A NEW BIOTYPING SCHEME FOR *SALMONELLA* *TYPHIMURIUM* AND ITS PHYLOGENETIC SIGNIFICANCE

J. P. DUGUID, E. S. ANDERSON, G. A. ALFREDSSON, RUTH BARKER
AND D. C. OLD

*Bacteriology Department, University of Dundee, Dundee, and Enteric Reference
Laboratory, Central Public Health Laboratory, London*

THE epidemiological value of different methods of distinguishing types within a bacterial species depends on their discriminating ability, reliability and ease of performance. Discriminating ability depends primarily on the number of types distinguished, but is poor if a large proportion of strains falls into a single type. Reliability depends on the reproducibility of each isolate's results in repeated tests and on the stability of the typing characters among different isolates from the same epidemic episode.

Probably the best single method for differentiating types in *Salmonella typhimurium* is the phage-typing system of Felix and Callow (1943, 1951) which, as extended by Callow (1959), distinguishes 34 phage-types and, as further extended by E. S. Anderson *et al.* (unpublished) now distinguishes 195 types.

A method of typing *S. typhimurium* by fermentation tests with nine substrates was developed by Kristensen, Bojlén and Faarup (1937). As extended by Hansen (1942) and Harhoff (1948) it distinguishes 21 biotypes (see table 1 of Alfredsson *et al.*, 1972). This method is less discriminating and more laborious than phage-typing, but it distinguishes different biotypes within individual phage-types, so that its combination with phage-typing gives a finer discrimination than either method alone (Kallings and Laurell, 1957; Rische and Kretzschmar, 1962; Lewis and Stocker, 1971).

In a combined phage-typing and biotyping study of *S. typhimurium* we found some serious defects in the Kristensen scheme.

(1) The fermentation tests with *d*-, *l*- and *m*- tartaric acids and citric acid proved unreliable and often gave different results on different occasions of testing the same isolate. Improved, more reliable methods were developed for the tartaric acids by Alfredsson *et al.* (1972) and these methods are used in the new scheme described in the present paper.

(2) In our series of strains, both the original and the modified tests identified several biotypes with patterns of reactions different from those of the twenty-one types already recognised, but the new types could not be accommodated in the Kristensen classification in such a way as to show their relations to the existing types. For example, a group of strains designated "type 19Xd" by Morgenroth and Duguid (1968) resembled biotype 6 except that it was rhamnose negative; if this new type had been added to the scheme as type 22, its close relation to typé 6 would not have been apparent.

(3) We found that discrimination in biotyping could be improved by the addition of tests for the fermentation of trehalose in peptone water, fermentation of rhamnose in Bitter's medium, fermentation of inositol at 25°C, gas production from glucose, growth-factor requirements and presence of flagella and fimbriae, but the results of these extra tests could not be shown in any convenient way within the Kristensen classification.

Mutational fermentation. Alfredsson *et al.* (1972) found that a major cause of unreliability in tests with the tartaric acids was the power of many non-fermenting strains to produce

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fermenting mutants and so give a positive reaction on prolonged incubation of the test. The time of mutational fermentation varied in replicate tests, and identical isolates commonly gave different results in tests read after a single selected period. In our present study with a wider range of substrates we read the tests at intervals during prolonged incubation and noted the occurrence and times of mutational fermentations. These observations enabled us to select for the definitive reading of each kind of test a period of incubation that was long enough to ensure a positive result with genotypically fermenting strains but not so long as to allow the development of mutational reactions by non-fermenting strains. In most tests a 24-hour period was found to be nearly optimal.

New biotyping scheme. A new scheme was required to accommodate the newly discovered biotypes and we designed one with the ability to incorporate any further types with new combinations of reactions that may be discovered in the future. A two-tier system is used (tables I and II). *Primary-type* numbers 1-32 are allocated to the 32 possible combinations of results in the five most reliable and discriminating tests, called the "primary tests". *Subtypes* are distinguished within the primary types by the results of the remaining, or "secondary tests". *Full biotypes* are designated by the primary-type numbers followed by small letters indicating the subtyping reactions.

This paper describes the methods used for the new scheme and the results obtained in typing 2030 strains of *S. typhimurium* isolated from a wide range of human and animal sources. Except where otherwise stated, all designations of biotype are according to the new scheme.

MATERIALS AND METHODS

Bacteria. The series of 2030 strains of *S. typhimurium*, which included most of those examined for fimbriae and Kristensen biotype by Duguid, Anderson and Campbell (1966), were isolated between 1920 and 1973 (most after 1950) in 54 different countries; 1171 were from Britain and Northern Ireland, 283 from 13 other countries in Europe, 217 from Australia, 48 from New Zealand, 135 from USA, 76 from 12 other countries in the Americas, 41 from 12 countries in Asia, 52 from 13 countries in Africa and 7 of unspecified origin. There were 1260 strains from man, mostly from the faeces of patients with gastroenteritis, 233 from cattle, 130 from 17 other mammalian species, 117 from fowls, 85 from 18 other avian species, 2 from turtles, 1 from a toad, and 202 from foodstuffs, sewage and unspecified sources.

Nearly two-thirds of the strains were collected at random, single strains being taken from different epidemic episodes. There were some groups of strains selected to represent a range of phage-types, e.g. (1) 187 phage-type strains of types 1-195, 12a, 15a and 20a, except types 26, 31, 33, 34, 48, 57, 77, 93, 98, 119 and 152, from the Enteric Reference Laboratory, (2) 26 phage-type strains representing 23 types and 3 subtypes in the scheme of Lilleengen (1948) from Professor L. O. Kallings, and (3) 30 strains of different phage-types in the scheme of Scholtens (1962). There were also some groups of strains collected from different patients or animals in the same epidemic episode, e.g. (1) 241 strains of phage-type 141 (formerly U65) isolated in Britain in 1972 and 1973, 192 of which were isolated in Scotland from what appeared to be a single epidemic broadcast, (2) 75 strains of phage-type 29 isolated in Britain between 1956 and 1973, most of which appeared to be from a single "vertical" epidemic, (3) 43 strains of phage-type 143 isolated in England in 1967, probably inter-related, and (4) 17 strains of phage-type 56 isolated in Britain in 1967-68, also probably interrelated.

Before admission to the series, most strains had been subcultured several times and then stored on Dorset's egg slopes at ambient temperature; some had been stored in nutrient-agar stabs and some freeze-dried. We accepted the identification of the strain as *S. typhimurium* made by the person supplying it to us, but we confirmed the serotype of all strains not

belonging to a recognised *S. typhimurium* phage-type and all those showing unexpected combinations of biotyping characters, e.g. non-fimbriate strains that fermented inositol or rhamnose. The culture received was plated on nutrient agar and a single colony was subcultured on a Dorset egg slope to serve as the stock for preparation of inocula for the biotyping tests.

Inocula. The stock culture was plated on nutrient agar and after growth for 18–24 h at 37°C the bacteria from two or three colonies (or from an 18–24-h agar subculture from one colony) were suspended in saline solution (0.85% NaCl) to a density of approximately 10^9 bacteria per ml. About 0.05 ml was inoculated with a capillary pipette or wire loop into each biotyping medium.

A stainless-steel inoculator with twelve blunt prongs was used to inoculate some of the tests in liquid media and most of those on agar. It was charged by dipping the prongs into the bacterial suspensions in a group of twelve 6-ml screw-capped bottles. The moist prongs were then lowered into the liquid test-media in a group of similar bottles or pressed gently to give twelve "spot" inoculations on the surface of a test plate.

Culture media

Nutrient broth was Oxoid Nutrient Broth no. 2 (pH 7.3) dispensed in 8–10-ml volumes in test-tubes (1.5 × 15 cm) stoppered with cotton-wool. *Nutrient agar* was Oxoid Nutrient Agar (pH 7.0). *Semi-solid agar* was a mixture of 6 volumes nutrient agar with 18 volumes nutrient broth. Except where stated otherwise, media were sterilised by autoclaving at 121°C for 15 min.

Sugar peptone waters were 1.5% Oxoid Peptone Water (i.e., 1% Oxoid L37 peptone, 0.5% NaCl in deionised water, pH 7.1) containing 0.002% bromocresol purple (purple at pH 6.8, yellow at pH 5.2) and 0.5% test carbohydrate. D-Glucose, *meso*-inositol, L-rhamnose, trehalose and D-xylose were prepared as 10% (w/v) solutions in sterile deionised water and steamed at 100°C for 1 h. The solutions were added to separate volumes of autoclaved peptone water with bromocresol purple and the mixtures dispensed in 5-ml volumes into sterile cotton-wool stoppered test-tubes or screw-capped bijou (6 ml) bottles, which were then steamed at 100°C for 30 min. The glucose test contained a Durham tube to detect gas production.

Bitter medium (Bitter, Weigmann and Habs, 1926) contained per litre of deionised water, Difco Bacto-Peptone 0.05 g, $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ 0.5 g, $(\text{NH}_4)_2\text{SO}_4$ 1.0 g, sodium citrate 2.0 g, NaCl 2.0 g and bromocresol purple 0.02 g; the pH was 7.1. The test sugar, sterilised separately, was added to a concentration of 0.5% and the completed medium was dispensed in 5-ml amounts and steamed at 100°C for 30 min.

Stern's (1916) *glycerol medium* (Cruickshank, 1968, p. 823) contained 1 litre of a solution of Lab-Lemco meat extract 10 g and Difco Bacto-Peptone 20 g in deionised water (pH 8.0) to which was added 2 ml of a 10% solution of basic fuchsin in ethanol, 16.6 ml of a fresh 10% solution of Na_2SO_3 in water and 10 ml of glycerol. It was dispensed in 5-ml volumes in 6-ml screw-capped bottles and sterilised at 121°C with the caps tightly closed.

Tartrate peptone waters for turbidity tests for the utilisation of *dextro* (*d*, i.e., +), *laevo* (*l*, i.e., –) and *meso* (*m*, i.e., inactive) tartaric acids were prepared as by Alfredsson *et al.* (1972). The tartaric acids were made up as 10% solutions in deionised water, neutralised with 5N-NaOH and autoclaved at 121°C. They were added to sterile peptone water so that the completed medium contained tartaric acid 10g, Oxoid L37 Peptone 6.7 g and NaCl 3.3 g in 1 litre deionised water, pH 7.2. This mixture was dispensed in 10-ml volumes into cotton-wool stoppered test-tubes (1.5 × 15 cm). For the growth of control cultures, part of the same batch of peptone water was brought to the same final concentration by the addition of deionised water instead of tartrate solution.

Minimal agar media. The basic medium (Davis and Mingioli, 1950) contained per litre of deionised water: K_2HPO_4 7g, KH_2PO_4 3 g, $(\text{NH}_4)_2\text{SO}_4$ 1 g, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.1 g, Oxoid Ionagar no. 2 12 g; pH 7.0. Sterile solutions of the following carbohydrates were added to separate lots of sterile melted basic medium to give the stated amount of sugar per litre of

complete medium: (1) glucose 3 g, (2) rhamnose 3 g, (3) xylose 3 g, (4) sodium citrate 2.5 g, (5) glycerol 5 g, (6) *d*-tartrate 5 g, (7) *l*-tartrate 5 g, (8) *m*-tartrate 5 g, (9) sodium citrate 2.5 g plus *m*-tartrate 5 g, and (10) glycerol 5 g plus *m*-tartrate 5 g. The completed media were poured in 20-ml volumes into dishes 8.5 cm in diameter.

Methods of tests

Incubation was aerobic, static and at 37°C. Caps were very loosely applied to screw-capped bottles to ensure free access of air. Inositol tests were done in parallel at 37°C and 25°C.

Fermentation in sugar peptone waters. Tests were examined for acid production (colour change to yellow) at intervals during the first 12 h, then at 24 h and, if still negative, daily for up to 40 days. The definitive reading for biotyping was that made at 24 h, except for the 25°C inositol test, which was read at 48 h. Gas production from glucose was read at 24 h.

Fermentation in Bitter media. Tests were examined for an acid colour change at 24 h. For auxotrophic strains, which grew poorly and gave unreliable results in Bitter media, the observation of "strong fermentation" (colour change before 10 h) in sugar peptone water was taken as equivalent to a positive result in the Bitter test and "weak fermentation" (colour change later than 10 h) in sugar peptone water as equivalent to a negative result.

Tartrate utilisation (turbidity) tests were done as described by Alfredsson *et al.* (1972). After incubation for 24 h the turbidities of the tartrate-containing and control cultures were compared by the naked eye and if the former culture was several times more turbid than the latter the test was read as positive. If there was no obvious difference the turbidities were measured with a Spekker photoelectric absorptiometer (no. 5 filter). Tests with *d*- or *l*-tartrate were read as positive if the turbidity of the tartrate-containing culture was more than 100% greater than that of the control culture, and as negative if less than 50% greater than that of the control. The few tests in which the difference was between 50 and 100% were read as doubtful and repeated. *m*-Tartrate cultures were read as positive if their turbidity was more than 35% greater than that of the control and as negative if more than 10% less than that of the control.

m-Tartrate plate-inhibition test. This was done as by Alfredsson *et al.* (1972). Parallel spot-inoculated cultures on (i) minimal medium with sodium citrate and (ii) minimal medium with sodium citrate plus *m*-tartrate were incubated for 48 h and observed for growth. Strains growing well on both media were read as positive for the utilisation of *m*-tartrate and strains inhibited on the medium with *m*-tartrate, though growing on the medium with citrate alone, were read as negative. The few strains failing to grow on the citrate medium were retested on media containing glycerol instead of citrate or, if auxotrophic, on media supplemented with the necessary growth factors. When, as happened with rare strains, the results of the plate-inhibition test differed from that of the turbidity test, the result of the former was accepted.

Stern's glycerol test was read after incubation for 48 h; a change of colour from yellow to deep red indicated a positive reaction.

Growth on glucose-ammonium minimal medium. Spot-inoculated plates were read at 48 h for the presence or absence of growth. The growth-factor requirements of auxotrophic strains were determined by further tests on plates of minimal media supplemented with different amino acids (10 µg per ml), nicotinamide (0.6 µg per ml) or other growth factors at the concentrations recommended by Meynell and Meynell (1965, pp. 36, 37).

Haemagglutinating activity and fimbriae. The methods were those of Duguid *et al.* (1966). All strains were cultured serially for 48-h periods in tubes of nutrient broth incubated under aerobic, static conditions. Each culture was killed with 0.1% (w/v) formaldehyde and centrifuged. A drop of packed bacterial deposit was tested for haemagglutinating activity on mixing for 30 min. with a drop of 2% (v/v) suspension of washed guinea-pig red blood cells. The mannose-sensitivity of the haemagglutinin, indicative of type-1 fimbriae, was observed in a parallel test in which 0.5% (w/v) D-mannose was added to the bacteria-erythrocyte mixture. Haemagglutinating strains usually gave positive reactions in the first broth culture, but strains were not read as negative until six successive cultures had been examined.

Nearly 300 strains were examined with the electron microscope for the presence of fimbriae. Washed, killed bacteria harvested from one or more of the series of 48-h broth cultures were prepared in films shadow-cast with gold-palladium. Hundreds of bacteria from each culture were examined carefully at high magnification and cultures containing either a small or a large proportion of bacteria bearing fimbriae were designated as fimbriate. Except for eight strains possessing type-2 (non-haemagglutinating) fimbriae, the presence of fimbriae was correlated with that of haemagglutinating activity. In strains not examined with the electron microscope, the presence or absence of fimbriae (type-1) was inferred from the presence or absence of haemagglutinating activity.

Motility and flagella. All strains were stab-cultured in semi-solid agar and observed at 6 and 24 h for diffusely spreading growth indicative of motility. Doubtfully spreading strains were examined microscopically for motility in wet films of 6-h broth cultures. Nearly 300 strains, including all 24 that were non-motile, were examined with the electron microscope for the presence of flagella.

RESULTS

Rhamnose and xylose tests

Sugar peptone water. Strains of *S. typhimurium* showed one of three reactions in rhamnose and xylose peptone waters: (1) *strong fermentation*, in which an acid (yellow) colour change appeared after incubation for less than 10 h, usually between 5 and 8 h, (2) *weak fermentation*, in which the change appeared after 10 but before 24 h, usually between 10 and 15 h, and (3) *non-fermentation* at 24 h, which was usually followed by late, mutational fermentation at between 2 and 40 days. These patterns of reaction with rhamnose were described by Morgenroth and Duguid (1968) who designated the strong, weak and negative reactors, Rha⁺, Rha^w and Rha⁻. Among our 2030 wild-type strains, 1677 were Rha⁺, 54 Rha^w and 299 Rha⁻; 935 were Xyl⁺, 1083 Xyl^w and 12 Xyl⁻. A few strains that fermented xylose between 24 and 48 h in the standard test, but did so in less than 24 h in tests tightly capped to limit access of air, were counted as Xyl^w.

In the biotyping scheme (tables I and II) the reactions in sugar peptone water are taken as read at 24 h, so that both the strongly and weakly fermenting strains are shown as positive, and the late, mutational fermenters as negative. The distinction between the strong and weak fermenters is shown by the results given for the tests in Bitter media.

Bitter tests. Except for auxotrophs, all strains that fermented rhamnose or xylose strongly in peptone water (i.e., within 10 h) also fermented that sugar within 24 h in Bitter medium, and all strains that gave weak or late fermentation in peptone water gave a negative reaction in 24 h in Bitter medium. The few strongly fermenting strains that were auxotrophic, i.e., failed to grow on unsupplemented glucose-ammonium minimal medium, gave unreliable, often negative results in the Bitter medium, which contains little peptone. For auxotrophs, therefore, we substituted the observation of whether or not the strain fermented the sugar in peptone water before 10 h for the result of the Bitter test.

Strong and weak fermenters on solid media. The strongly fermenting prototrophic strains grew well and the weakly fermenting prototrophs failed to grow on a minimal agar medium containing rhamnose or xylose as the

sole source of carbon and energy. The distinction was best seen when the bacteria were plated out in small numbers. When, however, as many as 10^7 – 10^8 bacteria of a Xyl^w strain were spot-inoculated on the medium, a moderate growth usually developed within 48 h, apparently due to the presence of some Xyl⁺ mutant bacilli in the inoculum. The strongly and weakly fermenting strains were also distinguishable when cultured for 24 h on DCA or EMB medium (Cruickshank, 1968, p. 821) containing rhamnose or xylose instead of lactose; colonies of the strong fermenters were red (acid) and those of the weak fermenters pale (non-acid).

Mutational fermentation of rhamnose. Every one of 299 Rha⁻ strains gave "late" fermentation when incubation of the rhamnose peptone water was

TABLE I

New scheme for biotyping Salmonella typhimurium: fermentation reactions of 32 primary biotypes and their correspondence with Kristensen's types

New primary biotype no.	Result of fermentation test* with					Kristensen type
	D-xylose in Bitter's medium	meso-inositol in peptone water	L-rhamnose in peptone water	d-tartrate turbidity test	m-tartrate plate test	
1	+	+	+	+	+	1, 7
2	+	+	+	+	—	...
3	+	+	+	—	+	6
4	+	+	+	—	—	...
5	+	+	—	+	+	...
6	+	+	—	+	—	...
7	+	+	—	—	+	(19Xd)
8	+	+	—	—	—	...
9	+	—	+	+	+	9
10	+	—	+	+	—	...
11	+	—	+	—	+	...
12	+	—	+	—	—	...
13	+	—	—	+	+	...
14	+	—	—	+	—	...
15	+	—	—	—	+	...
16	+	—	—	—	—	...
17	—	+	+	+	+	2, 3, 5, 8
18	—	+	+	+	—	4
19	—	+	+	—	+	...
20	—	+	+	—	—	...
21	—	+	—	+	+	19
22	—	+	—	+	—	...
23	—	+	—	—	+	14, 20
24	—	+	—	—	—	...
25	—	—	+	+	+	10, 11, 13
26	—	—	+	+	—	12
27	—	—	+	—	+	...
28	—	—	+	—	—	...
29	—	—	—	+	+	15
30	—	—	—	+	—	16
31	—	—	—	—	+	17, 18
32	—	—	—	—	—	...

* Readings at 48 h for m-tartrate and at 24 h for the other tests. For Bitter-xylose reaction of auxotrophs, see footnote to table II.

sufficiently prolonged. Most tests showed fermentation at some time between 3 and 10 days, but others took longer. When several tests were grown in parallel from inocula of the same strain, the majority fermented after periods ranging from 2 to 40 days, but a few remained unfermented. In all strains the late fermentation was shown to be due to the production of either Rha^w or Rha⁺ mutants. When a late-fermented test mixture was plated out on nutrient agar and separate colonies were tested in fresh tubes of rhamnose peptone water, some of the colonies (the mutants) fermented within 24 h and others (the parent strain) did not. The mutants later retained their Rha^w or Rha⁺ character through serial culture on rhamnose-free medium.

FIRN strains. Most (275) of our 299 Rha⁻ strains belonged to the FIRN group (Fimbriae, Inositol and Rhamnose Negative) defined by Morgenroth and Duguid (1968), i.e. to biotypes 29, 30, 31 and 32 in the new scheme. Of the remaining 24 Rha⁻ strains, 22 belonged to biotype 7 (type 19Xd of Morgenroth and Duguid) and 1 each to biotypes 21 and 23. We confirmed the difference in mutational behaviour between the FIRN and biotype-7 strains reported for their smaller series by Morgenroth and Duguid; FIRN strains produced only Rha^w mutants when first giving late fermentation in rhamnose peptone water, whereas biotype-7 strains produced Rha^w mutants in some tests and Rha⁺ mutants in others.

Rha^w and Rha⁺ mutation. The Rha^w mutants from all FIRN strains were capable of further mutation to the Rha⁺ phenotype; platings of the Rha^w culture on rhamnose minimal medium yielded a few Rha⁺ colonies after 2 or 3 days. In contrast, Rha⁺ mutants were rarely produced by the 54 wild-type Rha^w strains, most of which belonged to biotypes 1h, 2h, 17h, 25hi and 27hi.

Mutational fermentation of xylose. Only 12 of the 2030 strains were Xyl⁻ and each gave late fermentation of xylose in peptone water after 2-8 days.

TABLE II

New scheme for biotyping Salmonella typhimurium: results in secondary tests designating subtypes

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- a = Results in all secondary tests are positive; the strain is haemagglutinating (type-1 fimbriae), motile (flagellate) and grows on glucose-ammonium minimal medium
 - b = Non-fimbriate (Fim⁻) in haemagglutination test
 - c = Non-flagellate (Fla⁻) in motility test
 - d = *l*-Tartrate not utilised (IT⁻) in 24-h turbidity test
 - e = D-xylose in peptone water not fermented in 24 h (Xyl⁻)
 - f = Trehalose in peptone water not fermented in 24 h (Tre⁻)
 - g = Glycerol in Stern's medium not fermented to aldehyde in 24 h
 - h = L-rhamnose not fermented in Bitter's medium in 24 h, although fermented in peptone water in 24 h (Rha^w)*
 - i = *meso*-Inositol in peptone water not fermented in 48 h at 25°C
 - j = Gas not produced from glucose in peptone water in 24 hr
 - x = Nicotinamide required for growth on minimal medium
 - y = Cysteine required for growth on minimal medium
 - z = A growth factor required other than nicotinamide or cysteine
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* Auxotrophic strains give unreliable results in Bitter's medium and are recorded as positive or negative according to whether or not they ferment the sugar in peptone water before 10 h.

Platings from the late-fermented tests yielded a proportion of colonies that were either Xyl^w or Xyl⁺ mutants.

Inositol tests

The test for fermentation of inositol at 37°C, when done with exact control of the incubation temperature, gave a clear-cut distinction between 1081 strains that were "positive" (Inl⁺), i.e., gave an acid reaction before 24 h (usually in 8–12 h), and 949 that were "negative" at 24 h (Inl⁻). Rare borderline strains (5 in phage-type 2) produced acid early in the second day of incubation and were classified as "positive" (biotype 17). Most of the "negative" strains failed to ferment inositol during incubation for 40 days, but a few, particularly in biotype 9, gave late, mutational fermentation between 2 and 7 days. Late fermentation was observed with only 5 of the 275 FIRN strains in biotypes 29–32 and, in these strains, in only one or two out of 20–100 replicate tests; Inl⁺ mutants were isolated from them.

Many strains not fermenting inositol in the tests at 37°C, fermented it when incubated at 35°C or less. This temperature-sensitive inositol-fermenting function (Old, 1972), which was observed in tests incubated for 48 h at 25°C, was present in 865 of the 949 strains that were inositol negative at 37°C, including 268 of the 275 FIRN strains.

Trehalose tests

Of the 2030 strains, 1553 were trehalose positive (Tre⁺), giving an acid reaction within 24 h (mostly between 12 and 24 h) and 477 were trehalose negative at 24 h. All the Tre⁻ strains gave late fermentation after incubation for 2–7 days, and platings from the late fermented tests yielded mutant Tre⁺ organisms capable of fermenting trehalose in 24 h.

The trehalose character, whether positive or negative, appeared to be relatively stable during the epidemic spread of a strain through its natural hosts, for it was found to be the same in all recent isolates examined from different sources in the same epidemics. With some strains, however, it was variable during subculture in the laboratory, Tre⁺ mutants appearing in cultures of Tre⁻ strains on nutrient agar or Dorset's egg. Thus, in the first tests of the stock cultures of 192 isolates of phage-type 141 biotype 9f from different sources in a recent epidemic in Scotland, 14 gave a positive trehalose reaction in 24 h. When, however, these 14 "positive" stock cultures were examined by plating on nutrient agar and testing separate colonies, all 14 were found to contain a mixture of 80–90% Tre⁻ and 10–20% Tre⁺ bacteria. The Tre⁺ bacteria were apparently mutants from the Tre⁻ bacteria, which probably represented the original form of the isolate.

As our strains were maintained and tested by subculture from single colonies, trehalose mutation *in vitro* and the accidental picking of a mutant colony were the probable explanation of instances in which different results were obtained on different occasions of testing the same strain. These circumstances probably also accounted for the few cases in which different reactions were found in

groups of related strains tested long after their isolation; e.g., six strains of phage-type 178 biotype 17 isolated in Katowice in 1959 included five Tre⁺ (17a) and one Tre⁻ (17f), and nine strains of phage-type 118 biotype 1 isolated in Britain in 1963 included six Tre⁺ (1a) and three Tre⁻ (1f). In other groups the trehalose character was uniform despite prolonged maintenance *in vitro*; e.g., all 27 strains of phage-type 4 biotype 1 isolated in different countries between 1923 and 1969 were Tre⁻.

Stern, gas and tartrate tests

The Stern test for aldehyde production from glycerol distinguished 1911 positive strains giving a deep red colour within 48 h and 119 negative strains giving slight or marked reddening only after a longer period. The test was reliable in the sense that strains gave the same results on different occasions of testing in a correctly prepared medium. Since, however, the glycerol-fuchsin-sulphite medium is difficult to standardise and deteriorates on keeping, strains giving doubtful or unexpected results had to be retested in fresh medium.

Only 20 strains, 11 of which were phage-type 3 biotype 17j, were anaerogenic in glucose peptone water.

The reactions of the 2030 strains with *d*-, *l*- and *m*-tartrates were as described by Alfredsson *et al.* (1972) and are summarised in tables III and IV.

TABLE III

Number, of 2030 strains, of Salmonella typhimurium giving negative reactions in primary and secondary tests of the new scheme

Reaction in test	Number of strains
<i>Primary tests</i>	
D-xylose not fermented in Bitter's medium (Xyl ⁻ or Xyl ^o)	1095
<i>meso</i> -Inositol not fermented in peptone water at 37°C (Inl ⁻)	949
L-rhamnose not fermented in peptone water (Rha ⁻)	299
<i>d</i> -Tartrate not utilised (dT ⁻)	496
<i>m</i> -Tartrate not utilised (mT ⁻)	370
<i>Secondary tests*</i>	
b = Non-haemagglutinating (Fim ⁻)	319
c = Non-motile (Fla ⁻)	24
d = <i>l</i> -Tartrate not utilised (lT ⁻)	91
e = D-xylose not fermented in peptone water (Xyl ⁻)	12
f = Trehalose not fermented (Tre ⁻)	477
g = Glycerol not fermented	119
h = L-rhamnose not fermented in Bitter's medium though fermented in peptone water (Rha ^o)	54
i = <i>meso</i> -Inositol not fermented at 25°C	84
j = Gas not produced from glucose	20
x = No growth without nicotinamide	104
y = No growth without cysteine	24
z = No growth without growth factor other than nicotinamide or cysteine	29

* Strains gave a positive reaction in all secondary tests—878 in number—were designated subtype a.

Growth on glucose-ammonium minimal medium

Most strains were prototrophic and grew well in 48 h on glucose-ammonium minimal medium. Only 154 of the 2030 strains were auxotrophic and required supplementation of the medium with growth factors; 104 strains required nicotinamide, 24 required cysteine (1 also nicotinamide), 4 phenylalanine, 3 arginine, 3 pantothenic acid (1 also nicotinamide), 3 purine, 3 thiamine, 2 folic acid, 2 methionine (1 also nicotinamide), 1 leucine, 1 isoleucine plus valine, 1 histidine, 1 tyrosine, and 5 an undetermined factor. Because the requirements of nicotinamide and cysteine were relatively common (68 and 14% of the auxotrophs), their determination was included in the biotyping scheme (subtype characters x and y), but the other, less common requirements were indicated only collectively (subtype character z).

TABLE IV

Distribution of 2030 strains of Salmonella typhimurium among the observed primary biotypes and subtypes in the new scheme

Primary biotype no.	Number of strains	Subtypes (letters) observed in the primary biotype (and number of strains in subtype)	Number of full biotypes
1	428	{ a(246), b(2), d(3), f(145), g(7), h(4), j(1), x(3), z(6), bc(1), bf(3), cf(4), df(1), fz(2)	14
2	69	a(58), d(2), f(5), g(1), h(3)	5
3	194	a(178), b(4), f(8), g(1), h(1), df(1), fg(1)	7
4	1	df(1)	1
7	22	a(18), b(1), f(2), z(1)	4
9	220	a(3), b(1), f(199), i(15), di(1), ix(1)	6
12	1	bdhx(1)	1
17	333	{ a(154), b(11), d(8), e(2), f(19), g(70), h(18), j(15), x(2), be(2), bf(2), bg(1), bj(1), cf(1), df(2), dg(1), ef(1), eg(1), fg(11), fj(1), gh(1), dx(1), fz(2), gz(1), hy(1), hz(1), xy(1), fxz(1), fghz(1)	29
18	7	a(4), e(3)	2
19	25	a(23), f(1), h(1)	3
21	1	b(1)	1
23	1	by(1)	1
25	189	{ a(47), b(7), d(11), e(1), f(10), g(2), h(1), i(1), x(83), z(1), fi(1), hi(7), dx(1), fx(4), fz(1), gx(1), gz(1), hx(1), ix(2), xz(1), bcg(1), bhi(1), ghi(1), hix(1), fghi(1)	25
26	252	{ a(153), b(2), d(36), f(18), i(28), y(3), z(1), bd(1), cf(1), fg(1), fi(1), fj(1), gi(2), dz(1), iz(1), jz(1), dgi(1)	17
27	12	i(1), ei(1), hi(6), fhi(1), hiz(2), dfgi(1)	6
29	25	b(13), bc(2), bd(1), bf(8), bcf(1)	5
30	10	by(9), bfy(1)	2
31	210	b(161), bc(13), bd(16), bf(6), bg(8), bi(2), by(1), bz(1), bgz(2)	9
32	30	b(13), bf(3), bi(5), by(7), bz(1), bef(1)	6
Any	2030		144

Motility and flagella

Only 24 of the 2030 strains were non-motile and 22 of these 24 were found by electron microscopy to be non-flagellate. The exceptional flagellate non-motile strains were no. SW580 from the line of Friewer and Leifson (1952), which was biotype 29bcf, and no. S597, a bovine strain of biotype 26cf.

Thirteen of the non-flagellate strains were phage-type 14 biotype 31bc, and were isolated in different parts of England in 1961-63; they appeared to represent a non-flagellate line that for a time was successful in perpetuating itself in natural sources. The other nine non-flagellate strains occurred randomly in different biotypes and appeared to be Fla⁻ mutants that were unsuccessful in spreading widely.

Haemagglutination and fimbriae

Of the 2030 strains, 1711 (Fim⁺) showed mannose-sensitive haemagglutinating activity, indicative of the presence of type-1 fimbriae (Duguid *et al.*, 1966), and 319 (Fim⁻) were non-haemagglutinating. Electron microscopy demonstrated the presence of numerous fimbriae per bacterium in all of 146 of the haemagglutinating strains and the absence of fimbriae in all but 8 out of 150 of the non-haemagglutinating strains. We disregarded the occasional presence of single or very scanty filaments which may have been sex fimbriae.

FIRN strains. All 275 strains in the inositol-negative rhamnose-negative biotypes 29-32 were non-haemagglutinating and all of 106 of them examined with the electron microscope were non-fimbriate. These FIRN strains made up nearly 14% of our wild-type isolates. They were isolated between 1948 and 1973 from human and various animal, particularly avian, sources in many different countries in Europe, Africa, Australasia and North America.

Non-FIRN Fim⁻ strains. The remaining 44 non-haemagglutinating strains belonged to ten primary biotypes (1, 3, 7, 9, 12, 17, 21, 23, 25 and 26), most strains of which were haemagglutinating and fimbriate. Apart from six strains of biotype 17 isolated in Wellington, New Zealand, in 1969, and two pairs of strains, they had origins so separate as to preclude the possibility that they were epidemiologically related. Electron microscopy showed that 34 of the 44 strains were non-fimbriate but that 8 strains (in biotypes 12, 17 and 25) bore numerous fimbriae on a small proportion (1-3%) of the bacteria in a culture. These fimbriae resembled the non-haemagglutinating type-2 fimbriae described by Duguid *et al.* (1966) and Old and Payne (1971) in *S. paratyphi B*, *S. gallinarum* and *S. pullorum*. Because they lacked type-1 (haemagglutinating) fimbriae, the eight non-haemagglutinating fimbriate strains were included in the Fim⁻ category for biotyping.

Mutation in the Fim genotype. The Fim⁺ strains appeared to be relatively stable in their Fim genotype. Many of them were examined in several subcultures from separate colonies, but no Fim⁻ mutant colony was found. Because no method of culture selective for Fim⁻ bacteria is known, the possibility that some cultures contained small numbers of Fim⁻ mutant bacteria could not be excluded.

FIRN strains were highly stable in their *Fim*⁻ character and none gave spontaneous *Fim*⁺ mutants during serial culture in aerobic static broth, a procedure that is highly selective for bacteria with type-1 fimbriae (Old and Duguid, 1970). *Fim*⁺ mutants were, however, obtained by serial broth culture after treatment of the *FIRN* bacteria with mutagens, e.g., nitrous acid and ethyl methane sulphonate.

Nine of the 44 Non-*FIRN* *Fim*⁻ strains (in biotypes 1, 7, 9, 17 and 26) differed from the *FIRN* strains in sometimes giving spontaneous *Fim*⁺ mutants during serial culture in broth. Each mutant had the same fermentation characters as its parent strain. Mutant *Fim*⁺ bacteria were demonstrated in the originally received cultures of only two of the mutable strains, namely nos. S1566 (phage-type 49, biotype 26b) and S3449 (phage-type 29, biotype 26bd). Platings from these two cultures showed 30–50% of *Fim*⁺ colonies. When purified, the *Fim*⁻ line of strain S1566 gave rise to *Fim*⁺ mutants in only one out of 24 replicate series of three successive broth cultures.

DISCUSSION

Primary biotypes. The 32 primary biotypes of the new scheme were defined by the 32 possible combinations of positive and negative reactions in five primary tests (table I). These tests used five of the substrates of the Kristensen scheme, namely inositol, rhamnose, Bitter xylose, *d*-tartrate and *m*-tartrate. They were selected as primary tests because they distinguished the largest groups of strains giving the less common, i.e., negative, reaction (table III) and each of them discriminated between groups of strains not distinguished by the other tests. The 2030 strains of *S. typhimurium* included representatives of 19 of the 32 potential primary biotypes, the largest numbers being in biotypes 1, 3, 17, 25, 26 and 31. The 13 presently unrepresented types will accommodate any strains with new combinations of reactions to be discovered in the future.

Full biotypes. Of the ten secondary tests used to determine subtypes within the primary types, the trehalose, haemagglutination and glucose-ammonium tests differentiated fairly large numbers of strains with the less common, negative reaction, but the others differentiated fewer such strains (table III). The trehalose test was not selected as a primary test because the Tre character of many strains was mutable in stored stock cultures and the haemagglutination test was not selected because its performance is moderately laborious. Tests for fermentation of maltose, arabinose and sorbitol differentiated too few negatively reacting strains (16, 4 and 8 out of 852 tested) to justify their use even as secondary tests.

The results of the secondary added to those of the primary tests differentiated the 2030 strains into 144 full biotypes (table IV), so that the new scheme distinguished many more types than the 21 of the Kristensen scheme. A further advantage of the new, two-tier scheme is that further secondary tests can be added in the future and their results could be shown by the subtype symbols k–w.

Reliability of tests. The five primary and ten secondary tests proved quite reliable. They gave the same results on different occasions of testing the same strain and were not unduly sensitive to minor variations in technique. Difficulty was experienced only with a few strains that gave "borderline" reactions with inositol, xylose, trehalose or Stern's glycerol, fermenting the substrate sometimes just before and sometimes just after the period for the definitive reading. The main cause of unreliability in tests with tartrates had been found by Alfredsson *et al.* (1972) to be the irregular production of fermenting mutants during prolonged testing and we avoided this difficulty in the tartrate and other tests by making definitive readings after a short, carefully chosen period of incubation, which for most tests was 24 hours.

Stability of biotype. In all five primary and most of the secondary tests, mutants giving positive reactions commonly emerged when "negative" strains were subjected to the selective effect of prolonged culture in the test substrate. On unselective media, such as nutrient agar and Dorset's egg, the production of mutants, except for some in the trehalose character, was rarely observed. The stability of the biotype on unselective media is important for the usefulness of biotyping, because if mutants were commonly to emerge in cultures grown for the isolation and storage of strains, the method would be unreliable as a means of determining whether the strains had been similar or different while in their natural sources.

Our observations on series of strains isolated from different sources in the same epidemic showed that the biotypes were very stable under the conditions of growth in the natural sources. In most series the full biotype was identical in all isolates from the epidemic. In some series examined after several years' storage in the laboratory a minority of the isolates showed a difference in one of the subtype characters, most commonly in the trehalose reaction. Such differences were presumably due to mutants having been selected unknowingly during subculture.

The largest epidemic series examined was of 192 isolates of phage-type 141 from a multi-centred epidemic in Southern Scotland in 1972-73. It comprised cultures from human and animal sources in outbreaks on farms in six counties and from patients and food in food-poisoning outbreaks in Edinburgh and its environs. All these cultures were found to be of biotype 9f. (Of 49 other strains of phage-type 141 isolated in 1972-73 from British sources unrelated to the Scottish epidemic, 4 were of biotype 9f and 45 were of biotypes 1f, 31b and 31bd.) The negative characters in the inositol (37°C) and trehalose tests and the positive characters in the other tests were thus shown to be highly stable during growth of the 9f strain in its human and animal hosts.

Similarly, in other series, all of 43 strains of phage-type 143 isolated in different parts of England in 1967 were found to be of biotype 17a, all of 17 strains of phage-type 56 isolated in Britain in 1967-1968 were of biotype 17g, 3 strains of phage-type 173 from a family outbreak were of biotype 2a, 4 strains of phage-type 13 from an outbreak caused by infected pork pies were of biotype 31b, and 16 strains of phage-type 14 from fowls and turkeys in South-East Scotland in 1955 were of biotype 31b.

A few variations in full biotype were observed in a series of 70 cultures of phage-type 29, primary biotype 26, isolated from human and animal sources in Britain between 1956 and 1972. The epidemic was primarily in cattle and appeared to be caused by a single strain which, after 1962, successively acquired different antibiotic-resistance characters (Anderson, 1968, 1971). The early strains were of biotype 26a, but an *l*-tartrate-negative line, i.e., of biotype 26d, appeared in 1965 and thereafter became predominant, accounting for all but three of the 32 strains isolated between 1966 and 1972. A few strains showed variations in other subtype characters but did not become common; one strain was non-fimbriate, one was trehalose negative, nine were inositol negative at 25°C, four were Stern's glycerol negative and one was auxotrophic. Five strains of phage-type 29 isolated in Britain during the same period belonged to primary biotypes other than type 26, but their antibiotic-sensitivity characters suggested that they were unrelated to the epidemic strain, which therefore appeared to be stable in its primary biotype.

The FIRN group. The correlation reported by Duguid *et al.* (1966) between the characters of non-fimbriation (*Fim*⁻), non-fermentation of inositol (*Inl*⁻) and non-fermentation of rhamnose (*Rha*⁻) was confirmed in our larger series. All 275 strains in the *Inl*⁻ *Rha*⁻ biotypes 29-32, but only 44 of the 1755 strains in the *Inl*⁺ or *Rha*⁺ biotypes, were *Fim*⁻. The *Fim*⁻ *Inl*⁻ *Rha*⁻ (FIRN) strains were all alike in being Bitter-xylose negative and in giving the sequential mutations *Rha*⁻ to *Rha*^w to *Rha*⁺ described by Morgenroth and Duguid (1968), but they showed some differences in other characters and represented 22 full biotypes in the primary types 29-32 (table IV). Our findings support the view of Morgenroth and Duguid (1968) and Old (1972) that all FIRN strains are descended from a single ancestral clone that originated from a *Fim*⁺ strain of biotype 25 by successive mutations at two sites in the *rha* region of the chromosome and one or more sites in the *fim* region. Subsequently this clone must have diversified by mutation in the tartrate and other characters determining its membership of the 22 different full biotypes observed.

Other Fim⁻ or *Rha*⁻ strains. There were three *Fim*⁻ *Rha*⁻ strains that differed from FIRN strains by being *Inl*⁺. One of them, S3403, was biotype 7b and appeared to be a *Fim*⁻ mutant from a typical *Fim*⁺ strain of biotype 7a; it gave *Fim*⁺ mutants on prolonged selective culture in broth. The two others, no. S2338 in biotype 21b and no. S6925 in biotype 23by, were the only strains in our series belonging to primary biotypes 21 and 23. They resembled FIRN strains in being Bitter-xylose-negative, in giving the sequential mutations *Rha*⁻ to *Rha*^w to *Rha*⁺ and in failing to give spontaneous *Fim*⁺ mutants in broth cultures; they are thought to have originated as *Inl*⁺ mutants from FIRN strains in biotypes 29b and 31by.

Fim⁺ *Rha*⁻ strains, of which there were twenty-one formed a homogeneous group in biotype 7; eighteen were of type 7a, two of type 7f and one of type 7z. All showed the alternative patterns of mutation, *Rha*⁻ to *Rha*^w or *Rha*⁻ to *Rha*⁺, found in such strains by Morgenroth and Duguid (1968) who considered they were descended from a single *Rha*⁻ mutant clone of a strain of biotype 3.

Forty-one *Fim*⁻ *Rha*⁺ strains were heterogeneous in biotype and included

representatives of the primary types 1, 3, 7, 9, 12, 17, 25 and 26. Apart from a group of six strains of biotype 17 isolated in New Zealand in 1969 and two pairs of strains in other biotypes, they were from unrelated sources. They seem to have originated recently by Fim⁻ mutation from different Fim⁺ strains typical of their biotypes, but to have been relatively unsuccessful in perpetuating themselves in the host community.

Survival value of biotype characters

Chance circumstances permitting or preventing the dissemination of particular strains at particular times and places may play a large part in determining the relative numbers of isolates of different types in a randomly collected series. For this reason, the frequency distribution of the different typing characters cannot be taken as a measure of their survival values, though it seems reasonable to assume that properties absent from any widely distributed group of strains are unlikely to have a high survival value and that properties absent from only a few strains may be important for the perpetuation of the bacteria in the natural environment.

Types 1 and 1a are the primary and full biotypes containing the largest numbers of strains in the present series and this finding might suggest that possession of the genes determining positive reactions in all fifteen biotyping tests is advantageous to the bacteria. However, the fact that many strains gave negative reactions with inositol, rhamnose, Bitter xylose, *d*-tartrate, *m*-tartrate, trehalose and glycerol shows that lack of these activities had little effect on the ability of the bacteria to survive and spread in the host community. In contrast, very few strains lacked the power to form flagella, to produce gas from glucose or to ferment xylose in peptone water and the absence of these three properties may well be disadvantageous. A nutritional requirement for either nicotinamide or cysteine was no bar to prolonged epidemic spread, but strains with requirements for other growth factors were rare and apparently unsuccessful in perpetuating themselves.

It is possible that the survival value of one character may depend on the presence or absence of others. Thus non-fimbriate strains were rare except in the FIRN biotypes 29–32 and it appeared that Fim⁻ mutants arising in other biotypes were relatively unsuccessful in perpetuating themselves. When, however, the inability to form fimbriae was associated with rhamnose negativity and the other characters of the FIRN group, it was clearly no hindrance to successful parasitism and spread. The FIRN strains, indeed, made up 14% of our whole series. Possibly this group, which is very common in avian sources, is specially adapted by its Fim⁻ Rha⁻ character to particular niches within the wide range of habitats occupied by *S. typhimurium*.

Phylogeny

The serotype *S. typhimurium* probably evolved as a single clone possessing the specific typhimurium antigens and a single set of biochemical characters. This archetype would most probably be biotype 1a, the commonest type and

that in which all fifteen typing characters are positive. The other biotypes probably arose from the archetype by mutations that rendered particular gene functions inactive. The figure shows a hypothetical genealogical tree and a pattern of mutational pathways by which the observed primary biotypes could have evolved from biotype 1. The subtypes within the primary types could have arisen by later mutations determining the loss of particular secondary characters.

Besides that shown in the figure, there are, of course, many other combinations of mutational pathways by which the different biotypes might have been derived. It is also perfectly possible that different strains in the same biotype originated from different precursors. The suggested scheme, however, assumes the simplest possible series of transformations and agrees with available indications of the genetic relationships of strains in the different biotypes. Thus transduction studies have provided evidence that the sites of the *Fim*⁻ and *Rha*⁻ mutations are homologous in all FIRN strains and therefore, that these strains have a single, common origin (Morgenroth and Duguid, 1968;

FIGURE—Possible mutational routes whereby *Salmonella typhimurium* may have evolved eighteen observed primary biotypes (2-32) from the presumed archetypal biotype (1).*

* *Bxyl*⁻, *inl*⁻, *rha*⁻, *mta*⁻ and *dta*⁻ = mutations to forms giving negative reactions with Bitter xylose, inositol at 37°C, rhamnose, *m*-tartrate and *d*-tartrate; *fim*⁻ = mutation to a non-fimbriate form; *inl*⁺ = back mutation to a form fermenting inositol at 37°C. The mutations are cumulative in the successive steps in each route; e.g., biotype 2 is *m*-tartrate negative, whereas biotype 4 is both *m*-tartrate negative and *d*-tartrate negative.

Old, 1972). There is also similar evidence that all strains in biotype 7 have a common origin.

The proposed genealogical tree assumes changes by "loss" mutations in respect of the reactions with Bitter xylose, inositol, rhamnose, *d*-tartrate and *m*-tartrate and the production of fimbriae, but because of the absence of simple methods for selective culture of the deficient mutants we have not been able to demonstrate their production. For most of the proposed steps, however, we have observed the reverse mutation: e.g., biotype-7 strains mutating to biotype 3, biotype-2 strains mutating to biotype 1, FIRN strains giving Rha⁺ mutants in two stages and FIRN strains giving Fim⁺ mutants after mutagenesis. Thus, the strains in biotypes with negative characters were shown to possess genes capable of back-mutation to forms conferring the positive characters.

SUMMARY

A new, two-tier system for biotyping *Salmonella typhimurium* gives a finer and more reliable differentiation of strains than the Kristensen scheme and is capable of future extension by the addition of new types and new tests. Strains are allocated to a *primary type* (1–32) by their reactions in five primary tests with Bitter's xylose medium, *meso*-inositol, L-rhamnose, *d*-tartrate and *m*-tartrate. Subtypes are distinguished within the primary types by reactions in ten secondary tests, which include observations for flagella and type-1 (haem-agglutinating) fimbriae. *Full biotypes* are designated by letters indicating the subtype reactions appended to the primary-type numbers.

A series of 2030 strains of *S. typhimurium* collected from many different sources and countries during 53 years was classified into 19 of the 32 potential primary biotypes and into 144 full biotypes. Of the series, 14% (275) were non-fimbriate inositol-nonfermenting rhamnose-nonfermenting (FIRN) strain in primary biotypes 29–32. Most other strains were fimbriate and rhamnose fermenting.

Observations on several series of cultures isolated from different human or animal sources in the same epidemic showed that the biotype characters of a strain were generally stable during its growth in the natural environment and in the unselective media used for isolation and storage. Most non-fermenting strains gave rise to fermenting mutants on prolonged incubation in the substrate-containing—and therefore selective—test medium, and false-positive results from this cause were avoided by making the definitive readings of tests after a short, carefully chosen period of incubation.

A genealogical tree has been drawn to show how eighteen observed primary biotypes may have evolved from a presumed archetypal ancestor of biotype 1.

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