A biochemical subdivision of one phage type of *Salmonella typhimurium*

By M. J. LEWIS* and B. A. D. STOCKER†

The Guinness-Lister Research Unit,
Lister Institute, London

SUMMARY

A total of 1537 strains of *Salmonella typhimurium* belonging to seven prevalent phage types were examined on solid media for their ability to ferment rhamnose, xylose and inositol, for colicine production and for nutritional requirements. Most of the strains in each phage type were almost completely homogeneous, especially in their sugar fermentation reactions. However, strains of phage type 1a/2 were not homogeneous, but could be assigned to one of four subgroups on the basis of ability to ferment inositol, inhibition of growth by *meso*-tartrate and auxotrophy for nicotinic acid. The subdivision proved to have epidemiological value. The inhibition of growth by *meso*-tartrate was observed on a defined medium containing citrate as the energy source. Inhibition did not occur if glucose, casein hydrolysate or aspartic acid were added to the medium.

INTRODUCTION

Salmonella typhimurium is the commonest salmonella in the United Kingdom, causing some 50,000 human infections annually and attacking large numbers of farm livestock (Anderson, 1965). Investigation of the epidemiology of this organism has been made possible only by the development of methods of identifying different strains within the serotype. The most widely used of these methods have been the phage typing schemes devised by the late Miss Bessie Callow (Felix & Callow, 1943; 1951, Callow, 1959). The first of these schemes (scheme 1) entails the use of 10 typing phages to distinguish 12 types and subtypes of *S. typhimurium*. It was soon apparent that a greater number of epidemiologically valid types existed, and in 1959 Miss Callow published a new typing scheme (scheme 2) distinguishing at that time some 80 types. For some years both schemes have been used in parallel in the Enteric Reference Laboratory, Colindale, and the number of recognized phage types has further increased (Anderson, 1965). In this paper we will refer to phage types of *S. typhimurium* by their designations in both schemes; for example, type 1a/2 belongs to the type designated 1a in scheme 1

* Present address: Public Health Laboratory, City and Sherwood Hospitals, Nottingham, NG5 1PH.

† Present address: Department of Medical Microbiology, Stanford University School of Medicine, Stanford, California 94305.

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and 2 in scheme 2. A phage-typing scheme devised independently by Lilleengen (1948), depending on 12 anti-O phages to define 24 phage types, has been used in Scandinavia (Lundbeck, Plazikowski & Silverstolpe, 1955; Kallings & Laurell, 1957).

Strains of *S. typhimurium* differ in characters other than their reactions to typing phages. Kristensen, Bojlén & Faarup (1937) divided 361 strains into 18 types by tests of ability to ferment xylose, rhamnose, inositol and organic acids, and of ability to grow on defined media with citrate and tartrate isomers as sole carbon sources. This scheme was later extended to define 21 'fermentation types' which appeared stable and epidemiologically valid (Harhoff, 1948). Kallings & Laurell (1957) examined 393 strains from 120 outbreaks by both fermentation reactions and Lilleengen's typing phages. Some phage types correlated well with some fermentation types, but it was possible to subdivide 9 of 11 well-represented fermentation types by phage reactions, and 5 of the 14 well-represented phage types by fermentation pattern; this enabled strain identification to be carried beyond the point reached by either scheme alone.

In an investigation of strains of *S. typhimurium* of known phage type, provided by Dr E. S. Anderson (Enteric Reference Laboratory, Central Public Health Laboratory, Colindale) it was found (Stocker & Edgar, cited by Morgenroth & Duguid, 1968) that a minority of strains failed to grow on a defined medium with ammonium salts as sole nitrogen source, and that most of the nutritionally exacting strains grew well if provided with nicotinic acid or nicotinamide. Nearly all the strains requiring this growth factor were of Callow's phage type 1a/2. We describe here a survey of some variable biochemical characters in strains of *S. typhimurium* belonging to some of the commoner phage types, and the use of some of these characters, including nutritional character and the newly discovered property of sensitivity to *meso*-tartrate, for the subdivision of type 1a/2 into four biochemical subtypes – a subdivision which may have epidemiological value.

MATERIALS AND METHODS

Strains of Salmonella typhimurium

Dr E. S. Anderson kindly sent us 1537 strains of *S. typhimurium* selected from those submitted to the Enteric Reference Laboratory for phage typing in 1961 and 1962. These strains had been isolated from a variety of sources including cases of human and animal infection, drain and sewer swabs, and human and animal foods. They were selected from the seven more prevalent phage types and were as far as possible epidemiologically unrelated.

The strains were inoculated in a standard pattern on nutrient agar master plates, from which inocula were transferred by a multi-point replicator to solid media for the determination of sugar reactions, colicine production and nutritional character. Twenty-five test and two control strains were accommodated on each plate.

Media

Nutrient broth was prepared from a tryptic digest of beef, and was solidified with 15 g. Davis New Zealand agar per litre for plate culture, and 3·5 g./l. for soft agar.

The defined medium contained K_2HPO_4 10·5 g., KH_2PO_4 4·5 g., $MgSO_4$ 0·05 g., $(NH_4)_2SO_4$ 1 g., sodium citrate 0·47 g., glucose 2 g., Davis agar 15 g., and distilled water to 1 l. This medium was supplemented, when necessary, with nicotinic acid 1 μg./ml. In one series of tests the glucose was replaced by 5 g./l. of glycerol, to determine the ability of strains to use this substance as a source of carbon and energy. When testing strains for ability to grow in the presence of *meso*-tartrate, glucose was omitted from the defined medium, the concentration of sodium citrate was increased to 5 g./l. and 5 g./l. of sodium *meso*-tartrate were added.

Solid medium for the determination of sugar fermentation reactions contained Evans bacteriological peptone 20 g., NaCl 5 g. and Davis agar 15 g. in 925 ml. water. The initial pH was 7·2. Immediately before use this peptone agar was melted and the following solutions were added: 10 % sodium deoxycholate, 25 ml.; 1 % neutral red, 2·5 ml.; 20 % sugar, 50 ml. On this medium strains which ferment the sugar produce red colonies, while those which do not ferment the sugar produce opaque cream colonies. All plates were incubated at 37° C. and scored after 24 and 48 hr. incubation.

Tests for the production of colicine

Replicates of the master plate cultures were made on tryptic digest agar and incubated overnight at 37° C. The bacteria were killed with chloroform vapour and the plate covered with a layer of soft agar seeded with about 10^8 bacteria of an indicator strain. The indicator strains used, sensitive to all the common colicines (Ozeki, Stocker & Smith, 1962) were CL 142 (i.e. *E. coli* K 12-row of Fredericq) and CL 104 (a streptomycin-resistant derivative of *E. coli* strain ϕ).

RESULTS

Sugar fermentation tests

All 1537 strains of *S. typhimurium* were inoculated from the master plates to rhamnose, xylose and inositol fermentation plates.

Rhamnose fermentation

On rhamnose medium the strains were clearly differentiated into those able to ferment this sugar vigorously and those unable to ferment it at all. The rhamnose-fermenting strains gave deep red colonies, each surrounded by an opaque red zone about 0·5 cm. wide in which acid diffusing into the medium had changed the colour of the indicator and caused precipitation of deoxycholic acid. These strains have been termed 'Rha-A'. Those unable to ferment rhamnose (Rha⁻) showed no redness in the colony and no change in the surrounding medium. Of the 1537 strains tested, 1004 were Rha-A and 533 Rha⁻.

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Xylose fermentation

On xylose medium the results were more complex. One hundred and seventy strains gave a reaction like that described above as 'Rha-A', i.e. deep red colonies with a surrounding colour change and precipitate in the medium; these have been termed 'Xyl-A'. Three strains were unable to ferment xylose (Xyl⁻). The colonies of the remaining 1364 strains, although red, were not as deeply red as those of the Xyl-A strains, and there was no change in the appearance of the medium surrounding them. This appearance was attributed to a fermentation of xylose less vigorous than that effected by the Xyl-A strains, and was recorded as 'Xyl-a', to indicate the weaker acid reaction.

Inositol fermentation

None of the strains tested fermented inositol vigorously, as judged by appearances on the inositol indicator agar plates; to obtain consistent differentiation between fermenting and non-fermenting strains it was necessary to force the prongs of the replicator into the medium until they struck the bottom of the plate, so that growth occurred on the surface of the agar, in the depth of the stab and as a film in the interface between the agar and the bottom of the dish. Under these

Table 1. *Fermentation of Rhamnose, Xylose and Inositol*
by 1537 *strains of* Salmonella typhimurium

Sugar fermentation pattern			No. of strains
Rha	Xyl	Ino	
A	A	a	170
A	a	a	193
A	a	—	638
A	—	—	3
—	a	—	533
Total			1537

A, Strong acid production on sugar indicator agar plates. a, Weak acid production. —, No acid production.

conditions 363 strains gave red colonies corresponding to the 'weak' type of fermentation. The colour was most apparent in the film of growth in the interface between the agar and the bottom of the plate, but extended into the surface growth. Scoring was unequivocal when the colonies were viewed through the bottom of the dish. These strains were recorded as 'Ino-a'. It is likely that the stronger acid reaction in the depth of the medium results from the partially anaerobic conditions in that part of the colony. The remaining 1174 strains showed no evidence of acid production, and were recorded as 'Ino⁻'.

Table 1 records the fermentation of these sugars. Five fermentation patterns suffice to describe all the 1537 strains tested.

Nutritional characters

The nutritional requirements of these strains were examined by replicating from the master plates to three sets of plates of the defined medium. In one set the carbon source was 0·2 % (w/v) glucose with 0·047 % citrate. In another, the glucose was replaced by 0·5 % (w/v) glycerol. In the third, the glucose was omitted and the citrate concentration increased to 0·5 %. A total of 1334 strains were able to grow well on all these media. Six strains grew poorly or not at all on the plates containing glycerol, and one was unable to utilize citrate. Of the remaining 196 nutritionally exacting strains 174 needed nicotinic acid. These will be termed Nic⁻. A further 10 grew well when the media were supplemented with a pool which included thiamine, riboflavin, biotin, pantothenate and pyridoxin, and 12 when supplied with 0·1 % casein hydrolysate. We did not examine these last 22 strains for their ability to grow on media supplemented with individual growth factors or amino acids.

Colicinogeny

The incidence of colicinogeny in this series of strains was low, 1426 strains producing no colicine active on *E. coli* K 12 or on *E. coli* φ. Of the colicinogenic strains, 105 produced a colicine I, 3 colicine E 1, 2 colicine V and 1 strain an unidentified colicine.

The distribution of the characters we have been considering among the very small number of phage types represented in this series revealed that most of the phage types were remarkably homogeneous, especially in their sugar fermentation reactions. Thus, 152 of 154 strains recorded as belonging to phage type 2/12a had the fermentation pattern Rha-A Xyl-A Ino-a; 532 of 541 strains of type 2c/14 were unable to ferment rhamnose or inositol, and all gave the Xyl-a reaction; 206 of 209 strains belonging to type 1 var 5/U9 gave the pattern Rha-A Xyl-a Ino⁻. The incidence of colicinogeny was less than 18 % in any group. Except within phage type 1 a/2 the incidence of auxotrophy was so low as to make subdivision by this character unrewarding. However, it was also apparent that strains belonging to phage type 1 a/2 were not homogeneous in respect of the characters tested.

Biochemical differentiation within phage type 1 a/2

There were 315 strains belonging to phage type 1 a/2 in this series. All except one of these strains fermented rhamnose (Rha-A), and all gave the Xyl-a reaction. But only 38 strains were found to ferment inositol, and 163 were Nic⁻.

During an investigation of the ability of strains of *S. typhimurium* to utilize organic acids as sources of carbon it was noticed that some strains which grew well on a simple defined medium did not grow, or grew very poorly, when *meso*-tartrate was present in the medium. The degree of inhibition was greatly influenced by the composition of the basal medium, and the most consistent results were obtained by using the *meso*-tartrate medium described in the methods section. On this medium strains sensitive to *meso*-tartrate showed no growth after 48 hr.

incubation. Of 290 strains of phage type 1a/2 tested, 44 were inhibited by *meso*-tartrate.

When the characters we have been discussing were correlated four major groups emerged (Table 2). Thirty-six strains fermented inositol, were resistant to *meso*-tartrate and were Nic⁺ (group 1). Sixty-four strains were Ino⁻, *meso*-tartrate-resistant and Nic⁺ (group 2). Forty-two strains were Ino⁻, sensitive to *meso*-tartrate and Nic⁺ (group 3). One hundred and forty-four strains were Ino⁻, *meso*-tartrate-resistant and Nic⁻ (group 4).

Table 2. *Biochemical subdivision of* 290 *strains of*
Salmonella typhimurium *phage type* 1a/2

Group	Rha	Xyl	Ino	*Meso*-tartrate	Nic	No. of strains
1	A	a	a	R	+	36
2	A	a	—	R	+	64*
3	A	a	—	S	+	42†
4	A	a	—	R	—	144
Other	—	a	—	R	—	1
	A	a	—	?	—	1‡
	A	a	a	R	—	1
	A	a	a	S	+	1

Sugar reactions: A, Strong acid production on sugar indicator agar plates; a, weak acid production; —, no acid production.

Meso-tartrate: R, resistant; S, sensitive to inhibition by *meso*-tartrate, tested by ability to grow on a defined medium with citrate as the energy source and containing 5 g./l. sodium *meso*-tartrate.

Nic: +, able to grow on a medium devoid of nicotinic acid; —, unable to grow in the absence of nicotinic acid.

* One strain auxotrophic for thiamine.
† Two strains unable to utilize glycerol.
‡ This strain cannot utilize citrate.

All except four of the 290 strains fell into one or other of these groups. One of the four, which in all other respects belonged to group 4, was unable to ferment rhamnose. One was unable to use citrate as a carbon source, and thus could not be scored for tartrate sensitivity, but as it was Nic⁻ it probably belongs to group 4. The other two anomalous strains were both able to ferment inositol, but one was Nic⁻ and the other was inhibited by *meso*-tartrate. In addition, one strain in group 2 was unable to grow in the absence of thiamine, and two strains in group 3 were unable to utilize glycerol as sole carbon source. These results are shown in Table 2.

The suggestion that these groups represent epidemiologically valid subtypes of phage type 1a/2 received support from the examination of 74 additional strains of this type. All were isolated within a period of a few weeks from what at first appeared to be a single widespread outbreak involving adjacent areas of the counties of Lancashire and Yorkshire. Biochemical tests showed that 37 of these strains belonged to group 4, and all these had been isolated in Lancashire; 36

strains belonged to group 2, and had been isolated in Yorkshire; one strain, also from Yorkshire, belonged to group 3. Epidemiological assessment in the light of these findings suggested that we were observing two simultaneous outbreaks – a group 4 outbreak centred on the city of Manchester and a group 2 outbreak centred on Leeds, and that the one strain belonging to group 3 was from a sporadic case unconnected with either outbreak. We are grateful to Dr E. S. Anderson, of the Enteric Reference Laboratory, for giving us the opportunity of examining these strains during the development of the outbreak.

Inhibition of Salmonella typhimurium by meso-tartrate

The observation that some strains of *S. typhimurium* which are able to grow well on a simple defined medium are unable to grow when *meso*-tartrate is added demanded further investigation. No strains were found to use *meso*-tartrate as a sole source of carbon, and the inhibition of sensitive strains was to some extent dependent on the other carbon sources present. On defined media with citrate, glycerol, maltose or lactate as sole carbon sources there was consistent and complete inhibition, but inhibition was neither complete nor consistent when the medium contained glucose, glucose and citrate, or galactose.

Growth was not inhibited by *meso*-tartrate when as little as 0·01 % of casein hydrolysate was added to the medium, but it proved remarkably difficult to demonstrate that this effect was due to the action of any particular amino acid or combination of amino acids. The L isomers of alanine, glycine, serine, leucine, isoleucine, valine, phenylalanine, tyrosine, tryptophane, lysine, arginine, methionine and cysteine were added to the medium alone and in various combinations, in concentrations up to 0·1 %, but none caused any reversal of inhibition by *meso*-tartrate. When glutamic acid, proline or histidine were added to the medium sensitive strains were still inhibited after 18 hr. incubation, but showed some growth, not as vigorous as that of the uninhibited strains, after 48 hr. Aspartic acid, alone among the amino acids, caused a significant, although not complete, reversal of the effect of *meso*-tartrate within 18 hr., and complete reversal after 48 hr. incubation.

The reversal of tartrate inhibition by these amino acids may be related to the readiness with which bacteria can use them as sources of carbon and energy. Proline, glutamic acid and aspartic acid can all replace glucose in the minimal medium, although they do not induce as vigorous growth as does glucose. But proline is superior to aspartic acid in this respect, although much less effective in reversing inhibition by *meso*-tartrate, while histidine is a very poor carbon and energy source.

DISCUSSION

A common and widespread bacterial species can be expected to evolve into subspecies varying in any of a number of different characters, such as ability to ferment a sugar or to be attacked by a phage or to produce different antigens. Different epidemiologically valid typing schemes can be devised using any variable

characters of a bacterial species to define groups of strains (types) which are alike in the characters studied.

Duguid, Anderson & Campbell (1966) drew attention to a correlation between the characters of fimbriation and rhamnose fermentation in *S. typhimurium* which led to the description of two groups of Rha⁻ strains of this organism. In the first group, designated FIRN and of frequent occurrence, the Rha⁻ character is accompanied by failure to ferment inositol and inability to produce fimbriae. The second, or non-FIRN, group contains all other Rha⁻ strains; these are relatively uncommon. Morgenroth & Duguid (1968) have shown that the sites of the Rha⁻ mutations differ in the FIRN and non-FIRN groups, but that within each group the mutation appears to be located at the same site in every strain.

In another study Stocker & Edgar (1959) showed that 15 wild Nic⁻ strains of *S. typhimurium* of phage type 1a/2 all appeared to be mutant at the same site, different from but linked to the site of mutation of a Nic⁻ line of *S. typhimurium* LT 2 derived in the laboratory.

It is likely that all strains in any one of these groups are descendants of a single bacterium (that is, each group is a clone), and this supposition is strengthened by the fact that the strains have other characters in common. However, mutations, even at identical loci, can occur in different lines at different times, and a single genetic change might result in alteration in several apparently unrelated characters; for example, a mutation affecting carbohydrate metabolism may alter the antigenic structure of a bacterium, and consequently affect its sensitivity to phages. Such a change may be reflected in differently based typing schemes, suggesting a spuriously close relationship between strains of diverse origins.

The reactions of bacterial strains to typing phages may be determined, at least in part, by the carriage of temperate phages, or of bacterial plasmids such as R factors and colicinogenic factors, or of the transfer factors often associated with such plasmids (Anderson & Lewis, 1965; Anderson, 1966). Acquisition or loss of these agents provides another means by which originally similar strains may come to differ, or different strains come to resemble one another.

The use of two or more typing methods may often allow a greater precision in strain differentiation than can be achieved by using one method alone, at the expense of an increase in complexity which may become self-defeating. Typing schemes must be practical. We suggest, however, that a limited use of biochemical characters may be of value in subdividing common phage types of *S. typhimurium*, and we describe one such subdivision for one phage type of this serotype.

The use of the multipoint replicator greatly reduced the work involved, a consideration of importance when many strains have to be typed in an epidemic, and the testing of sugar fermentation reactions on a solid indicator medium permitted the differentiation of 'strong' and 'weak' fermentation of xylose, a distinction which cannot be made when using peptone-water sugar media.

REFERENCES

ANDERSON, E. S. (1965). The Phage typing of Salmonellae other than *S. typhi*. In *The World Problem of Salmonellosis*, pp. 89–110. Ed. E. van Oye. The Hague: Dr W. Junk, Publ.

ANDERSON, E. S. (1966). Influence of the Δ transfer factor on the phage sensitivity of salmonellae. *Nature, London* **212**, 795–9.

ANDERSON, E. S. & LEWIS, M. J. (1965). Characterization of a transfer factor associated with drug resistance in *Salmonella typhimurium*. *Nature, London* **208**, 843–9.

CALLOW, B. R. (1959). A new phage-typing scheme for *Salmonella typhimurium*. *Journal of Hygiene* **57**, 346–59.

DUGUID, J. P., ANDERSON, E. S. & CAMPBELL, I. (1966). Fimbriae and adhesive properties in salmonellae. *Journal of Pathology and Bacteriology* **92**, 107–38.

FELIX, A. & CALLOW, B. R. (1943). Typing of paratyphoid B bacilli by means of Vi bacteriophage. *British Medical Journal* ii, 127–30.

FELIX, A. & CALLOW, B. R. (1951). Paratyphoid B Vi-phage typing. *Lancet* ii, 10–14.

HARHOFF, N. (1948). *Gastroenteritis baciller af Salmonellagruppen i Danmark*. Copenhagen: Nyt Nordisk Forlag.

KALLINGS, L. O. & LAURELL, A.-B. (1957). Relation between phage types and fermentation types of *Salmonella typhimurium*. *Acta pathologica et microbiologica scandinavica* **40**, 328–42.

KRISTENSEN, M., BOJLÉN, K. & FAARUP, C. (1937). Bacteriologisk-epidemiologiske Erfaringer om Infektioner med Gastroenteritis baciller af Paratyfusgruppen. *Bibliotek for Laeger* **129**, 310–75.

LILLEENGEN, K. (1948). Typing of *Salmonella typhimurium* by means of bacteriophage. *Acta pathologica et microbiologica scandinavica*, Supplement 77.

LUNDBECK, H., PLAZIKOWSKI, U. & SILVERSTOLPE, L. (1955). The Swedish Salmonella outbreak of 1953. *Journal of Applied Bacteriology* **18**, 535–48.

MORGENROTH, A. & DUGUID, J. P. (1968). Demonstration of different mutational sites controlling rhamnose fermentation in FIRN and non-FIRN rha⁻ strains of *Salmonella typhimurium*: an essay in bacterial archaeology. *Genetical Research* **11**, 151–69.

OZEKI, H., STOCKER, B. A. D. & SMITH, SYLVIA (1962). Transmission of colicinogeny between strains of *Salmonella typhimurium* grown together. *Journal of General Microbiology* **28**, 671–87.

STOCKER, B. A. D. & EDGAR, J. B. (1959). *Annual Report of the Board of Governors of the Lister Institute*, p. 13.