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Variations in biochemical phenotypes and phage types of *Salmonella enteritidis* in Germany 1980–92

M. KATOULI[1], R. H. SEUFFER[2], R. WOLLIN[3], I. KÜHN[1,3] AND R. MÖLLBY[1]

[1] *Department of Bacteriology, Karolinska Institute, Box 60400, S-104 01 Stockholm, Sweden*

[2] *Laboratory for Microbiology and Epidemiology, D-7410 Reutlingen 11, Germany*

[3] *Department of Bacteriology, the National Bacteriology Laboratory, S-105 21 Stockholm, Sweden*

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SUMMARY

The Phene Plate system for typing *Salmonella* serotypes (PhP-S) is a simple automated typing method based on biochemical fingerprinting. It gives a quantitative value of the metabolism of various substrates by measuring the speed and intensity of each reaction. The 'biochemical fingerprint' of each isolate is used to calculate similarities among the tested strains with a personal computer program. We used this system to examine a collection of 86 strains of *Salmonella enteritidis* isolated from human sporadic cases in Germany between 1980 and 1992. Twenty-three biochemical phenotypes (BPTs) consisting of 9 common (C) and 14 single (S) BPTs were identified. BPTs C2 and C4 containing 20 and 36 strains respectively accounted for 65% of the isolates. Strains of BPT C2 were found over a wide period of time whereas strains of BPT C4 were isolated during the period between 1988 and 1992. With phage typing, 11 discrete phage types (PTs) and 18 strains designated as non-specific type (NST) were identified. PTs 4 and 8 with 39 and 17 strains respectively were the dominant PTs. Strains of PT 8 were isolated over a wide period of time whereas all (except one) strains of PT 4 were isolated between 1988 and 1992. Combination of biochemical fingerprinting and phage typing divided the strains into 25 phenotypes (BPT : PTs). Whilst phenotype C2 : 8 was found over a number of different years, phenotype C4 : 4 was isolated only between 1988 and 1992. These findings indicate the presence of one persistent and one recently emerged phenotype among *S. enteritidis* strains in Germany. Although both methods identified the presence of both major and less common types, biochemical fingerprinting with the PhP-S system also provided information about relationships between the strains.

INTRODUCTION

Salmonella enterica serotype Enteritidis is an increasing cause of food-borne outbreaks in the United States [1–3], the United Kingdom [4, 5] and central parts of Europe [6, 7]. Poultry meat [8, 9] and hens' eggs [10–13] are regularly identified as sources of infection. Phage typing provides a rapid method for strain

discrimination and has been used extensively in epidemiological studies [6, 14]. During the past few years, phage type (PT) 4 has been responsible for a large proportion of outbreaks and sporadic cases in Europe [15–17]. To confirm the epidemiological relationship between different strains other methods such as antimicrobial-susceptibility testing and plasmid profile analysis have been employed [18–20]. These methods, although they are discriminatory, may not always be useful since many *S. enteritidis* strains show little or no resistance to antimicrobial drugs [5] or contain, if present, plasmids of similar size [21, 22].

An easy to perform and computerized biochemical fingerprinting method for typing of *Salmonella* serotypes has been developed (the PhP-S system). The system measures the kinetics of several biochemical reactions of bacteria grown in liquid medium in microtitre plates and yields, for each strain, a set of quantitative data which can be used to establish an identity level among the tested strains using a personal computer program. For each *Salmonella* serotype a battery of specific tests with optimal discrimination has been chosen. The system has been previously evaluated for discriminatory power, reproducibility [23] and stability of the biochemical markers [24]. It has also been used in epidemiological studies of various serotypes of *Salmonella* [25, 26]. The aim of the present study was to investigate the usefulness of this system alone and together with phage typing for studying the phenotypic variations among a selection of *S. enteritidis* strains isolated over 12 years in Germany.

MATERIALS AND METHODS

Bacterial strains

From a collection of *S. enteritidis* strains, isolated in Reutlingen, from sporadic cases of salmonellosis which had occurred in Baden-Württemberg, Germany between 1980 and 1992, 10 strains from each year were randomly (where possible) selected. No strains were available from years 1985 and 1987. Another five strains isolated between 1970 and 1973 in Hamburg, Germany (kindly provided by Dr S. Aleksic, Hamburg) were also included, yielding altogether 86 strains (Table 1).

Phage typing

The strains were phage typed according to the method of Ward and colleagues [27]. Nutrient broth tubes were inoculated, incubated in a rotary shaker for 2 h at 37 °C and nutrient agar plates flooded with the cultures. The plates were allowed to absorb the fluid and the surface was then dried in an incubator for 15–20 min. A drop of each phage was applied on the agar surface and the plates were incubated over night at 37 °C. Isolates showing lytic patterns consistent with recognized phage types were so designated, whereas isolates with a discrete pattern not consistent with that of any recognized phage type were designated as non-specific types (NST). The typing phages were kindly provided by Dr B. Rowe, PHLS, London.

Biochemical fingerprinting

The Phene Plate (PhP) system for biochemical fingerprinting of *Salmonella* serotypes (the PhP-S, Biosys inova, S-113 51 Stockholm, Sweden) consists of microplates containing three parallel sets of 32 dried substrates, specifically chosen for biochemical fingerprinting of *Salmonella* strains of different serotypes.

Table 1. *Year and number of strains of* Salmonella enteritidis *isolated in Germany and tested for their biochemical fingerprint and phage types*

Year of isolation	Number of strains	Mode of storage*
1970†	2	mwb −70 °C
1971†	1	mwb −70 °C
1973†	2	mwb −70 °C
1980	3	mwb −20 °C
1981	5	mwb −20 °C
1982	7	mwb −20 °C
1983	2	wsc room temp.
1984	10	wsc room temp.
1986	4	wsc room temp.
1988	10	sb −20 °C
1989	10	sb −20 °C
1990	10	sb −20 °C
1991	10	sb −20 °C
1992	10	sb −20 °C

mwb, Meat water broth; wsc, Wax sealed cultures; sb, standard I-broth.
† Isolated in Hamburg.

For each serotype a set of tests with optimal degree of discriminations for the respective serotype has been selected and the computer program automatically excludes tests not wanted in the calculations. Tests chosen for typing of *S. enteritidis* strains include: D-mannoic-lactone, D-xylose, melibiose, inosine, sorbitol, deoxyribose, β-methyl-glucoside, malonate, pyruvate, L-tartarate, saccharate and L-glutamate.

One colony of the strains to be tested was suspended in 10 ml Proteose Peptone (Difco) 0·1 % w/v containing bromthymol blue 0·01 % w/v and aliquots (150 μl) inoculated to the wells in the pre-prepared microtitre plates containing the substrates (3·75 g/l in each well). To allow proper rehydration of substrates, plates were stored at 4 °C overnight and incubated at 37 °C the following morning. The absorbance$_{(A620)}$ of each reaction was measured at 4, 7, 24, and 48 h on a microplate reader. The absorbance values, transferred automatically to a personal computer, were multiplied by 10, thus yielding scores ranging from 0 to 30 for each reaction; low values indicated acid (yellow) reactions and high values alkaline (deep blue) reactions. After the final reading, the mean value of 4 readings was calculated providing 12 different numbers ranging 0–30 for each strain constituting the biochemical fingerprint for each strain. Similarities between strains were calculated as correlation coefficients (r), as described before [28] and clustered according to the unweighted-pair group method with arithmetic averages (UPGMA) [29] into a dendrogram. Strains with r values higher than the identity level used for the PhP-S system (0·980) were assigned to the same biochemical phenotype (BPT). BPTs with > 1 isolate were called common (C) BPTs and those with only one isolate were called single (S) BPTs.

The handling of data, including optical readings, was performed with the PhP software (BioSys, inova).

RESULTS

Altogether 23 BPTs consisting of 9 common (C) and 14 single (S) BPTs were identified (Table 2). BPTs C2 and C4 containing 20 and 38 strains respectively

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Table 2. *Variations in biochemical phenotypes (BPTs) of 86 strains of* Salmonella enteritidis *isolated in Germany between 1982 and 92**

Common BPTs (no. of strains)	Year of isolation (no. of strains)	Single BPTs	Year of isolation
C1 (2)	1970 (1), 1971 (1)		
		S1	1973
		S2	1972
C2 (20)	1970 (1), 1980 (1)		
	1981 (4), 1982 (5)		
	1984 (3), 1988 (1)		
	1989 (4), 1990 (1)		
		S3	1983
		S4	1984
		S5	1981
C3 (2)	1981 (1), 1982 (1)		
		S6	1986
C4 (38)	1984 (1), 1988 (5)		
	1989 (7), 1990 (8)		
	1991 (8), 1992 (8)		
	1973 (1)		
		S7	1984
		S8	1988
		S9	1991
		S10	1991
C5 (2)	1990 (1), 1992 (1)		
C6 (2)	1984 (2)		
		S11	1984
		S12	1984
C7 (2)	1980 (2)		
C8 (2)	1983 (1), 1992 (1)		
C9 (2)	1986 (1), 1988 (1)		
		S13	1986
		S14	1986

* Five strains isolated during 1970–3.

accounted for 67 % of the isolates. Strains of BPT C2 had been isolated over a wide period of time and consisted of 1 strain from year 1970, 13 strains from the period 1980–4, and 6 strains isolated between 1988 and 1990. In contrast, 36 strains of BPT C4 had been isolated during 1988–92 (Table 2).

Altogether 12 phage types (PTs) (including strains designated as non-specific types 'NST') were identified (Table 3). The two most common PTs, i.e. PT 4 with 39 strains and PT 8 with 17 strains, accounted for 65 % of the isolates. There were also 18 strains which could not be assigned to any recognized phage types and were thus considered as non-specific type (NST) (Table 3). PT 8 contained strains isolated over periods 1981–4 (13 strains) and 1988–90 (4 strains). In contrast, all (except three) strains of PT 4 had been isolated during the period 1988–92 (Table 3).

Combination of biochemical fingerprinting and phage typing divided the strains into 10 common and 25 single phenotypes (BPT : PTs) (Fig. 1). Again, two major phenotypes were identified. Phenotypes C2 : 8 (9 isolates) was very common among the material isolated in the early 1980s (7/9 isolates) (Fig. 1). This phenotype was

Table 3. *Variations in phage types (PTs) of 86 strains of* Salmonella enteritidis *isolated in Germany between 1980 and 1992*

Common PTs (no. of strains)	Year of isolation (no. of strains)	Single PTs	Year of isolation
		1	1981
		3	1988
4 (39)	1970 (1), 1980 (2), 1988 (4), 1989 (7), 1990 (8), 1991 (8), 1992 (9)		
		6	1988
		6a	1989
8 (17)	1981 (1), 1982 (5), 1984 (7), 1988 (1), 1989 (2), 1990 (1)		
11 (3)	1973 (1), 1980 (1), 1981 (1)		
		13a	1981
		14	1988
		14b	1992
15 (2)	1970 (1), 1973 (1)		
NST* (18)	1971 (1), 1981 (1), 1982 (2), 1983 (2), 1984 (3), 1986 (4), 1988 (2), 1990 (1), 1991 (2)		

* Non-specific type.

also isolated during 1989 (two isolates). Two BPTs C3 and S4 closely related to BPT C2 (similarity level = 0·960) also belonged to PT 8; 2 of them had been isolated in the early 1980s and 1 in 1988 (Fig. 1). Phenotype C4:4 contained 31 strains, all of them isolated during 1988–92. Three strains of PT 4, also isolated during this period, were closely related to BPT C4 (Fig. 1). On the other hand, there were also strains of PT 4 isolated during 1992 (i.e. BPT C8) or 1989 (i.e. BPT C2) which had a totally different and unrelated biochemical fingerprint (Fig. 1).

DISCUSSION

Increasing reports of infections by *Salmonella* serotypes and in particular *S. enteritidis* in Europe [4–6], and other parts of the world [30, 31], make serotyping alone less effective as an epidemiological marker [32]. Phage typing together with plasmid profile analysis [20, 22] and antimicrobial resistance pattern [21] have been used extensively for discrimination of *S. enteritidis* strains. Biochemical markers have also been used for typing of different *Salmonella* serotypes [33–35]. However, to our knowledge, their application for typing of *S. enteritidis* strains has not yet been documented. In the present study biochemical fingerprinting with the PhP-S system proved to be an excellent method for typing such strains. Of the 23 BPTs identified with this method, 2 accounted for 67% of the isolates. These two BPTs were each dominant during certain periods of time and although phage typing also detected the presence of these major and other less common phenotypes among our collection of *S. enteritidis* strains, biochemical fingerprinting provided more information about the similarities between the strains. Strains isolated during the early 1970s were unique and belonged to biochemical phenotypes (BPTs) totally different from the others. However, one particular strain had the same BPT as those found during the early 1980s (i.e. BPT C2). This

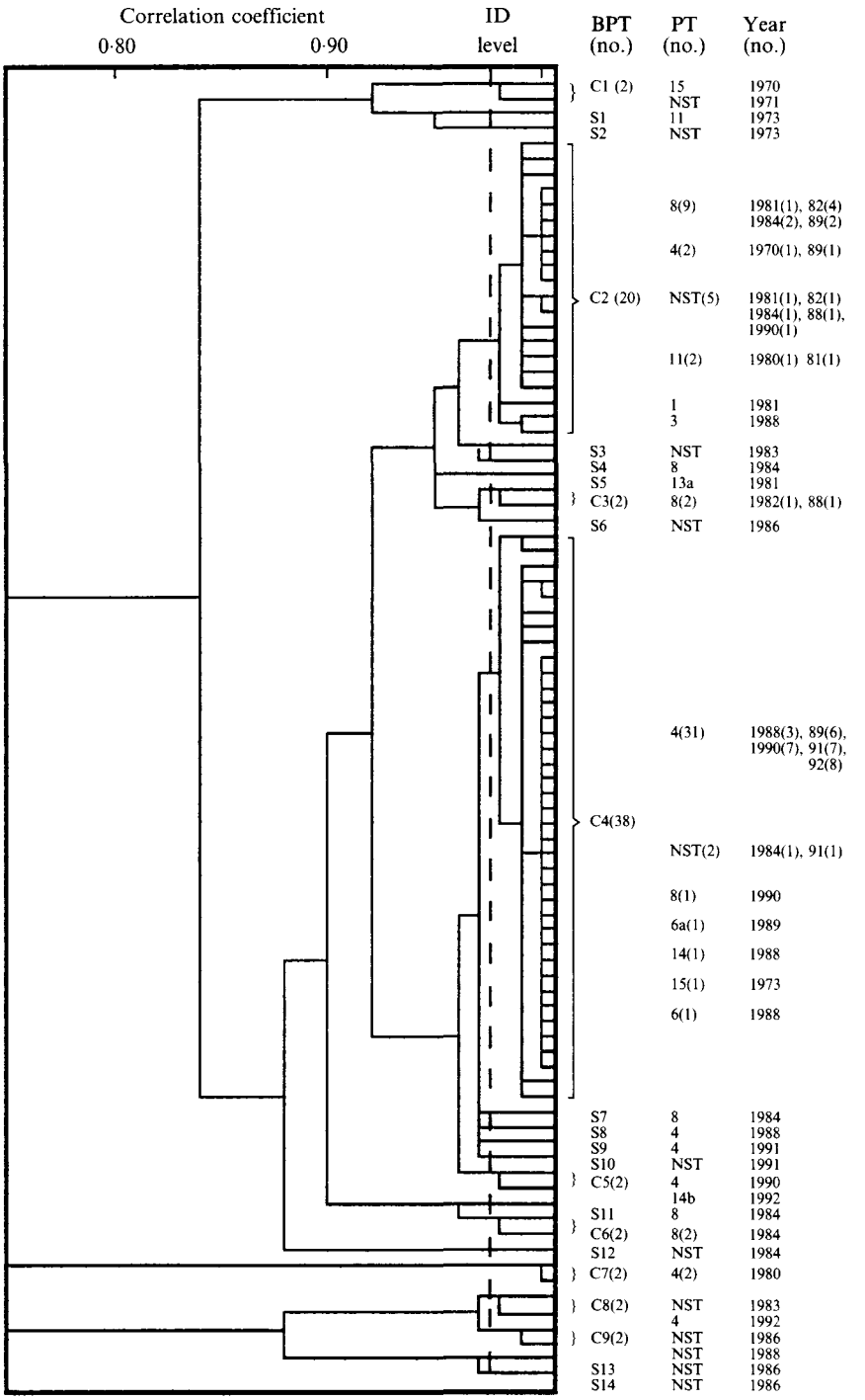

Fig. 1 Dendrogram showing variations among biochemical phenotypes (BPTs) and phage types (PTs) of 86 strains of *Salmonella enteritidis* isolated in Germany between 1980 and 1992 (5 strains from 1970 to 1973). ID-level indicates the chosen identity level ($r = 0{\cdot}980$) for strain assigned to the same BPT. NST, non-specific type.

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BPT was one of the two major BPTs identified among the whole material and consisted of isolates mainly from 1980–4 (13/20). Interestingly, strains of this BPT were also found among the material collected between 1988 and 1992 indicating the persistence of this particular BPT over time. Unfortunately strains from 1985 and 1987 are missing but since 1988 a totally new BPT (i.e. C4) emerged and became dominant. This BPT comprised 62% of the isolates from 1988 to 1992.

Phage typing also revealed the presence of two major groups in our collection with phage type (PT) 4 being the most common PT. All strains of this PT (except 3 strains isolated in 1970 and 1980) were from the period 1988–92. This is by no mean surprising and indicates that as in other parts of Europe [36, 37], *S. enteritidis* strains of PT 4 are responsible for a high percentage of human cases of food-borne diseases in Germany. This is also in agreement with what has been reported earlier in this country [6, 7]. Combination of biochemical fingerprinting and phage typing indicated that only 31/39 strains of PT 4 were of similar BPTs. The other eight strains which had been isolated over a wide period of time (including the period 1988–92) belonged to BPTs quite unlike the others (e.g. BPTs C7 and C8). These findings suggest that the strains of the phenotype C4:4 which were prevalent during 1988–92 are in fact 'offsprings' of a recent and unique 'clone', different from those of PT 4 seen in the early 1980s, and that combining biochemical fingerprinting with the PhP-S system and phage typing is useful in identifying non-related *S. enteritidis* strains of PT 4 in epidemiological studies.

Another major PT (i.e. PT 8) consisted of 17 strains, 13 of which were isolated between 1980 and 1984. This PT was also found among strains isolated during 1988–92. PT 8 is the most prevalent PT isolated from human sporadic cases and outbreaks caused by *S. enteritidis* strains in north America [30] and is, after PT 4, the most common PT isolated between 1981 and 1986 in the United Kingdom [27]. Recent epidemiological investigation of the salmonella situation in Germany [6] indicates that PT 8 is the third most prevalent PT isolated from human and food sources and the second most prevalent PT isolated from poultry. Biochemical fingerprinting of the *S. enteritidis* PT 8 strains in the present study also showed that during 1980–4 there were different BPTs of this PT in Germany but one particular BPT (i.e. C2) was the most prevalent and has persisted over time since it was also found among the material isolated during 1988.

One of the main characteristics of a good typing system is typability. With phage typing there are always strains that react in patterns which do not conform to the currently recognized patterns. Since such strains are not epidemiologically significant they have not been assigned to definitive type [27]. In the present study we also found 18 strains, isolated over different periods of time, which could not be assigned to any of the known phage types and were thus regarded as non-specific type (NST). Biochemical fingerprinting showed that whilst seven of these strains shared similar BPTs to those of the major BPTs in this study, the rest belonged to totally different distantly related BPTs (Fig. 1).

In conclusion, our data showed the presence of two major phenotypes (BPT:PTs) among a collection of *S. enteritidis* strains isolated between 1980 and 1992 in Germany. The phenotype C2:8, which was common among strains isolated

during 1980–84, was also seen among those isolated in the early 1970s and between 1988 and 1992, indicating the persistence of this particular phenotype over time in Germany. Another phenotype, representing a more newly emerged and spread 'clone', contained strains isolated during 1988–92. We also conclude that biochemical fingerprinting with the PhP-S system is a useful method for epidemiological analysis of *S. enteritidis* strains. It can not only detect identical strains but also shows existing relationships among them, information which might be very useful in tracing the origin of strains in epidemiological studies.

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