the rate of apparent recombination. The mean values for groups of crosses are given as the number of rha$^+$ bacteria (colonies) obtained per one bacterium plated.

(vi) *Determination of the frequency of mutation to rha$^+$*

The frequency of mutation of the rha$^-$ bacteria to the rha$^+$ genotype was observed in the control (no-phage) platings of the recipient bacteria in the transduction experiments and in further similar platings of the same bacteria. About $1{\cdot}3 \times 10^8$ bacteria from a $3\frac{1}{2}$ h broth culture were inoculated per two plates of enriched rhamnose minimal medium and the plates were scored for colonies after 65 h. For comparison with the rate of recombination in the transductional platings, the mutant frequency is stated as the number of rha$^+$ bacteria (colonies) obtained per rha$^-$ bacterium plated. This value is probably considerably greater than the rate of mutation per bacterium per generation. Viable counts showed that the small amount of enriching broth in the minimal medium enabled the $0{\cdot}65 \times 10^8$ rha$^-$ bacteria inoculated on each plate to multiply about 50-fold in the first 24 h of incubation. Since the plates were scored for rha$^+$ colonies after 65 h and since rha$^+$ bacteria can form fair-sized colonies within 48 h on rhamnose minimal medium, it is probable that any rha$^+$ mutants arising from the bacterial population present at 24 h would have formed recognizable colonies within 65 h. The number of rha$^+$ colonies obtained per rha$^-$ bacterium plated at 0 h was therefore divided by 50 to give an estimate of the number of rha$^+$ mutant bacteria produced per rha$^-$ bacterium present at 24 h. This latter rate is taken as possibly approximating to the mutation rate per bacterium per generation.

3. RESULTS

(i) *Distinction of rha$^+$ transduction from rha$^+$ back-mutation*

The occurrence of recombination in transductions between different rha$^-$ strains of *Salmonella typhimurium* was determined by observation of the development of rha$^+$ colonies in platings of the transduction mixture on enriched rhamnose minimal medium. After incubation for 65 h the rha$^+$ colonies were seen in clear contrast against the thin film of growth of parental rha$^-$ bacteria supported by the small amounts of enriching broth contained in the medium and added with the inoculum. Evidence that the colony-forming rha$^+$ bacteria had arisen by

recombination, and not by spontaneous back-mutation, was provided by the demonstration that the recipient-strain bacteria either did not mutate to the rha$^+$ genotype in control platings without phage or did so only at a rate too low to account for the number of colonies obtained in the transduction platings. Since all the twenty-three FIRN and fourteen non-FIRN rha$^-$ strains were able to grow well on minimal medium containing glucose instead of, or in addition to, rhamnose, the absence of growth in transduction or control platings on the rhamnose medium could be attributed to absence of recombinant or mutant bacteria capable of utilizing rhamnose (i.e. rha$^+$).

Table 2. *Frequency of rha$^+$ mutants in control (no phage) platings of 14 non-FIRN rha$^-$ and 23 FIRN strains of* Salmonella typhimurium *on enriched rhamnose minimal medium*

Strain(s) plated	No. of platings of $1{\cdot}3 \times 10^8$ bacteria	No. of rha$^+$ colonies obtained in all platings	Mutant frequency expressed as no. of rha$^+$ colonies per bacterium plated
Non-FIRN rha$^-$			
1179	60	2	$2{\cdot}6 \times 10^{-10}$
1180	24	1	$3{\cdot}2 \times 10^{-10}$
1541	20	2	$7{\cdot}7 \times 10^{-10}$
1992	20	2	$7{\cdot}7 \times 10^{-10}$
2002	28	1	$2{\cdot}8 \times 10^{-10}$
2037	60	0	$< 1{\cdot}3 \times 10^{-10}$
2212	20	1	$3{\cdot}8 \times 10^{-10}$
2213	36	1	$2{\cdot}1 \times 10^{-10}$
2241	45	4	$6{\cdot}8 \times 10^{-10}$
2323	24	2	$6{\cdot}4 \times 10^{-10}$
2368	58	1	$1{\cdot}3 \times 10^{-10}$
2382	20	1	$3{\cdot}8 \times 10^{-10}$
6768	34	2	$4{\cdot}5 \times 10^{-10}$
6899	20	2	$7{\cdot}7 \times 10^{-10}$
All 14 strains	469	22	$3{\cdot}6 \times 10^{-10}$
FIRN			
2306	38	0	$< 2{\cdot}0 \times 10^{-10}$
2309	30	0	$< 2{\cdot}6 \times 10^{-10}$
Each other strain	20	0	$< 3{\cdot}8 \times 10^{-10}$
All 23 strains	488	0	$< 1{\cdot}6 \times 10^{-11}$

(ii) *Frequency of mutation to rha$^+$*

Non-FIRN rha$^-$ strains. This group of fourteen strains, all of which were fim$^+$ inl$^+$ rha$^-$ and biotype 19Xd, were tested in a total of 469 double-platings of $1{\cdot}3 \times 10^8$ bacteria on enriched rhamnose minimal medium, each strain in 20–60 such platings. All but one strain, no. 2037, produced at least one colony of rha$^+$ mutant bacteria, and a total of twenty-two such mutants were obtained from the total of *c.* $6{\cdot}1 \times 10^{10}$ rha$^-$ bacteria plated (Table 2). In individual strains, excluding no. 2037, the number of mutants produced ranged from $1{\cdot}3 \times 10^{-10}$ to $7{\cdot}7 \times 10^{-10}$

per bacterium plated, and the average rate for the 14 strains was $3{\cdot}6 \times 10^{-10}$ per bacterium plated. Since the number of bacteria present on the plate after the first 24 h of incubation was about 50 times the number plated, the average mutant frequency per bacterium present at 24 h was about $7{\cdot}2 \times 10^{-12}$; this value may approximate to the mutation rate per bacterium per generation (see Materials and Methods).

Table 3. *Transduction of the rha⁺ character to non-FIRN rha⁻ and FIRN strains of* Salmonella typhimurium *with phage propagated on the rha⁺ strain SL375 as donor*

Recipient strain, group and no.	No. of rha⁺ colonies obtained per $1{\cdot}3 \times 10^8$ bacteria plated*	Mean transduction rate for group, as no. of rha⁺ recombinants: Per bacterium plated	Per donor phage virion
Non-FIRN rha⁻			
1992	17		
2002	22		
2037	22		
2212	15		
2213	17	$1{\cdot}4 \times 10^{-7}$	$2{\cdot}6 \times 10^{-8}$
2241	19		
2323	14		
2368	17		
2382	17		
FIRN			
1879	104		
1881	97		
2306	221	$1{\cdot}5 \times 10^{-6}$	$2{\cdot}8 \times 10^{-7}$
2309	231		
2322	314		

* Number of rha⁺ colonies obtained on two plates of enriched rhamnose minimal medium plated with $1{\cdot}3 \times 10^8$ bacteria infected with 7×10^8 phage virions. Results for strains 2037, 2241 and 2322 are means for two double-platings.

FIRN strains. This group of twenty-three fim⁻ inl⁻ rha⁻ strains were tested in 488 double-platings, each strain in at least twenty double-platings. The mutational behaviour of the group was clearly different from that of the non-FIRN rha⁻ group. Not a single rha⁺ mutant was obtained from any strain (Table 2). The aggregate results show that the frequency of rha⁺ mutants was less than $1{\cdot}6 \times 10^{-11}$ per bacterium plated, i.e. less than $3{\cdot}2 \times 10^{-13}$ per bacterium present at 24 h. The absence, or extremely low frequency, of rha⁺ mutation in the FIRN group was confirmed by our failure to obtain any rha⁺ mutants in similar platings, incubated for 5–7 days, of another 150 FIRN strains isolated from different sources.

(iii) *Transduction from a wild-type rha⁺ strain as donor*

The efficiency of the transducing system was tested in experiments in which nine non-FIRN rha⁻ and five FIRN strains were transduced with phage that had

been propagated on the inl$^+$ rha$^+$ strain SL 375 as donor. Rha$^+$ recombinants were obtained from each of the recipients, the rate being consistently about ten-fold higher in the FIRN than in the non-FIRN rha$^-$ strains (Table 3). The rate of production of rha$^+$ bacteria per phage-infected bacterium plated, even in the strain (2323) with the lowest rate ($1{\cdot}1 \times 10^{-7}$), was much higher than the highest rate of production of rha$^+$ mutants observed in the control platings of that or any other strain ($7{\cdot}7 \times 10^{-10}$ in strains 1541, 1992 and 6899 Table 2). It is concluded therefore, that most of the rha$^+$ bacteria produced in the platings with phage were transductional recombinants and not spontaneous mutants.

A number of the rha$^+$ transductant colonies were picked from the platings of the FIRN recipients and pure subcultures of all of them were found to have the inl$^-$ character of the recipient strain together with the rha$^+$ character from the donor. This finding shows that the inl$^+$ and rha$^+$ characters of the donor were not transduced jointly and that the rha$^+$ colonies did not arise from donor-strain bacteria that had survived the treatment of the phage preparation with chloroform.

(iv) *Transduction from rha$^-$ donors to rha$^-$ recipients*

Phages propagated on 12 non-FIRN rha$^-$ and 7 FIRN strains as donors were tested with 14 non-FIRN rha$^-$ and 23 FIRN strains as recipients in a total of 1075 transduction experiments. The results, as the mean number of rha$^+$ colonies obtained in the different tests of each cross, are shown in Table 4. The means of the rates of apparent transduction per plated bacterium observed for the different pairs of strains in each of the four classes of cross, non-FIRN rha$^-$ to non-FIRN rha$^-$, FIRN to non-FIRN rha$^-$, FIRN to FIRN, and non-FIRN rha$^-$ to FIRN, are shown in Table 5.

Crosses of non-FIRN rha$^-$ with FIRN. Every strain in the non-FIRN rha$^-$ and FIRN groups produced rha$^+$ bacteria, presumed to be recombinants, in at least some of its crossings with members of the other group; most strains did so in a majority of such crossings (Table 4). Each of the 14 non-FIRN rha$^-$ strains tested as recipients gave rha$^+$ bacteria in crosses with at least 2 FIRN strains as donors, and each of the 23 FIRN strains tested as recipients gave rha$^+$ bacteria in crosses with at least 1 non-FIRN rha$^-$ strain as donor. Conversely, each of 12 non-FIRN rha$^-$ strains tested as donors gave rha$^+$ bacteria from at least 1 FIRN recipient and each of 7 FIRN strains tested as donors gave rha$^+$ bacteria from at least 1 non-FIRN rha$^-$ recipient.

It is thought that most or all of the rha$^+$ bacteria obtained in these crosses were recombinants, and not mutants, because the average rate of their production in the transduction platings of each recipient strain was considerably greater than the average rate of production of rha$^+$ mutants in the control platings of the strain (cf. Tables 2 and 4). The difference between the transduction rate and the mutant frequency was much greater in the crosses in which the recipient was FIRN than in those in which it was non-FIRN rha$^-$. The FIRN recipients gave the higher rates of transduction (every strain $> 7{\cdot}0 \times 10^{-9}$ per bacterium plated, most strains *c.* $1{\cdot}0 \times 10^{-7}$) and the lower frequencies of mutation (every strain $< 3{\cdot}8 \times 10^{-10}$ per

Table 4. *Production of rha⁺ recombinants in transductions made to different rha⁻ strains of* Salmonella typhimurium *as recipients with phage propagated on different rha⁻ strains of* S. typhimurium *as donors*

Recipient strain	Mean no. of rha⁺ colonies obtained per $1{\cdot}3 \times 10^8$ recipient bacteria plated when the donor strain was: non-FIRN rha⁻												FIRN						
	1179	1180	1541	1992	2002	2037	2212	2241	2323	2368	6768	6899	706	735	2306	2309	2571	2578	2581
Non-FIRN rha⁻																			
1179	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	2
1180	0	0	0	0	0	0	0	0	0·5	0	0	1	6	1	4	4	17	27	3
1541	0	0	0	0	0	0	1	0	0·5	0	0	0	1	0	0	0	0	2	0
1992	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0
2002	0	0	0	0	0·5	0	0	0	0	0	1	0	0	1	0	2	0	1	0
2037	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	2	1	0
2212	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	1	1
2213	0	0	0	0	0	0	0	0	0·5	0	0	0	0	0	0	0	1	1	1
2241	0·5	0·5	1	0	0	0	0	0·5	0	0	0	0	0	1	0	0	1	3	1
2323	0	0	0	0	0·5	0	0·5	0	0	0	0	0	0	0	0	0	0	2	1
2368	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	1	1
2382	0	0	0	0	1	0	0	0	0	0	0	0	1	1	0	0	2	1	0
6768	0	0	0	0	0·5	0	0	0	0	0	0	0	1	0	0	0	0	2	0
6899	0	0	1	0	0	0	1	0	0	0	0	0	0	1	0	1	1	1	0
FIRN																			
706	6	0·5	13	10	9	10	18	7	2	7	3	2	0	0	.	.	.	.	0
735	21	0	11	16	17	32	20	51	2	6	17	34	.	.	0	.	0	0	.
1289	30	0	3	12	22	16	16	47	2	4	6	31	.	.	0	.	0	0	.
1294	2	0	3	5	8	6	13	16	1	6	4	1	0	0	.	.	.	.	0
1723	.	1	.	.	14	.	.	.	.	.	.	.	0	0	.	.	.	.	0
1804	6	0	8	26	36	39	45	18	5	26	8	3	0	0	.	.	.	.	0
1877	.	4	.	.	19	.	.	.	.	.	.	.	0	0	.	.	.	.	0
1879	6	0·5	5	12	26	23	33	13	5	10	9	4	.	.	0	.	0	0	.
1881	5	0·3	6	31	29	37	27	19	5	17	13	2	.	.	0	.	0	0	.
2025	.	0	.	.	11	.	.	.	.	.	.	.	0	0	.	.	.	.	0
2205	12	1	2	21	43	31	41	26	9	21	21	6	0	0	.	.	.	.	0
2235	2	0	2	5	13	8	14	15	0	15	6	5	0	0	.	.	.	.	0
2306	24	0·3	8	15	28	23	29	90	0·2	6	16	38	0	0	0	0	0	0	0
2309	6	0·5	2	15	36	23	27	28	3	15	12	6	.	.	0	.	0	0	.
2321	.	0	.	.	39	.	.	.	.	.	.	.	0	0	.	.	.	.	0
2322	25	1	9	16	30	13	33	61	3	7	13	18	.	.	0	.	0	0	.
2333	12	0·5	6	14	17	21	13	78	2	4	19	30	0	0	.	.	.	.	0
2540	22	0·5	5	17	23	28	20	62	3	12	17	29	0	0	.	.	.	.	0
2555	8	0	6	23	21	28	26	22	4	13	10	4	0	0	0	0	0	0	0
2565	2	1	0·5	1	5	0	4	4	4	1	1	0	.	.	0	.	0	0	.
2571	1	0·5	0	0	3	0	0·5	4	2	0	0·5	0	.	.	0	.	0	0	.
2578	3	0	2	7	17	8	13	9	3	13	8	2	.	.	0	0	0	0	.
2581	7	1	2	5	18	24	17	15	4	10	4	5	0	0	.	.	.	.	0

The fractional results 0·5, 0·3 and 0·2 are average values in crosses in which one rha⁺ recombinant was obtained in, respectively, 2, 3 or 4 platings of $1{\cdot}3 \times 10^8$ bacteria. Average values greater than one are rounded off to the nearest whole number.

bacterium plated). Most of the non-FIRN rha$^-$ recipients gave a transduction rate of *c.* $4{\cdot}0 \times 10^{-9}$ and a mutant frequency of *c.* $4{\cdot}0 \times 10^{-10}$, and so showed a difference of only about ten-fold between the two rates. The lowest inter-group transduction rate obtained with any recipient strain was $2{\cdot}2 \times 10^{-9}$ (non-FIRN rha$^-$ strain 1992), yet even this minimum transduction rate was substantially higher than the highest mutant frequency shown by any strain ($7{\cdot}7 \times 10^{-10}$, strains 1541, 1992 and 6899). If the conclusion is accepted that most of the rha$^+$ bacteria produced in each cross were recombinants, the findings are evidence that any non-FIRN rha$^-$ strain is capable of giving rha$^+$ recombinants when crossed with any FIRN strain and, therefore, that the sites of the rha$^-$ mutations in the non-FIRN rha$^-$ strains are non-identical and non-overlapping with the sites of the rha$^-$ mutations in the FIRN strains.

Table 5. *Rates of apparent transduction of rha$^+$ in four classes of crosses of FIRN and non-FIRN rha$^-$ strains compared with the rates of production of rha$^+$ mutants in control platings of the recipient strains*

(The transduction values are calculated from the aggregated results for the different pairs of strains in each class of cross (Table 4) and the mutation values from the aggregated results for the different recipient strains (Table 2).)

Class of cross		Mean no. of rha$^+$ bacteria obtained	
		Per bacterium plated	Per donor phage virion
Donor strain	Recipient strain		
Non-FIRN rha$^-$	FIRN	$1{\cdot}0 \times 10^{-7}$	$1{\cdot}7 \times 10^{-8}$
FIRN	FIRN	$< 9{\cdot}9 \times 10^{-11}$	$< 1{\cdot}7 \times 10^{-11}$
None (control)	FIRN	$< 1{\cdot}6 \times 10^{-11}$	.
FIRN	Non-FIRN rha$^-$	$8{\cdot}9 \times 10^{-9}$	$1{\cdot}6 \times 10^{-9}$
Non-FIRN rha$^-$	Non-FIRN rha$^-$	$5{\cdot}5 \times 10^{-10}$	$1{\cdot}0 \times 10^{-10}$
None (control)	Non-FIRN rha$^-$	$3{\cdot}6 \times 10^{-10}$	.

The rate of production of rha$^+$ transductants in the FIRN × non-FIRN rha$^-$ crosses was only about a fifteenth as great as that in the crosses of the same FIRN and non-FIRN rha$^-$ strains as recipients with the rha$^+$ strain SL 375 as donor (cf. Tables 3, 4). A likely explanation of this difference is that in 14 out of 15 occasions of transduction in the FIRN × non-FIRN rha$^-$ crosses the rha$^-$ site (or sites) of the donor was incorporated into the recipient's genome along with the positive allele corresponding to the rha$^-$ site of the recipient. We have no explanation of why the rha$^+$ transduction rates were generally about ten-fold higher in the crosses with FIRN recipients, than in those with non-FIRN rha$^-$ ones. In the class of cross, non-FIRN rha$^-$ donor × FIRN recipient, there was a marked variation in the yield of rha$^+$ recombinants from the different pairs of strains (Table 4). This variation may be attributable in part to some of the strains being lysogenic for phages homologous or related to the transducing phage, and to differences between donor and recipient bacteria in respect of the restriction-modification character.

Crosses of FIRN with FIRN. Rha$^+$ recombinants were not obtained in any cross between 78 different pairs of strains selected from 7 FIRN strains as donors and 23

FIRN strains as recipients (Table 4). This uniformly negative finding, which confirms that of Old (1963), suggests that the rha$^-$ mutation is located at the same site or at overlapping sites in all FIRN strains.

Crosses of non-FIRN rha$^-$ with non-FIRN rha$^-$. Single rha$^+$ bacteria were obtained in a few (17 out of 168) of the crosses made between 12 non-FIRN rha$^-$ strains as donors and 14 non-FIRN rha$^-$ strains as recipients (Table 4). These rha$^+$ bacteria probably arose by spontaneous mutation in the recipient-strain bacteria, not by recombination, because the frequency of their production (average for group: $5{\cdot}5 \times 10^{-10}$ per bacterium plated) was not significantly greater than the frequency of production of rha$^+$ mutant bacteria in the control platings of the recipient strains (average $3{\cdot}6 \times 10^{-10}$). Although the non-FIRN rha$^-$ strains were less competent as recipients of rha$^+$ than the FIRN strains, they gave rha$^+$ recombinants at a fairly high rate ($1{\cdot}1$–$1{\cdot}7 \times 10^{-7}$) in crosses with the donor strain SL 375 (Table 3). The level of their general competence as recipients therefore does not appear to be low enough to account for the extreme infrequency of rha$^+$ bacteria in their crosses with non-FIRN rha$^-$ donors. It may be concluded, rather, that rha$^+$ recombination is impossible in crosses between any two strains in the non-FIRN rha$^-$ group because the rha$^-$ mutation is located at the same site or at overlapping sites in all members of the group.

(v) *Rhamnose-weak (rha^{-w}) mutation*

FIRN rha^w mutants. Although FIRN bacteria do not give rise to colonies of rha$^+$ mutants when plated on enriched or unenriched rhamnose minimal medium, they give rise to 'weakly' rhamnose-fermenting (rha^w) mutants which grow out selectively during prolonged culture in rhamnose peptone water. The FIRN strains invariably failed to ferment rhamnose in peptone water within 24 h at 37 °C.

Table 6. *Times of acid production in cultures of FIRN strain 2306 and non-FIRN rha$^-$ strain 2213 grown from inocula of 1 or 2 bacteria in 3 ml volumes of rhamnose peptone water at 37 °C*

	No. of cultures producing acid within stated time of incubation			No. of cultures producing acid within stated time of incubation	
Time of incubation (days)	Strain 2306 (40 cultures tested)	Strain 2213 (60 cultures tested)	Time of incubation (days)	Strain 2306 (40 cultures tested)	Strain 2213 (60 cultures tested)
1	0	0	7	29	9
1·5	0	1	8	33	12
2	0	1	9	34	14
2·5	0	2	10	34	16
3	1	2	12	35	16
3·5	6	3	14	36	16
4	12	3	16	38	16
4·5	14	3	18	.	20
5	19	5	20	.	20
6	27	7			

Every strain, however, gave 'late' fermentation when incubation was prolonged for a sufficient period, which varied from 3 to 40 days. The time of fermentation differed widely even in repeated tests of the same strain. For example, 40 cultures of FIRN strain 2306 were grown in bottles of 3 ml rhamnose peptone water from inocula of one or two bacteria taken from the same 10^7-fold diluted 20 h broth culture and were observed daily for 16 days. Acid was produced by one of the 40 cultures in 3 days, by 19 within 5 days and by 38 within 16 days (Table 6).

The late fermentation of rhamnose in peptone water was due to the production and selective outgrowth of rha^w mutant bacteria. Examination of the colonies obtained in platings on nutrient agar showed that rha^w bacteria made up about 50 % of the population in the fermented cultures, but were absent from the unfermented ones of the same age. The rha^w colonies were recognized because pure subcultures from them gave fermentation in rhamnose peptone water within 24 h. In tests inoculated with about 10^7 rha^w bacteria the times taken by the mutants of different FIRN strains to produce acid varied from 10 to 24 h. In similar tests, different wild-type and mutant rha$^+$ strains produced acid in periods of from 4 to 9 h. The time taken for fermentation by rha^w and rha$^+$ bacteria also varied with the size of the inoculum (Table 7).

Table 7. *Times of acid production in cultures of rha$^+$ strain SL375 and rhamnose-weak strain 2306 rha^w-1 grown from different-sized inocula in 3 ml volumes of rhamnose peptone water at 37 °C*

No. of viable bacteria inoculated	Time (h) of acid production by	
	SL375 (rha$^+$)	2306 rha^w-1
10^8	3·5	10
10^7	4·5	13
10^6	5·5	13
10^5	6·5	15·5
10^4	8	19·5
10^3	10·5	21
10^2	11·5	22
10^1	13	24–36

If it is assumed that the generally observed failure of a small proportion of the cultures of a FIRN strain in rhamnose peptone water to give late fermentation is due to the failure of these cultures to produce even a single rha^w mutant bacterium it may be concluded that the rate of mutation of FIRN bacteria to the rha^w form was not much greater than about 3×10^{-10} per bacterium per generation. This calculation is based on the observation that an unfermented culture of FIRN bacteria in 3 ml rhamnose peptone water yields, in about 48 h, a maximum viable population of about 10^9 per ml.

The ability of the rha^w bacteria to ferment rhamnose in peptone water was associated with their having the ability to utilize rhamnose for growth in this medium. The viable counts and turbidity values of rha^w cultures grown for 24 h in rhamnose peptone water were 2–3-fold higher than those of cultures grown in the

same medium without rhamnose. This ability to utilize rhamnose as an extra source of energy was presumably the reason why the small number of rha^{w} bacteria that originated by mutation in cultures of FIRN bacteria in rhamnose peptone water were able selectively to outgrow the rha^{-} parental bacteria. Surprisingly, however, the rha^{w} bacteria were unable to utilize rhamnose for growth on minimal agar media. Cultures grown for 24 h on enriched rhamnose minimal medium showed little if any greater multiplication of bacteria than that, *c.* 50-fold, observed in parallel cultures grown on the same medium without rhamnose. As well as thus differing from rha^{+} bacteria in giving slower fermentation of rhamnose in peptone water and failing to utilize rhamnose in minimal medium, the rha^{w} bacteria also differed from rha^{+} bacteria in not giving a positive reaction in Bitter rhamnose medium.

Rha^{w} to rha^{+} mutation. The rha^{w} mutants of all FIRN strains were capable of undergoing a further mutation to the strongly rhamnose-fermenting rha^{+} genotype. A majority of their platings on enriched rhamnose minimal medium yielded a few rha^{+} colonies when incubated for 3 days. The frequency of mutation in a series of platings of strain 2306 rha^{w}-1 was $1{\cdot}6 \times 10^{-7}$ per rha^{w} bacterium plated, or $3{\cdot}2 \times 10^{-9}$ per rha^{w} bacterium present after 24 h. These rha^{+} mutants of FIRN strains had the same rhamnose-fermentation properties as wild-type rha^{+} strains and rha^{+} mutants derived from non-FIRN rha^{-} strains. They differed, however, from the latter mutants in being capable of derivation only by two successive mutations and two stages of selection, the first in peptone water (for rha^{w}) and the second on minimal medium (for rha^{+}).

Non-FIRN rha^{w} mutants. Like the FIRN strains, all the non-FIRN rha^{-} strains gave late fermentation in rhamnose peptone water. The time of fermentation varied widely in different tests of the same strain, but usually lay between 2 and 14 days. The results for a series of tests inoculated with 1 or 2 bacteria taken from the same broth culture of strain 2213 are shown in Table 6. Some of the late-fermented cultures of each non-FIRN rha^{-} strain were found to contain rha^{w} mutant bacteria with similar properties to FIRN rha^{w} mutants, but others contained rha^{+} mutants.

Stability of mutants. The rha^{w} and rha^{+} mutant genotypes derived from FIRN and non-FIRN rha^{-} strains appeared to be fairly stable. The rhamnose-fermentation properties remained unchanged when the mutants were carried through ten or more serial subcultures on nutrient agar not containing rhamnose.

(vi) *Transduction to FIRN rha^{w} recipients*

Three independently isolated rha^{w} mutants of FIRN strain 2306, namely mutants 2306 rha^{w}-1, 2306 rha^{w}-2 and 2306 rha^{w}-3, were tested as recipients in transductions with phages propagated on these three mutants, on wild-type rha^{+} strain SL 375, non-FIRN rha^{-} strains 2002 and 2241, and FIRN strains 706, 735 and 2306. The numbers of rha^{+} colonies obtained in platings of the rha^{w} bacteria treated with phages from rha^{w} donors and FIRN donors were no greater than the numbers obtained in control platings without phage. These colonies presumably

arose from spontaneous mutations, so that there was no evidence that rha$^+$ recombination took place in any of the crosses between the different rha^w strains or between rha^w recipients and FIRN donors. The crosses of the rha^w recipients with the non-FIRN rha$^-$ and wild-type rha$^+$ donors gave ten times as many rha$^+$ colonies as the control platings and the majority of these rha$^+$ bacteria are presumed to have been recombinants.

(vii) *Transduction from FIRN rha^w donors*

The FIRN (rha$^-$) strain 2306 was tested as recipient in transductions with phages propagated on strain 2306 rha^w-2 as donor. Platings on enriched rhamnose minimal medium did not give colonies, presumably because rha^w bacteria cannot grow on this medium. The production of rha^w transductants was demonstrated instead by culture in rhamnose peptone water, which is selective for rha^w bacteria. An overnight broth culture of the recipient strain was diluted 1 in 50 in rhamnose peptone water and ten 3 ml volumes of the diluted culture were incubated in 6 ml bottles for $3\frac{1}{2}$ h, to yield *c.* $3{\cdot}5 \times 10^8$ bacteria per ml. Donor phage was added to a concentration of 6×10^9 virions per 3 ml and the cultures were re-incubated for several days and observed daily for production of acid. Similarly treated cultures of the recipient bacteria without the addition of phage served as negative controls, and cultures of the recipient bacteria to which about 15 rha^w bacteria of the donor strain were added served as positive controls. The negative control cultures of the rha$^-$ bacteria gave late, mutational fermentation at different times between 70 h, when the first of them fermented, and 9 days, when eight out of ten had fermented. Fermentation took place much earlier in the rha$^-$ cultures treated with phage from the rha^w donor, presumably as a result of the early production of rha^w transductant bacteria, and in the positive controls; all these cultures formed acid within 73 h and seven out of ten of the transduction cultures did so in 48–54 h. Rha^w bacteria were isolated from the fermented transduced cultures.

The finding that the rha^w character may be transduced into FIRN rha$^-$ recipients from a FIRN rha^w mutant as donor and the rha$^+$ character into FIRN rha^w recipients from a wild-type rha$^+$ donor agrees with the suggestion that FIRN strains bear two mutations at different sites in their rhamnose region. The absence of rha$^+$ recombinants in the crosses tested between independently derived rha^w mutants of a FIRN strain is in keeping with the view that these mutants arise by back-mutation at the same rha$^-$ site in different FIRN strains and not by suppressor mutations at different sites.

4. DISCUSSION

The results of the mutation and transduction studies of the non-rhamnose-fermenting FIRN and non-FIRN rha$^-$ groups of *Salmonella typhimurium* suggest that the site, or sites, of the rha$^-$ mutation, or mutations, are identical or overlapping in all strains in either group, but are not identical or overlapping as between the two groups. In the tests of mutation to rha$^+$, all the twenty-three

FIRN strains behaved in the same way: all failed to produce colonies of rha$^+$ mutant bacteria when plated directly on enriched rhamnose minimal medium, all produced weakly fermenting rha^w mutants in ageing cultures in rhamnose peptone water, in which they thereby caused 'late' (> 2 days) fermentation, and all produced colonies of rha$^+$ mutants in platings of their rha^w mutants. The pattern of mutation appeared uniform also among the strains of the non-FIRN rha$^-$ group, but the pattern in this group differed from that in the FIRN group. All but one of the fourteen non-FIRN rha$^-$ strains produced one or more colonies of rha$^+$ mutant bacteria in direct platings on enriched rhamnose minimal medium, and all strains produced rha$^+$ bacteria in some late-fermenting cultures in rhamnose peptone water and rha^w bacteria in other such cultures. The fact that restoration of the rha$^+$ genotype required two successive mutations in FIRN strains (rha$^-$ to rha^w to rha$^+$) but only one mutation in non-FIRN rha$^-$ strains (rha$^-$ to rha$^+$) suggests that FIRN strains bear two mutational defects at different sites in the rhamnose region of the chromosome and non-FIRN rha$^-$ strains one such defect at a third site. In FIRN strains the rha$^-$ to rha^w mutation may be a back-mutation at one of the two sites, which partially restores fermenting activity, and the rha^w to rha$^+$ mutation may be a back-mutation at the second site, which is expressed only when the first site is also active. In the non-FIRN rha$^-$ strains the rha$^-$ to rha$^+$ mutation may be a back-mutation at the single rha$^-$ site and the rha$^-$ to rha^w mutation may be a partially effective suppressor mutation. Power (1967) has shown that in *Escherichia coli* the fermentation of rhamnose is controlled by a cluster of four genes, three of which appear to be structural genes for L-rhamnose isomerase, L-rhamnulokinase and L-rhamnulose 1-phosphate aldolase, and the fourth an activator-producing regulatory gene. *S. typhimurium* probably possesses a similar rhamnose operon, but we have not determined which of its genes contain the rha$^-$ sites in FIRN and non-FIRN rha$^-$ bacteria.

The rha^w biotype, which permits slow (10–24 h) fermentation of rhamnose in peptone water but not the utilization of rhamnose in minimal medium, has not hitherto been clearly defined. Although it is not included among the twenty-one wild-type biotypes recognized by Kristensen *et al.* (1937), Harhoff (1948), and Kallings & Laurell (1957), it is not confined to laboratory mutants. Dr B. A. D. Stocker identified and sent to us five wild-type rha^w strains isolated in 1962, and one of us (J.P.D.) and Dr E. S. Anderson have detected several other such strains in a large series of isolates of *S. typhimurium*. All these wild-type rha^w strains were fim$^+$ and *d*-tartrate-positive, and so differed in one or other character from the rha^w mutants derived from FIRN and non-FIRN rha$^-$ strains. The reason why rha^w bacteria cannot utilize rhamnose in minimal medium, although they are able to utilize it in peptone water, is unknown.

The finding that rha$^+$ recombinants were produced in transductional crosses between any one FIRN strain and any one non-FIRN rha$^-$ strain is clear evidence that the sites of the rha$^-$ mutations are non-identical and non-overlapping between the two groups. The consistent absence of rha$^+$ recombinants in crosses between different FIRN strains confirms and extends the findings of Old (1963) and

suggests that the sites of the rha$^-$ mutations are identical or overlapping in all members of the FIRN group. The significance of the finding in crosses between different strains in the non-FIRN rha$^-$ group is less clear. These strains produced small numbers of rha$^+$ bacteria by mutation and it was impossible to determine with certainty whether the few rha$^+$ bacteria obtained in the transduction platings were mutants or recombinants. Since, however, the rate of production of rha$^+$ bacteria in the non-FIRN rha$^-$ × non-FIRN rha$^-$ crosses was not significantly higher that the rate of production of rha$^+$ mutants in the control platings, it is probable that rha$^+$ recombination did not take place in any of the crosses and, therefore, that the sites of the rha$^-$ mutations are identical or overlapping in all members of the non-FIRN rha$^-$ group. This conclusion would be in keeping with the observed similarity of mutational behaviour in the non-FIRN rha$^-$ strains.

The conclusion drawn from the present findings and those of Old (1963) that all FIRN strains have their rha$^-$ mutations located at identical sites and the similar conclusion drawn from the findings of Duguid *et al.* (1962) and Old (1963) that all FIRN strains have their fim$^-$ mutations located at identical sites, suggest strongly that all these strains have a common ancestry. FIRN strains have probably originated from the much commoner fim$^+$ inl$^-$ rha$^+$ strains of *S. typhimurium* by successive mutations in the fim and rha genes. If different lines of FIRN strains had originated independently at different times and places, they would almost certainly have differed from one another in the sites of their mutations. The apparent identity of the mutational sites in all FIRN strains, including ones isolated in several different countries, argues that all of them have descended from a single ancestral FIRN bacterium, which itself was derived from a fim$^+$ inl$^-$ rha$^+$ bacterium. One may suppose that the latter bacterium first acquired one of the two characters, fim$^-$ and rha$^-$, by mutation and that after an unknown number of generations one of its descendants acquired the other character by a second mutation. The progeny of the double-mutant, or FIRN bacterium, must then have spread widely throughout the world and become diversified in biotype and phage-type by mutation and lysogenic conversion. The intermediate, single-mutant class of bacteria, which would have been either fim$^-$ inl$^-$ rha$^+$ or fim$^+$ inl$^-$ rha$^-$, apparently failed to proliferate widely, since only two strains of the former type and none of the latter were found among 775 wild-type strains from different sources examined by Duguid *et al.* (1966). If, as our mutational studies suggest, a FIRN bacterium bears two rha$^-$ mutations at different sites, the derivation of the first FIRN bacterium from a fim$^+$ inl$^-$ rha$^+$ ancestor must have involved three, not two, mutations and the production of an additional intermediate class of mutant.

For reasons similar to those adduced in regard to the FIRN group, the apparently negative results of the transductional crosses between different strains in the non-FIRN rha$^-$ group suggest that all strains in this latter group have descended from a single non-FIRN rha$^-$ bacterium that originated by rha$^-$ mutation in a bacterium of the common fim$^+$ inl$^+$ rha$^+$ type of *S. typhimurium*. The ancestral bacterium was presumably biotype 6, which, except for its being rha$^+$, resembles

in all biotyping characters the biotype 19Xd, to which all the non-FIRN rha⁻ strains belong. The identity of the rha⁻ sites in the different non-FIRN rha⁻ strains is not likely to be due to a special mutability of this site, since there is no obvious reason why such a specially mutable site should be present only in strains of biotype 6. Our fourteen non-FIRN rha⁻ strains included isolates from Britain, Australia and the U.S.A., and representatives of eight phage-types, and it is apparent that this race, like the FIRN race, has become widely disseminated from its place of origin and widely diversified in phage-type.

Although the conclusions we have drawn are speculative, our results suggest that transduction studies of wild-type strains with mutational deficiencies may make it possible to elucidate the recent evolutionary history of particular strains and types of bacteria.

SUMMARY

Observations of mutation and phage-mediated transduction of rhamnose-fermentation properties were made on two groups of wild-type rhamnose-non-fermenting (rha⁻) strains of *Salmonella typhimurium* isolated in several different countries. The 'FIRN' group included twenty-three strains in the biotypes 15, 16, 17 and 18, and the 'non-FIRN rha⁻' group included fourteen strains in a new biotype, provisionally designated 19Xd. The two groups showed different patterns of mutational behaviour and the pattern was uniform for the different strains in each group. Non-FIRN rha⁻ strains gave rise directly to colonies of rha⁺ mutant bacteria in platings on rhamnose minimal media. FIRN strains were unable to do this, but produced rha⁺ bacteria by two successive mutations, the first of which yielded weakly fermenting (rha^w) bacteria capable of fermenting rhamnose in peptone water, but not of utilizing it for growth on minimal medium.

Rha⁺ recombinant bacteria were produced in transductional crosses between any one FIRN strain and any one non-FIRN rha⁻ strain, but not in any cross between one FIRN strain and another FIRN strain or between one non-FIRN rha⁻ strain and another non-FIRN rha⁻ strain. These findings suggest that although the sites of the rha⁻ mutations are different between the FIRN and non-FIRN rha⁻ groups, they are located at identical sites on the chromosome in all strains in either group. It is concluded that all FIRN strains have probably descended from a single ancestral strain which underwent mutations at two sites in the rhamnose region of the chromosome and that all non-FIRN rha⁻ strains have descended from a single ancestral bacterium which underwent a mutation at a third site in the rhamnose region.

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