J. Hyg., Camb. (1982), **88**, 413
Printed in Great Britain

Differential typing of *Salmonella agona*: type divergence in a new serotype

By RUTH BARKER, D. C. OLD
Bacteriology Department, University of Dundee Medical School, Ninewells Hospital, Dundee DD1 9SY

AND ZOFIA TYC
Bacteriology Department, National Institute of Hygiene, Warsaw 36, Poland

(*Received* 20 *November* 1981; *accepted* 1 *December* 1981)

SUMMARY

An international collection of 419 isolates of *Salmonella agona* was phage typed, biotyped and colicine typed. Of 16 recognized phage types, 15 were represented. Three phage types (I, V and XVI) accounted for 84% of all isolates, were widely distributed and may be interconvertible. Biotyping afforded little type differentiation; thus 92·6% of the isolates belonged to biotype 1a. A rhamnose non-fermenting variant line (of biotype 5a) became established in Zaire from 1979 to 1980. A maltose late-fermenting line of biotype 1a, isolated in Scotland in 1974, did not thereafter become established. Two Col^+ lines (producing colicine Ib) accounted for 45 of 68 colicinogenic isolates. The implication of type diversification and the phylogenetic significance of these findings are discussed.

INTRODUCTION

About 90% of the more than 1700 recognized serotypes of salmonella are isolated so infrequently and so intermittently throughout the world that they are considered to be 'exotic' serotypes (Turnbull, 1979). However, it is clear from the long-term surveillance of infections due to salmonellas that even rare serotypes can suddenly emerge from obscurity to become established epidemic strains of world-wide significance. Thus, *Salmonella agona* (serotype 4, 12; fgs:-), first isolated from cattle in Ghana and described by Guinée, Kampelmacher & Willems (1961), was seldom isolated thereafter until, in 1971, it spread in epidemic fashion throughout several countries simultaneously (Clark *et al.* 1973). Its appearance in Israel, the Netherlands, UK and USA was attributed to imported Peruvian fish meal used in the preparation of animal feeds (Lee, 1974). Its persistence in livestock, such as pigs and poultry, has probably resulted from the recycling of treated animal wastes as feed (Turnbull, 1979) and its spillover to man has been vast. Now, in the decade since 1971, *S. agona* has become clearly established as a major epidemic serotype, regularly in the 'top ten' of serotypes isolated worldwide from man and animals (Turnbull, 1979; Rowe *et al.* 1980).

Each of the carbohydrates D-fructose, D-mannose, D-melezitose, D-melibiose, D-raffinose, D-ribose and L-sorbose was tested for its ability to serve as sole carbon and energy source for growth in mineral salts agar medium (Davis & Mingioli, 1950) with 0·3 % (w/v) carbohydrate.

Colicine typing

Colicine-producing cultures were detected on Nutrient Agar (Oxoid CM 3) by an agar overlay method (Ozeki, Stocker & Smith, 1962) with indicator strain CL 104 of *Escherichia coli*. Colicines were identified in tests with ten indicator strains of *E. coli* of known specific resistance to different colicine(s) (Barker, 1980).

RESULTS

Phage typing

Phage typing divided 398 isolates of *S. agona* into 15 phage types (Table 2); there was no representative in this series of phage type XIV and 21 cultures were untypable (NT). The three commonest phage types, each containing between 17 and 40 % of the cultures, were I (168 isolates), V (110) and XVI (73). Together, these three types, widely distributed in most countries (Table 2), accounted for 84 % of all isolates. The remaining 47 isolates belonged to one of twelve phage types, none of which contained more than 10 cultures. The culture of the index strain from Ghana was the sole representative of phage type IX.

Biotyping

There were 388 cultures, including that from Ghana, that gave positive results in tests with each of the primary and secondary substrates of the biotyping scheme (Duguid *et al.* 1975) and, hence, were of biotype 1a. Among the remaining 31 isolates, only six other biotypes were represented: one culture was non-fimbriate (biotype 1b); one was non-motile (1c); of the seven auxotrophs four required cysteine (1y) and one each required arginine, or methionine or another, unidentified, growth factor (1z); one was *d*-tartrate non-fermenting (3a); and 21 were rhamnose non-fermenting (5a).

When tested for their ability to utilize or ferment the additional 11 sugars or sugar alcohols not included in the standard biotyping scheme, each of the 419 cultures utilized arabinose, dulcitol, fructose, mannose, melibiose, raffinose, ribose and sorbitol; none utilized melezitose or sorbose. In tests in peptone water medium, 383 cultures fermented maltose in 24 h; the other 36 cultures, 35 of which belonged to biotype 1a and one to 1y, did not ferment maltose in 24 h, but did so in 48 h.

Colicine typing

There were 68 cultures (16·2 %) that produced colicines which were identified as: Ia (by 9 cultures), Ib (56), E1 (2) and B(1).

Correlation of phage type, biotype and colicine type with source

The phage types of the 419 cultures of *S. agona* and their correlation with biotypes and colicine types are shown in Table 3. The few isolates of the variant

Table 2. *Distribution of phage types of* S. agona *from different countries*, 1961–80

Period of isolation	Country	Number of cultures of phage type																	
		I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV	XV	XVI	NT	All
1961	Ghana	—	—	—	—	—	—	—	—	1	—					—	—	-	1
1973–80	Scotland	109	—	—	1	49	—	—	—	—	1	3	2	—	—	—	23	2	190
1976–80	Sweden	—	—	—	—	13	—	1	—	—	1	—	1	1	—	—	4	2	23
1976–9	Denmark	2	—	—	—	—	—	—	—	—	—	—	—	—	—	—	5	1	8
1976–7	Poland	3	3	—	3	3	2	—	3	—	—	—	3	3	—	—	—	—	23
1977	Czechoslovakia	—	—	—	—	2	—	—	—	—	—	—	—	—	—	—	—	2	4
1978–9	Japan	4	—	—	—	4	—	—	—	—	—	—	—	—	—	—	1	1	10
1978–9	West Germany	6	—	—	—	1	—	—	—	—	—	—	4	—	—	—	2	—	13
1979–80	Belgium	13	-	1	—	12	—	—	—	—	2	—	—	—	—	1	6	1	36
1979–80	England	12	—	1	—	5	—	1	—	—	—	—	—	—	—	—	2	1	22
1979–80	France	10	—	—	—	11	—	—	—	—	—	—	—	—	—	—	1	8	30
1979–80	Zaire	2	—	—	—	6	—	—	—	—	—	—	—	—	—	7	20	1	36
1979	Switzerland	3	—	—	—	1	—	—	—	—	—	—	—	—	—	1	5	1	11
1980	Bulgaria	—	—	—	—	1	—	—	—	—	—	—	—	—	—	—	—	—	1
1980	Netherlands	4	—	—	—	2	—	—	—	—	—	—	—	—	—	—	4	1	11
	All	168	3	2	4	110	2	2	3	1	4	3	10	4	0	9	73	21	419
	Poland and Roumania*	587	111	—	202	49	173	0	321	—	—	—	12	106	14	0	0	...	1575

NT = not typable with phages; — = no representative.

* From Tyc (1980).

Table 3. *Phage types of* 419 *cultures of* S. agona *and their correlation with biotypes and colicine types*

Phage type	Biotype (and no. of cultures)	Colicine type* (and no. of cultures)
I	1a (164), 1b (1), 1c (1), 1y (2)	Ia (3), Ib (23), E1 (2), B (1), 0 (139)
II	1a (2), 3a (1)	0 (3)
III	1a (2)	0 (2)
IV	1a (3), 1z (1)	Ia (1), 0 (3)
V	1a (73), 1aML (34), 1yML (1), 1z (2)	Ia (3), Ib (8), 0 (99)
VI	1a (2)	0 (2)
VII	1a (2)	0 (2)
VIII	1a (3)	0 (3)
IX	1a (1)	0 (1)
X	1a (4)	0 (4)
XI	1a (3)	0 (3)
XII	1a (10)	0 (10)
XIII	1a (4)	0 (4)
XV	1a (9)	Ib (1), 0 (8)
XVI	1a (52), 1aML (1), 5a (20)	Ia (1), Ib (20), 0 (52)
NT	1a (19), 1y (1), 5a (1)	Ia (1), Ib (1), 0 (19)

NT = not typable with available phages.
ML = fermented maltose in 48 h, but not in 24 h.
* 0 = no colicines detected.

Table 4. *Characters of two biotype variant groups of* S. agona*

Origin	Biotype	Phage type	Colicine type	No. of isolates
Zaire (1979–80)	5a	XVI	Col^+ (Ib)	19
	5a	XVI	Col^-	1
	5a	NT	Col^+ (Ib)	1
	1a*	I	Col^+ (Ib)	2
	1a*	V	Col^+ (Ib)	6
	1a*	XV	Col^-	7
Scotland (1978–80)	1aML	V	Col^-	34
	1yML	V	Col^-	1
	1aML	XVI	Col^-	1
	1a*	I	Col^-	42
	1a*	I	Col^+ (E1)	2
	1a*	V	Col^-	9
	1a*	XVI	Col^-	11

ML = fermented maltose in 48 h but not in 24 h.
* For comparison, characters of presumed biotype ancestors.

biotypes 1b, 1c, 1y and 1z were found mostly in the common phage types I or V. From epidemiological evidence, it seemed likely that each of the four cysteine auxotrophs, which were from unrelated foci, probably arose independently. Furthermore, the members of biotype 1z were also independent as judged by their different growth factor requirements. Tests with three human isolates of phage

https://doi.org/10.1017/S0022172400070273 Published online by Cambridge University Press

type II isolated in Poland in 1977 showed that two were of biotype 1a and the third was *d*-tartrate non-fermenting (3a).

From human and environmental sources in an outbreak in Scotland in 1974, 36 maltose late-fermenting cultures of biotype 1a (or its auxotrophic variant, 1y) were recovered; 35 were of phage type V, and one was of phage type XVI.

Nineteen of 21 rhamnose non-fermenting cultures of biotype 5a isolates in Zaire in 1979–80 were of phage type XVI and Col^+ (1b). Of the two atypical cultures of biotype 5a, one was of phage type XVI and Col^-; the other was Col^+ (1b) but not typable with the typing phages.

DISCUSSION

In a previous phage-typing study of 1575 isolates of *S. agona* from Poland and Roumania, Tyc (1980) established the potential epidemiological value of phage typing for discriminating different types of *S. agona*, and demonstrated the constancy of the lytic patterns obtained on repeated testing of cultures and the *in vitro* stability of the phage types on storage. The number of isolates of the different phage types in that earlier series is shown in a footnote to Table 2. Though the present series of 419 cultures was isolated in 15 different countries, additional information from some donors suggested that many patients had become infected in countries other than those of isolation. Thus, 31 source countries were implicated: 13 in Europe, 4 in Central or South America; 6 in Africa; and 8 in Asia, indicating the international nature of the collection. This study revealed seven new phage types (III, VII, IX, X, XI, XV and XVI) not present among the Polish isolates (Tyc, 1980). In most countries the common phage types were I, V and XVI which, together, accounted for 88% of the typable isolates. In each country from which reasonable numbers of isolates were tested, there were representatives of from 5 to 9 phage types, even among isolates recovered over short periods (Table 2). Thus, in the Scottish series, collected over eight years and including some of our earliest isolates, the seven phage types present seemed to be well-established types.

Each of the other two typing systems used in isolation was less successful than phage typing for type discrimination in *S. agona*. Nevertheless, they provided further information about diversity in the serotype and indicated possible relationships among cultures of different phage types.

The biotyping scheme originally devised for *S. typhimurium* (Duguid *et al.* 1975), though convenient for biotyping *S. agona*, afforded little type differentiation. Thus 92·6% of the isolates, including cultures of the index strain, were of biotype 1a; the remainder (7·4%) belonged to only six other biotypes. The likeliest explanation of those cultures (usually single isolates) which differed in one biotype character (i.e. 1b, 1c, 1y, 1z, 3a) from the majority type (1a) is that they are variants derived by mutation to loss of functions from a strain of biotype 1a during its epidemic spread *in vivo*. The only well-represented biotype variant (in the classical scheme) was 5a; the 21 isolates from Zaire (1979–80) were probably replicate isolates of the same epidemic clone, and the finding that > 95% of them were of phage type

XVI is in line with that suggestion. Other isolates recovered from human and animal sources in Zaire at the same time were examined; their characters and those of cultures of biotype 5a are shown in Table 4. If, as seems probable, the rhamnose non-fermenting line arose from an ancestral bacterium of biotype 1a of contemporary origin, it is of interest that phage type XVI, characteristic of biotype 5a, was not a phage type (I, V and XV) encountered among Zaire isolates of biotype 1a. Since the putative event that rendered biotype 5a cultures phage type XVI might have occurred any time after the emergence of the first biotype 5a culture, the phage-type anomaly among cultures of biotypes 1a and 5a should not be overstated. Furthermore, because isolates of biotype 5a from before 1979 were not available, the problem remains unresolved. However, among this group of isolates from Zaire, colicinogeny was common (80%), but rare in the serotype generally (Table 3). Thus, the finding that these isolates of types 1a and 5a were usually Col^+ (1b) indicates further their relatedness.

Among the additional substrates tested for dicrimination of minority types of *S. agona*, only maltose was useful. Maltose, originally proposed by Edwards (1936) for the discrimination of types in *S. typhimurium*, differentiated too few (2%) strains of the non-fermenting type among the large series of *S. typhimurium* examined by Duguid *et al.* (1975) to justify its use even as a secondary test. It discriminated *ca.* 8·5% of the isolates in this series of *S. agona* as maltose late-fermenting (ML) but should not be included for routine biotyping because each of the 36 ML isolates of biotype 1a (or 1y) was recovered in Scotland in 1974, i.e. were probably members of the same clone. This group of isolates, all but one of which was of phage type V, formed a distinct cluster (Table 4). Other isolates of the presumed ancestral type (1a) present in Scotland at the same time were of phage type I; later isolates were of phage types V and XVI (Table 4). Thus, the presence of isolates of biotype 1a and phage type V can be explained: either by the reversion of the ML line to prompt maltose fermentation, or by a change of phage type (from I to V) of the maltose-prompt fermenters. If the latter explanation is correct, the 1aML line, though successful for 6 months, failed to establish itself permanently as an epidemic strain.

The detection of relatively few variant biotypes of *S. agona*, often represented by single isolates, suggests that this serotype is of recent origin, a conclusion in keeping with its recognition in only the past twenty years. That finding is in marked contrast to that of *S. typhimurium*, a serotype of ancient origin and in which there has been considerable biotype diversification as judged by the existence of at least 166 full biotypes (Duguid *et al.* 1975; Barker, Old & Sharp, 1980). Furthermore, because so many of the variant biotypes of *S. typhimurium* themselves subsequently became established epidemic types, probable relationships among cultures of different phage types within closely related biotypes could be predicted (Anderson *et al.* 1978; Barker, Old & Sharp, 1980; Barker & Old, 1980). Although there has been little biotype diversification in *S. agona* in its short history, it is of interest, nevertheless, that among each of the only two biotype variant groups of possible importance (biotypes 1aML and 5a), and their presumed ancestors (Table 4), members generally belonged to phage types I, V and XVI. That finding might

reflect only that these are the common phage types; it may, however, suggest that these are three closely related, interconvertible phage types. Furthermore, Col Ib seems not to be the determinant of phage types I, V or XVI, as judged by the presence of Col^- variants of each (Table 4).

Colicine typing was helpful for another group of isolates, namely 30 isolates of biotype 1a (phage type I) isolated in Dundee, Scotland (1978–80); 17 were Col^+ (Ib) and 13 were Col^-. In the earlier stages of that outbreak, Col^+ and Col^- isolates were found; after mid-1979, all isolates were Col^+ (Ib), suggesting, perhaps, the establishment of the Col^+ line. Thus, although the percentage of Col^+ isolates of *S. agona* (16%) was slightly higher than that reported for *S. typhimurium* (12%; Barker, 1980), only two lines accounted for nearly 70% of the Col^+ isolates. Other Col^+ isolates of *S. agona* apparently did not become established.

We have applied multiple typing techniques in epidemiological studies of other enterobacteria: *S. typhimurium* (Duguid *et al.* 1975; Anderson *et al.* 1978; Barker *et al.* 1980); *E. coli* (Crichton & Old, 1980; Old *et al.* 1980); and *Shigella sonnei* (Helgason & Old, 1981; Old, Helgason & Scott, 1981). In these examples, accurate strain identification with two or more differential typing techniques indicated probable relationships among strains which, during their epidemic spread, had undergone variation in their type characters.

With *S. typhimurium*, biotyping allowed the proposal of a hypothetical genealogical tree that attempted to explain the relationships of the different biotypes of *S. typhimurium* (Duguid *et al.* 1975). A series of physiological and genetic studies confirmed that the relationships of biotypes as outlined in the tree were essentially correct (Morgenroth & Duguid, 1968; Old & Duguid, 1979; Old & Mortlock, 1979; May & Old, 1980; Old, Dawes & Barker, 1980). That analysis, however, was retrospective, and the hypothesis that all biotypes of *S. typhimurium* were descendants of an ancestral bacterium of biotype 1a could not be proved in the absence of known cultures of the index isolate of that serotype.

This study suggests that epidemic strains of *S. agona* during their spread may show variation *in vivo* not only in their biotype characters: for example single isolates of biotypes 1b, 1c and 1y among Scottish strains of 1a; but also in phage-type characters, such as single isolates of the biotype 5a clone from Zaire and the 1aML clone from Scotland had become, respectively, untypable, NT, with the phages (from XVI) and type XVI (from V). Thus, although it might seem in the short term that phage typing will be the method most likely to aid the rapid tracing of types of *S. agona* in outbreaks, it must be remembered that the combined use of several techniques is invaluable because it detects such *in vivo* variation. In the longer term, therefore, it will be of interest to monitor in this serotype the establishment of future biotype variants as epidemic strains, and note that they were derived from an ancestral bacterium of biotype 1a.

We thank Professor J. P. Duguid and Dr S. Kałużewski for helpful discussions and encouragement. For the gifts of strains and information about them, we gratefully acknowledge the co-operation of: Professor H. Brandis, Dr B. Brest Nielsen, Dr G. Ghysels, Dr R. W. A. Girdwood, Dr P. A. M. Guinée, Professor G.

Lebek, Dr L. Le Minor, Professor A. A. Lindberg, Dr D. Mollov, Dr R. Sakazaki, Dr J. Šourek, and Dr C. Wray. We thank Mrs J. Robertson and Miss T. Mainou for technical assistance.

REFERENCES

ANDERSON, E. S. (1964). The phage typing of salmonellae other than *S. typhi*. In *The World Problem of Salmonellosis* (ed. E. van Oye), pp. 89–110. The Hague: Dr W. Junk.

ANDERSON, E. S. WARD, L. R., DE SAXE, M. J., OLD, D. C., BARKER, R. & DUGUID, J. P. (1978). Correlation of phage type, biotype and source in strains of *Salmonella typhimurium*. *Journal of Hygiene* **81**, 203–217.

BARKER, R. M. (1980). Colicinogeny in *Salmonella typhimurium*. *Journal of General Microbiology* **120**, 21–26.

BARKER, R. & OLD, D. C. (1980). Biotypes of strains of *Salmonella typhimurium* of phage types 49, 204 and 193. *Journal of Medical Microbiology* **13**, 369–371.

BARKER, R., OLD, D. C. & SHARP, J. C. M. (1980). Phage type/biotype groups of *Salmonella typhimurium* in Scotland 1974–6: variation during spread of epidemic clones. *Journal of Hygiene* **84**, 115–125.

CLARK, G. M., KAUFMANN, A. F., GANGAROSA, E. J. & THOMPSON, M. A. (1973). Epidemiology of an international outbreak of *Salmonella agona*. *Lancet* ii, 490–493.

CRICHTON, P. B. & OLD, D. C. (1980). Differentiation of strains of *Escherichia coli*: multiple typing approach. *Journal of Clinical Microbiology* **11**, 635–640.

DAVIS, B. D. & MINGIOLI, E. S. (1950). Mutants of *Escherichia coli* requiring methionine or vitamin B12. *Journal of Bacteriology* **60**, 17–28.

DUGUID, J. P., ANDERSON, E. S., ALFREDSSON, G. A., BARKER, R. & OLD, D. C. (1975). A new biotyping scheme for *Salmonella typhimurium* and its phylogenetic significance. *Journal of Medical Microbiology* **8**, 149–166.

EDWARDS, P. R. (1936), Fermentative varieties of *Salmonella aertrycke*. *Journal of Infectious Diseases* **58**, 225–229.

GUINÉE, P. A. M. & VAN LEEUWEN, W. J. (1978). Phage typing of Salmonella. In *Methods in Microbiology*, vol. XI (ed. T. Bergan and J. R. Norris), pp. 157–191. London, New York, San Francisco: Academic Press.

GUINÉE, P. A. M., KAMPELMACHER, E. H. & WILLEMS, H. M. C. C. (1961). Six new *Salmonella* types, isolated in Ghana. *Antonie van Leeuwenhoek* **27**, 469–472.

HELGASON, S. & OLD, D. C. (1981). Comparison of four methods of differential typing of isolates of *Shigella sonnei*. *Journal of Hygiene* **87**, 339–356.

LEE, J. A. (1974). Recent trends in human salmonellosis in England and Wales: the epidemiology of prevalent serotypes other than *Salmonella typhimurium*. *Journal of Hygiene* **72**, 185–195.

MAY, S. G. & OLD, D. C. (1980). *Meso*-tartrate resistance and phylogenetic relationships of biotypes of *Salmonella typhimurium*. *Genetical Research* **36**, 327–337.

MORGENTROTH, 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**, 151–169.

OLD, D. C. & DUGUID, J. P. (1979). Transduction of fimbriation demonstrating common ancestry in FIRN strains of *Salmonella typhimurium*. *Journal of General Microbiology* **112**, 251–259.

OLD, D. C. & MORTLOCK, R. P. (1979). Phylogenetic relationships between different D-xylose biogroups in wild-type *Salmonella typhimurium* strains and a suggested evolutionary pathway. *Journal of Applied Bacteriology* **47**, 167–174.

OLD, D. C., DAWES, P. F. H. & BARKER, R. M. (1980). Transduction of inositol-fermenting ability demonstrating phylogenetic relationships among strains of *Salmonella typhimurium*. *Genetical Research* **35**, 215–224.

OLD, D. C., CRICHTON, P. B., MAUNDER, A. J. & WILSON, M. I. (1980). Discrimination of urinary strains of *Escherichia coli* by five typing methods. *Journal of Medical Microbiology* **13**, 437–444.

Old, D. C., Helgason, S. & Scott, A. C. (1981). Discrimination by multiple typing of isolates of *Shigella sonnei* in Dundee (1971–6). *Journal of Hygiene* **87**, 357–368.

Ozeki, H., Stocker, B. A. D. & Smith, S. M. (1962). Transmission of colicinogeny between strains of *Salmonella typhimurium* grown together. *Journal of General Microbiology* **28**, 671–687.

Rowe, B., Hall, M. L. M., Ward, L. R. & de Sa, J. D. H. (1980). Epidemic spread of *Salmonella hadar* in England and Wales. *British Medical Journal* **280**, 1065–1066.

Turnbull, P. C. B. (1979). Food poisoning with special reference to Salmonella – its epidemiology, pathogenesis and control. *Clinics in Gastroenterology* **8**, 663–714.

Tyc, Z. (1980). Schemat bakteriofagowego różnicowania pałeczek *Salmonella agona*. *Medycyna doświadczalna i mikrobiologia* **32**, 17–22.