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Application of physico-chemical typing methods for the epidemiological analysis of *Salmonella enteritidis* strains of phage type 25/17

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SUMMARY

Eighty-nine *Salmonella enteritidis* phage type 25/17 strains isolated from a localized outbreak in the German state Nordrhein-Westfalen (outbreak NWI) could not be further differentiated by biochemotyping and plasmid pattern analysis. They were submitted to a complex typing system consisting of modern physico-chemical analytical procedures. In lipopolysaccharide pattern analysis the strains proved to be homogeneous. In multilocus enzyme electrophoresis, outer membrane and whole cell protein pattern (WCPP) analysis, and Fourier-transform infrared (FT–IR) spectroscopy (increasing extent of differentiation in the given order) strains deviating from each basal pattern were found. The extent of correspondence in these deviations was satisfactory.

Forty-six strains of the same sero- and phage type, however, obtained from different outbreaks, were additionally typed. The results obtained with them indicate that the data of the first group were not restricted to strains from outbreak NWI, but of general validity.

It was found that both WCPP and FT–IR represent valuable methods for the sub-grouping of bacteria.

INTRODUCTION

Salmonella enteritidis strains are able to cause feverish gastroenteritides. In 1992 in an old people's home in Nordrhein-Westfalen (Germany) a large outbreak with some deaths took place presumably resulting from food poisoning caused by bacteria [1]. The bacterial strains we obtained from this outbreak exclusively proved to belong to the serovar *S. enteritidis*. Using classical methods of microbiology (serology, biochemotyping, phage typing) and additionally plasmid pattern analysis the strains could not be differentiated further (Rabsch, personal communication) and thus were considered to belong to the same clone [clone definition see ref. 2].

In recent years, in our laboratory, a system for complex typing of bacteria was developed using modern physico-chemical analytic procedures. It contains methods of diverse differentiation powers [3].

The system consists of: (i) estimation of the lipopolysaccharide (LPS) and outer

membrane protein (OMP) patterns and of whole cell protein profiles (WCPP), respectively, in each case by means of polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulphate (SDS); (ii) multilocus enzyme electrophoresis (MLEE) in starch gel; (iii) estimation of fatty acid profiles by gas liquid chromatography (FA–GC), and (iv) Fourier-transform infrared spectroscopy (FT–IR).

This system was applied for complex typing of the above-mentioned salmonellae.

This was done for several reasons. Firstly, we intended to compare the classical and the modern typing methods, secondly, the efficacy of our typing systems to differentiate related strains should be tested, and thirdly, the question should be investigated, whether or not in the course of the outbreak characteristics of the strains had changed.

To check, whether or not our results were typical of the above-mentioned event and if other strains of this serovar and phage type behaved similarly, additional *S. enteritidis* strains of the same phage type obtained from several unrelated outbreaks in Germany were typed in subsequent investigations.

MATERIALS AND METHODS

Bacterial strains

All the investigated *S. enteritidis* strains were taken from the collection of the Salmonella Reference Centre of the Bundesgesundheitsamt (Bereich Wernigerode) and have been listed in Table 1. The strains were proven to belong to phage type 25/17, i.e. in the typing systems of Ward and colleagues [4] to type 25, in that of Laszlo and co-workers [5] to type 17 (Rabsch, personal communication). Eighty-five strains were obtained from the above-mentioned outbreak; the remaining 46 strains were isolated from 8 territorially different outbreaks in Germany in the years 1991 and 1992 for the purpose of comparative investigations (Table 1). Each of the four isolates 4588/92, 26/93, 49/93, and 53/93 on Endo-agar surfaces showed two differently coloured colony types and thus proved to be mixtures of two *S. enteritidis* phage type 25/17 strains.

Disintegration of the bacteria

Each 5 ml of a preculture (3·5 h) were poured onto trypticase soy agar plates (Difco Lab., Detroit, USA); $\varnothing$ = 140 mm) and incubated for 18 h at 37 °C. Then the bacteria were harvested by washing off with each 10 ml Tris-buffer (10 mM, pH 7·8), the obtained suspensions centrifuged (10000 g, 10 min), resuspended, and centrifuged again. The sediments were suspended in 10 ml Tris buffer and the bacteria disrupted by sonication (100 W, 20 kHz, 1·5 min). To remove non-disrupted or insufficiently disrupted bacteria the sonicates were centrifuged (10000 g, 10 min). The sediments were removed, from each of the supernatants 1·0 ml taken for MLEE and 0·1 ml for WCPP. Each of the remaining solutions was divided into two equal parts. All the first parts were centrifuged for 1 h at 41000 g and the sediments obtained thus used for the LPS pattern analysis (Sed. 1). All the second parts after this first centrifugation were suspended in a 1·67% Sarkosyl solution, incubated for 15 min at room temperature, and after that centrifuged for

Table 1. *Origin of the investigated strains*

Outbreak	Strain numbers
Nordrhein-Westfalen I (NWI)	4583/92–4602/92; 4760/92–4771/92; 8/93–14/93; 16/93; 18/93; 20/93; 23/93–27/93; 29/93–31/93; 33/93–46/93; 48/93–51/93; 53/93; 54/93; 56/93; 58/93; 60/93–64/93; 66/93–73/93
Nordrhein-Westfalen II (NWII)	5341/92; 5344/92
Bayern I (BI)	839/91; 842/91; 843/91; 846/91–849/91; 851/91; 854/91; 855/91; 861/91; 863/91; 866/91.
Bayern II (BII)	1225/91; 1227/91
Bayern III (BIII)	2565/92–2569/93
Baden-Württemberg I (BWI)	2116/91; 2119/91–2122/91; 2124/91; 2125/91
Baden-Württemberg II (BWII)	2104/91–2109/91
Baden-Württemberg III (BWIII)	2409/92–2413/93
Mecklenburg-Vorpommern (MV)	721/92–726/92

In our laboratory strains 4588/92, 26/93, 49/93 and 53/93 were found to be mixtures of two colony variants (see Table 2).

1·5 h at 41000 g. The sediments (Sed. 2) were applied for the OMP pattern analysis.

OMP pattern analysis

Each Sed. 2 was boiled for 5 min at 100 °C in 0·5 ml sample buffer (10 ml 0·5 M-Tris-buffer pH 6·8, 8 ml 10% sodium dodecyl sulphate (SDS), 4 ml glycerol, 5 mg bromphenol blue, 2·5 ml 3-mercapto-propane-1,2-diol, water 50 ml). The OMPs were separated according to Lugtenberg and colleagues [6] in a stacking gel composed of 3% acrylamide and 0·08% methylene bisacrylamide (pH 6·8) and a running gel of 11% acrylamide and 0·2% bisacrylamide (pH 8·8) each in the presence of 0·1% SDS and 4 M-urea; the gel dimensions were 0·08 × 20 × 20 cm. Electrophoresis was performed with a constant current of 30 mA in the stacking gel and 60 mA in the running gel. The proteins were stained with 0·25% Coomassie blue in methanol:acetic acid:water = 50:7:43, unbound dye removed with the same solvent.

LPS pattern analysis

Each Sed. 1 was solved in 0·5 ml sample buffer, incubated with 0·25% Proteinase K, and after this treated in the same manner as the OMP samples. After electrophoresis the gels were stained with alkaline $AgNO_3$-solution according to Tsai & Frash [7].

FA–GC analysis

The strains were cultivated for 24 h at 37 °C on trypticase soy agar plates (Becton Dickinson Ltd, Cockeysville, USA). Both the free and bound fatty acids were transformed into the methyl esters (FAME) by the direct transesterification method of Müller and colleagues [8] with trimethylsulphonium hydroxide (TMSOH; Macherey-Nagel, Düren, Germany). The fatty acid profiles were taken according to Miller and colleagues [9] using the capillary column Ultra 2 (crosslinked 5% Ph Me Silicone; Hewlett–Packard, Palo Alto, USA) and the gas liquid chromatograph model HP 5890 A (Hewlett–Packard). The individual fatty

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acid methyl ester peaks were identified by calculating the equivalent chain lengths (ECL-values) according to Miller and colleagues [9] using a FSME-standard (Subelco, No-4-7080, Bellefonte, USA). The data were clustered using the algorithms 'average linkage' and 'Ward'.

Multilocus enzyme electrophoresis (MLEE)

The supernatants obtained by sonication of the bacteria and low-speed centrifugation were separated according to Selander and colleagues [10] by horizontal electrophoresis in 10% starch-gel (Smithies; Serva, Heidelberg, Germany), 4 h at 15 V/cm, using Tris-citrate pH 8·0 throughout as buffer. After separation each gel was cut twice parallel to its largest surface and thus three identical parts of the gel obtained. After this the individual enzymes were localized by specific staining as described by Selander and colleagues [10]. The following enzymes were tested: aconitase (ACO), adenylate kinase (ADK), alcohol dehydrogenase (ADH), acid phosphatase (ACP), alkaline phosphatase (ALP), catalase (CAT), esterases (EST), fumarase (FUM), glucose 6-phosphate dehydrogenase (G6P), glutamic oxaloacetic transaminase (GOT), indophenol oxidase (IPO), isocitrate dehydrogenase (IDH), lactate dehydrogenase (LDH), leucine aminopeptidase (LAP), malate dehydrogenase (MDH), malic enzyme (ME; = decarboxylating malate dehydrogenase), phosphoglucomutase (PGM), 6-phosphogluconate dehydrogenase (6PG), phosphoglucose isomerase (PGI).

Whole-cell protein profile (WCPP)

The supernatants obtained by ultrasonic disruption of the bacteria and low-speed centrifugation were submitted to the polyacrylamide gel electrophoresis under the same experimental conditions as described for OMP and LPS pattern analysis. The gels were stained with alkaline $AgNO_3$ solution as described by Rabilloud [11]. They were interpreted visually.

FT–IR measurements

The germs were cultivated for 24 h at 37 °C on caso-agar plates (Merckoplate®, Merck, Darmstadt, Germany) using a four-quadrant streaking pattern. For the preparation of the bacterial films one loop-full (1 mm ∅) from the confluent growth of the third quadrant (about 10–60 μg) was suspended in 80 μl distilled water. After this an aliquot (35 μl) was transferred to a preformed sample area on a zinc selenide-optical plate of a multisample cuvette and was dried in vacuo at about 3·5 kPa to get a transparent film. The spectra were recorded in the region from 4000 cm^{-1} to 500 cm^{-1} (wave numbers) on an FT–IR spectrometer IFS-25/B (Bruker, Karlsruhe, Germany). One hundred and twenty-eight interferograms were co-added and averaged for each spectrum. The spectral resolution was 6 cm^{-1}. The measurements were performed in triplicate and subsequently averaged after minimum–maximum normalization between 0 and 2 A of the spectra and a combination of spectral windows. To get better resolutions the cluster analyses were performed using the first or the second derivative. It was carried out by weighting (W) and use of the specific information content at 3000–2800 cm^{-1} (W = 1·0; fatty acid region I), at 1200–900 cm^{-1} (W = 3·0, polysaccharide region), and at 900–700 cm^{-1} (W = 1·0; true 'fingerprint' region) using the algorithms 'average linkage' and 'Ward' [12].

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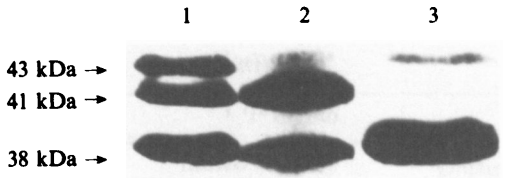

Fig. 1. Patterns of major OMP. Lane 1: strain 4586/92; lane 2: strain 854/91; lane 3: strain 4588/1/92. For details see text.

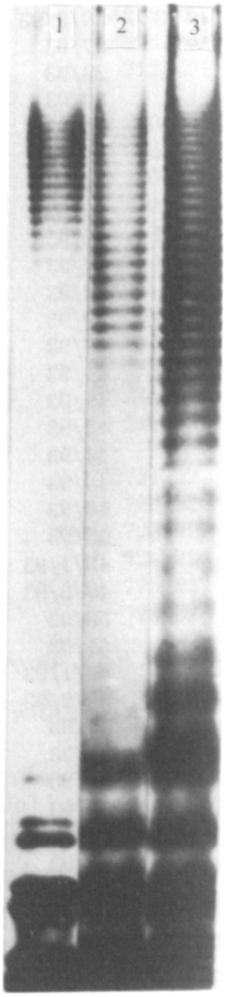

Fig. 2. Patterns of LPS. Lane 1: strain 38/93; lane 2: strain 4586/92; lane 3: strain 72/93. For details see text.

RESULTS

The investigations were started with 89 strains of the outbreak NWI (see Table 1; group 1). According to the results of phage typing[1] (all strains belonged to phage type 25/17) and plasmid pattern analysis[2] (all strains contained the

[1] Phage typing was performed in the laboratory of W. Rabsch.
[2] Plasmid pattern analysis was performed in the laboratory of H. Tschäpe.

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Table 2. *Results of complex typing of* Salmonella enteritidis *phage type 25/17 strains*

Group 1									
Strain no.	OMP	FT–IR	WCPP	MLEE	Strain no.	OMP	FT–IR	WCCP	MLEE
	Outbreak NW1					Outbreak NWI			
4583/92	1	1	1	1	25/93	2	2	2	1
4584/92	1	1	1	1	26/1/93	1	1	1	1*
4585/92	1	1	1	1	26/2/93	1	1	1	1*
4586/92	1	1	1	1	27/93	1	2	1	1
4587/92	1	1	1	1	29/93	1	1	1	1
4588/1/92	1	1	1	1	30/93	1	2	1	1
4588/2/92	3	2	4	2	31/93	1	2	1	1
4589/92	1	1	1	1	33/93	1	1	1	1
4590/92	1	2	1	1	34/93	1	1	1	1
4591/92	1	1	1	1	35/93	1	2	1	1
4592/92	1	1	1	1	36/93	1	2	1	1
4593/92	1	1	1	1	37/93	3	2	4	2
4594/92	1	1	1	1	38/93	1	2	1	1
4595/92	1	1	1	1	39/93	1	1	1	1
4596/92	1	1	1	1	40/93	1	2	1	1
4597/92	1	2	2	1	41/93	1	2	1	1
4598/92	1	2	2	1	42/93	1	1	1	1
4599/92	1	1	1	1	43/93	1	1	1	1
4600/92	1	1	1	1	44/93	1	1	1	1
4601/92	1	2	1	1	45/93	1	1	1	1
4602/92	1	1	1	1	46/93	1	2	2*	1
4760/92†	1	1	1	1	48/93	1	2	2*	1
4761/92†	1	1	1	1	49/1/93	1	2	2*	1
4762/92†	1	1	1	1	49/2/93	1	1	1	1
4763/92†	1	1	1	1	50/93	1	2	2*	1
4764/92†	1	1	1	1	51/93	1	2	1	1
4765/92†	1	1	1	1	53/1/93	1	1	1	1
4766/92†	1	1	1	1	53/2/93	2	2	2	1
4767/92†	1	1	1	1	54/93	1	2	1	1
4768/92†	1	1	1	1	56/93	1	2	1	1
4769/92†	1	1	1	1	58/93	1	1	1	1
4770/92†	1	1	1	1	60/93	1	1	1	1
4771/92†	1	1	1	1	61/93	1	1	1	1
8/93	1	1	1	1	62/93	1	1	1	1
9/93	1	2	1	1	63/93	1	1	1	1
10/93	1	1	1	1	64/93	1	1	1	1
11/93	1	2	1	1	66/93	1	1	1	1
12/93	1	1	1	1	67/93	1	1	1	1
13/93	1	2	1	1	68/93	1	1	1	1
14/93	1	1	1	1	69/93	1	1	1	1
16/93	1	2	1	1	70/93	1	1	1	1
18/93	1	1	1	1	71/93	1	1	1	1
20/93	1	2	1	1	72/93	2	2	2	1
23/93	1	1	1	1	73/93	1	1	1	1
24/93	1	1	1	1					

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Table 2. (*cont.*)

Group 2

Strain no.	OMP	FT-IR	WCPP	MLEE	Strain no.	OMP	FT-IR	WCCP	MLEE
Outbreak NWII					Outbreak BWI				
5341/92	1	1	1	1	2116/91	1	1	1	1
5344/92	1	1	1	1	2119/91	1	1	1	1
Outbreak BI					2120/91	1	1	1	1
839/91	1	1	1	1	2121/91	1	1	1	1
842/91	1	1	1	1	2122/91	1	1	1	1
843/91	1	1	1	1	2124/91	1	1	1	1
846/91	1	1	1	1	2125/91	1	2	1	1
847/91	2	2	2	1	Outbreak BWII				
848/91	1	1	1	1	2104/91	1	1	1	1
849/91	1	1	1	1	2105/91	1	1	1	1
851/91	1	1	1	1	2106/91	1	1	1	1
854/91	2	2	2	1	2107/91	1	1	1	1
855/91	1	1	1	1	2108/91	2	1	1	1
861/91	1	1	1	1	2109/91	2	2	1	1
863/91	1	1	1	1	Outbreak BWIII				
866/91	1	1	1	1	2409/92	1	1	2	1
Outbreak BII					2410/92	1	2	2	1
1225/91	1	2	1	1	2411/92	2	2	3	1
1227/91	1	2	1	1	2412/92	1	1	1	1
Outbreak BIII					2413/92	1	1	1	1
2565/92	1	1	1	1	Outbreak MV				
2566/92	1	1	1	1	721/92	2	2	2	1
2567/92	1	1	1	1	722/92	2	1	1	1
2568/92	1	1	1	1	723/92	1	1	1	1
2569/92	1	1	1	1	724/92	1	1	1	1
					725/92	1	1	1	1
					726/92	1	1	1	1

NW, Nordrhein-Westfalen; B, Bayern; BW, Baden-Württemberg; MV, Mecklenburg-Vorpommern.
*, Very similar to, but not identical with type 2.
†, Pathological material.

plasmids 37, 4·7, 3·7 and 1·5 MDa) belonged to the same clone. The typing results of our system likewise pointed to a clonal character of the strains, but additionally to the existence of subclones. To learn whether these results were characteristic of strains from outbreak NWI or of other strains of the same phage type, too, we in the same way typed 46 *S. enteritidis* phage type 25/17 strains from outbreaks, which were epidemiologically completely unrelated to the above-mentioned one (group 2).

OMP pattern

Most strains from outbreak NWI formed patterns of the major OMP which were similar, but not completely identical with that found by Helmuth and colleagues [13] to be typical of *S. enteritidis* (pattern 1 in Fig. 1). It consists of three bands of molecular masses of 43, 41, and 38 kDa (the latter representing the OmpA protein [13]). In the case of the strains 25/93, 53/2/93, and 72/93 the intensity of the middle band was distinctly higher and that of the upper band distinctly lower

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(pattern 2); the mobilities were unchanged. The patterns of the strains 4588/2/92 and 37/93 besides very small amounts of the 43kDa protein contain the OmpA protein as a strong band (pattern 3). The additionally investigated strains (group 2) behaved similarly (Table 2); however, 7 strains from 46 proved to form pattern 2 and no strain pattern 3.

LPS pattern

The LPS patterns of all investigated strains showed the typical ladder-like structure (for examples see Fig. 2). The distance of the 'rungs' were in all cases identical, indicating the presence of 'repeating units' of the same size. The distribution of the bands is bimodal [14]; in most cases we found in the S-specific region chain lengths in the range from 20 to 33 'repeating units' (lane 1). In some cases the control of the chain length during LPS-biosynthesis seemed to be reduced. In these cases the chain length distribution was less restricted (range from 14 to 33 repeating units; lane 2). This, however, was without any epidemiological significance. In one case (strain 72/93; lane 3) the length control obviously was completely abolished. The reason for this will be investigated.

FA–GC analysis

The fatty acid patterns of the primarily investigated strains showed at most small differences against each other. By this reason 32 strains from the outbreak NWI were tested to judge the resolving power of this method in differentiation of *S. enteritidis* strains. To estimate the reproducibility of this method each strain was cultivated separately three times and each culture analysed in double estimation. Thus a reproducibility of 6·9% was obtained. The dendrograms calculated from these analyses using the Ward algorithm showed variances of only 4·2%. Though seven strains clustered off, the differences were within the noise level. Therefore the estimation of the fatty acid profiles seemed to be unsuitable for the differentiation of these strains.

MLEE

The results of MLEE typing are, among others, presented in Table 2. As the patterns of the investigated strains were (with the exception of strains 4588/1/92 and 37/93) identical the results of the individual enzymes are not presented in detail. It must be mentioned that enzyme patterns of the predominant electrotype (named type 1) differ from that of the additionally occurring (type 2) in 17 from 19 tested enzymes; only phosphoglucomutase and 6-phosphogluconate dehydrogenase showed identity. This indicates a high extent of diversity among both types, though in biotyping, phage and plasmid pattern analysis differences had not been found.

WCPP

SDS–PAGE of the whole cell protein extracts of salmonellae yielded pherograms containing at least 40 individual bands, each indicating the presence of one polypeptide or protein and thus of an equal amount of genes. Thus the number of checked parameters is higher than that of MLEE and therefore the resolving power more distinct.

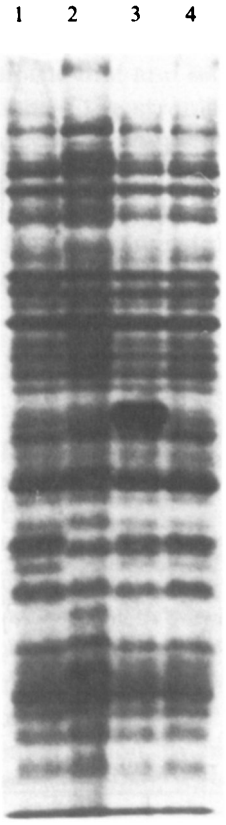

Fig. 3. Patterns of the four WCPP types. Lane 1: strain 51/93; lane 2: strain 53/2/93; lane 3: strain 2411/93; lane 4: strain 37/93. For details see text.

The WCPP of 78 from 89 strains of outbreak NWI (group 1) proved to be absolutely identical (Table 2; Fig. 3, lane 1). This pattern was named type 1. The patterns of nine of the residual strains were identical or very similar (types 2 and 2*; Fig. 3, lane 2) among each other and resembled pattern 1. The patterns of strains 4588/2/92 and 37/93 again proved to be different (pattern 4; Fig. 3, lane 3).

The additionally investigated strains (group 2) clustered in the same manner: 40 from 46 strains formed pattern 1 and 5 strains pattern 2. Strain 2411/92, however, formed a sole pattern (named 3) which was very similar to, but not identical with, pattern 1.

FT–IR

The primarily investigated 89 strains from outbreak NWI (group 1) after cluster analysis of the normalized average FT–IR spectra gave two subgroups of 60 (type 1) and 29 (type 2) strains, respectively. This grouping is mainly caused by differences in characteristic polysaccharide vibrations observed in the spectral region from 1200 to 900 cm^{-1} [15]. In the spectral area from 1185 to 1015 cm^{-1} (second derivative; for an example see Fig. 4) three band areas ($\sim$ 1173, $\sim$ 1153, and $\sim$ 1024 cm^{-1}) can be seen the intensities of which differ characteristically

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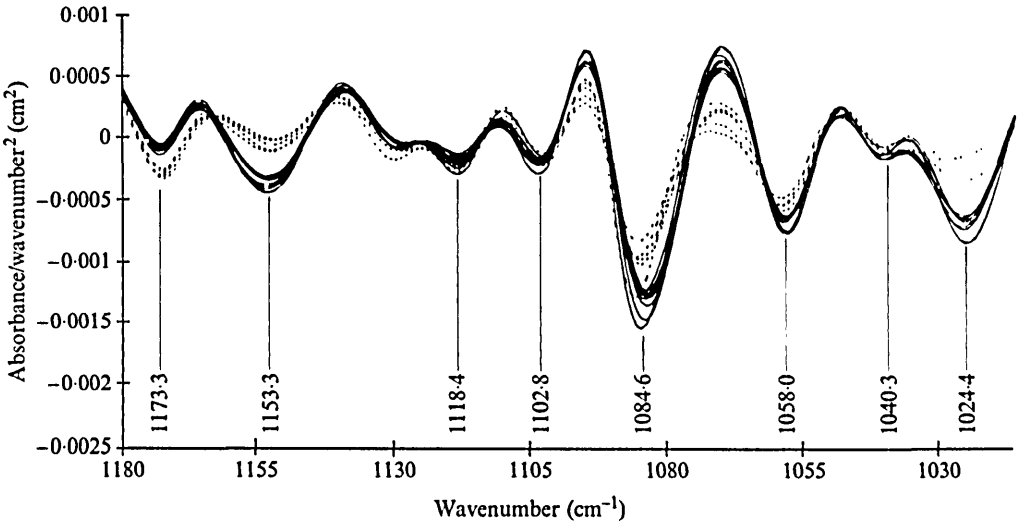

Fig. 4. Normalized FT–IR spectra (2nd derivative) of ten strains of type 1 (full lines; strains 4584/92, 4600/92, 4766/92, 4885/92, 8/93, 12/93, 42/93, 49/2/93, 53/1/93, 60/93) and five of type 2 (dashed lines; strains 4597/92, 4598/92, 25/93, 49/1/93, 53/2/93). Differences in the minima of such curves represent indications for differences in the original spectral curve (see the differences at ~ 1173 cm^{-1}, ~ 1153 cm^{-1}, and ~ 1024 cm^{-1}).

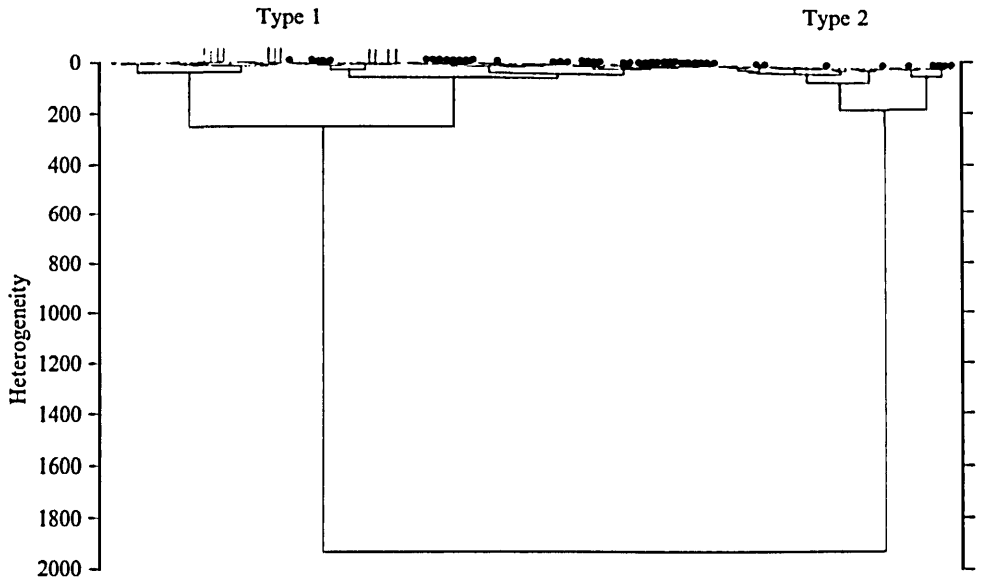

Fig. 5. Dendrogram obtained from the FT–IR spectra of all 135 strains. Unlabelled: group 1 strains; –, group 1 strains isolated from pathological material; ●, group 2 strains.

between the strains of the first and the second type while the band shifting is very slight (~ 1 cm^{-1}). Cluster analyses on the basis of the spectral region from 1185 to 1120 cm^{-1} yielded dendrograms with very high spectral distances (475) and heterogeneities (16600; cluster analysis not shown). The additionally investigated strains of group 2 behaved similarly (Table 2), e.g. they also could be classified into

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the two above-mentioned types. In total from 135 investigated strains 73% belonged to type 1 and 27% to type 2. The dendrogram calculated from the FT–IR spectra of the strains from the first and second group (Fig. 5) clearly shows a rather homogeneous distribution of the strains from the two groups.

To learn whether or not the spectral differences in the polysaccharide region are caused by the O-antigenic LPS we isolated these substances from each strain of both types (4584/92 and 4597/92) according to Westphal & Jann [16] and investigated them. Contrary to those of the total cells the FT–IR spectra of the LPS proved to be highly homogeneous, thus indicating up to now unknown cell components to be responsible for the observed subdifferentiation (data not shown).

DISCUSSION

The 89 *S. enteritidis* strains (group 1; Table 2) from the outbreak NWI which we typed at the beginning of our investigations proved to be identical in sero-, biochemo-, phage and plasmid type and thus were regarded as belonging to the same clone. LPS and fatty acid pattern analysis, both performed by us, yielded no significant differences. On the other hand FT–IR spectroscopy (29 deviations, being identical to each other), WCPP (11 deviations, forming 2 subgroups), and OMP pattern (5 deviations, forming 2 subgroups) indicated differences among the strains. All the 11 strains deviating in WCPP also differed in FT–IR from the basal pattern. Similarly the 5 strains deviating in OMP pattern differed both in FT–IR spectrum and in WCPP, 2 of them additionally in MLEE (4588/2/92 and 37/93). The latter strains were the only two which differed not only in OMP pattern, FT–IR spectrum, and WCPP but significantly also in MLEE patterns from all the other strains. It must be mentioned, however, that both in phage type and plasmid pattern such differences have not been observed. All strains isolated from pathological material did not deviate from the basal type in any of the applied methods.

As shown in Fig. 1 strains 4588/2/92 and 37/93 in their outer membrane contain OmpA as the only protein. Though this protein forms non-specific diffusion channels in the outer membrane [17] the penetration of solutes is drastically lower than through porin pores. Thus the supply with nutrients of such strains should be drastically reduced. As strains of phage type 25/17 are not too rare [18] it seems to be rather probable that these two strains do not belong to the outbreak-causing clone and (though having been isolated from specimens obtained from the same old people's home) are of different origin.

As mentioned above, four of the NWI isolates were found to be mixtures of each of the two strains. In our tests these 'pairs' behaved differently. In the case of pair 4588/92 the two strains were found to be highly different to one another, in the case of pairs 49/93 and 53/93, respectively, slightly different, and in the case of 26/93 nearly identical. In the latter case only in MLEE three slight differences (MDH, ME, and PGI, data not shown) were observed.

All the strains of group 1 were isolated from the patients in 1992. Some of them (4583/92–4771/92) had been sent immediately to our institute, the other strains (8/93–73/93) after some delay. Comparing the percentage of deviation in each one of the used methods from the basal type of the former and the latter strains (Table

2) one finds rather big differences. From the strains 1992 3% differed in OMP pattern, 15% in FT–IR spectrum, and 9% in WCPP pattern; the corresponding data in the case of strains 1993 are 7, 42 and 14%, i.e. they are clearly higher.

We could not exclude the notion that these differences in subgrouping of the strains could be typical only of the outbreak NWI and attributed for instance to different environmental conditions in the course of the epidemic process. For this reason further strains of phage type 25/17 were investigated that were obtained from outbreaks which were epidemiologically clearly different from outbreak NWI (group 2). These strains, too, by the use of biochemotyping, plasmid and LPS pattern analysis, and MLEE yielded identical results, i.e. they all belonged to the same clone. On the other hand in FT–IR, WCPP, and OMP pattern analyses again comparable differentiations were observed. However, in this case the percentage of the strains deviating in the OMP pattern from the basal type were distinctly higher (15%) than in group NWI. Four of these strains deviated from the basal type in FT–IR and WCPP, too, an additional strain only in FT–IR. In FT–IR deviated 9 strains (20%), in WCPP 6 strains (13%) from the corresponding basal type; we found corresponding deviations of both these methods at 5 strains. As in the case of the strains from outbreak NWI the deviations in FT–IR were uniform among each other, in WCPP again two subgroups were found. None of these strains differed in MLEE from the basal type.

The significance of OMP, LPS, and fatty acid patterns as well as of MLEE as epidemiological typing methods is generally accepted. However, the importance of WCPP for this purpose is differently assessed in the literature. Costas and colleagues [19] think it valuable, Olsen and co-workers [20] found it to be not very convincing at least in the case of *S. berta*. Similarly the efficiency of FT–IR spectroscopy is not yet unambiguously clarified.

The results of our investigations indicated in this case study a higher resolving power of both the FT–IR and the WCPP technique compared with the other methods used. The discriminatory index (DI), calculated on the basis of the data given in Table 2 by the formula [21]

$$DI = 1 - \frac{1}{N(N-1)} \sum_{j=1}^{N} a_j$$

(N, number of the investigated strains; a_j, number of strains indistinguishable from the type of the jth strain) is in the case of FT–IR = 0·408; WCPP = 0·227; OMP = 0·165; and MLEE = 0·029.

The subgrouping obtained by both FT–IR and WCPP was predominantly in parallel. In total in FT–IR spectroscopy 38 and in WCPP 17 strains deviated from the basic pattern; in the case of 16 (=94%, related to the WCPP deviations) of these strains the deviations ran in parallel. This high extent of correspondence represents an indication that the subgrouping obtained by FT–IR and WCPP is not method-conditioned and therefore not more or less fortuitous; however, FT–IR represent the more sensitive method. Thus both methods turned out to be suitable subgrouping procedures and to complement each other.

It has been published [22] that not host-adapted serovars as e.g. *S. enteritidis* under natural conditions form a much larger number of clones than host-adapted salmonella serovars as e.g. *S. typhi*. The results presented in this paper show that

even in a seemingly uniform outbreak of *S. enteritidis* (NWI) two groups of strains could be differentiated and that this differentiation was not restricted to strains of this outbreak. From this fact and from the high extent of correspondence among the results of all our methods one must conclude that the observed deviations in the patterns represent objective phenomena rather than artefacts or an 'over-typing'. On the other hand the results of phage typing and plasmid pattern analysis (see above) and of the MLEE (Table 2) favour the assumption that all strains (perhaps without strains 4588/2/92 and 37/93) belong to the same clone. This is in agreement with the statements of both Stubbs and colleagues [23] and Usera and co-workers [24] who subdivided *S. enteritidis* phage type 8 strains into several groups without giving them the status of a clone. Thus, at present two possible explanations for the subgrouping of the *S. enteritidis* phage type 25/17 strains can be given: either the strains tend to split into two sub-clones [25] or a new clone starts to separate from the main clone. Investigation of *S. enteritidis* strains from future outbreaks possibly could provide us with arguments for one or the other possibility.

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REFERENCES

1. Kühn H, Rabsch W, Gericke B, Reissbrodt R. Infektionsepidemiologische Analysen von Salmonellosen, Shigellosen und anderen Enterobacteriaceae-Infektionen. Bundesgesundhbl 1993; **36**: 324–33.
2. Ørskov F, Ørskov I. Summary of a workshop on the clone concept in the epidemiology, taxonomy, and evolution of the Enterobacteriaceae and other bacteria. J Infect Dis 1983; **148**: 346–57.
3. Seltmann G, Beer W, Voigt W. Die komplexe Typisierung von Bakterien mit Hilfe moderner physikochemischer Analysenverfahren. Bundesgesundhbl 1993; **36**: 313–18.
4. Ward LR, de Sa JDH, Rowe B. A phage typing scheme for *Salmonella enteritidis*. Epidemiol Infect 1987; **99**: 291–4.
5. Laszlo VG, Csorian ES, Paszti J. Phage types and epidemiological significance of *Salmonella enteritidis* strains in Hungary between 1976 and 1983. Acta Microbiol Hung 1985; **32**: 321–40.
6. Lugtenberg B, Meiers J, Peters R, van der Hoek P, van Alphen L. Electrophoretic resolution of the 'major outer membrane protein' of *Escherichia coli* K12 into four bands. FEBS letters 1975; **58**: 254–8.
7. Tsai C-M, Frash CE. A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. Analyt Biochem 1982; **119**: 115–19.
8. Müller D, Husmann H, Nalik HP. A new and rapid method for the assay of bacterial fatty acids using high resolution capillary gas chromatography and trimethylsulfonium hydroxide. Zbl Bakt 1990; **274**: 174–82.
9. Miller L, Berger T. Bacteria identification by gas chromatography of whole cell fatty acids. Hewlett–Packard Application Note 1985; **228–41**: 1–8.
10. Selander RK, Caugant DA, Ochmann H, Musser JH, Gilmour MN, Whittam TS. Methods of multi-locus enzyme electrophoresis for bacterial population genetics and systematics. Appl Environment Microbiol 1986; **51**: 873–4.
11. Rabilloud T. A comparison between low background silver diamine and silver nitrate protein stains. Electrophoresis 1992; **13**: 429–39.

12. Helm D, Labischinski H, Schallehn G, Naumann D. Classification and identification of bacteria by Fourier-transform infrared spectroscopy. J Gen Microbiol 1991; **137**: 69–79.
13. Helmuth R, Stephan R, Bunge C, Hoog B, Steinbeck A, Bulling E. Epidemiology of virulence-associated plasmids and outer membrane protein patterns within seven common *Salmonella* serotypes. Infect Immun 1985; **48**: 175–82.
14. Bastin DA, Stevenson G, Brown PK, Haase A, Reeves PR. Repeat unit polysaccharides of bacteria: a model for polymerization resembling that of ribosomes and fatty acid synthetase, with a novel mechanism for determining chain length. Mol Microbiol 1993; **7**: 725–34.
15. Naumann D, Helm D, Labischinski H, Giesbrecht P. The characterization of microorganisms by Fourier-transform infrared spectroscopy (FT–IR). In: Nelson WH, ed. Modern techniques for rapid microbiological analysis. Weinheim, New York: Verlag Chemie 1991: 34–96.
16. Westphal O, Jann K. Bacterial lipopolysaccharides. Extraction with phenol/water and further applications of the procedure. In: Whistler RL, ed. Methods in carbohydrate chemistry, vol. 5. New York: Academic Press, 1965: 83–91.
17. Sugawara E, Nikaido H. Pore-forming activity of OmpA protein of *Escherichia coli*. J Biol Chem 1992; **267**: 2507–11.
18. Kühn H, Rabsch W, Liesegang A. Gegenwärtige epidemiologische Situation bei der Salmonellose des Menschen in Deutschland. Immun Infekt 1994; **22**: 4–9.
19. Costas M, Holmes B, Sloss LL. Numerical analysis of electrophoretic protein patterns of *Providencia rustiganii* strains from human diarrhoea and other sources. J Appl Biol 1987; **63**: 319–28.
20. Olsen JE, Brown DJ, Baggesen DL, Bisgaard M. Biochemical and molecular characterization of *Salmonella enterica* serovar *berta*, and a comparison of methods for typing. Epidemiol Infect 1992; **108**: 243–60.
21. Nørrung B, Gerner-Smidt P. Comparison of multilocus enzyme electrophoresis (MEE), ribotyping, restriction enzyme analysis (REA) and phage typing for typing of *Listeria monocytogenes*. Epidemiol Infect 1993; **111**: 71–9.
22. Selander RK, Beltran P, Smith NH. Evolutionary genetics of *Salmonella*. In: Selander RK, Clark G, Whittam TS, eds. Evolution at the molecular level. Sunderland, Mass.: Sinauer Ass. 1991: 24–57.
23. Stubbs AD, Hickman-Brenner FW, Cameron D, Farmer III JJ. Differentiation of *Salmonella enteritidis* phage 8 strains: evaluation of three additional phage typing systems, plasmid profiles, antibiotic susceptibility patterns, and biotyping. J Clin Microbiol 1994; **32**: 199–201.
24. Usera MA, Popovic T, Bopp CA, Strockbine NA. Molecular subtyping of *Salmonella enteritidis* phage 8 strains from the United States. J Clin Microbiol 1994; **32**: 194–8.
25. Achtman M, Mercer A, Kusecek B, et al. Six widespread bacterial clones among *Escherichia coli* K1 isolates. Infect Immun 1983; **39**: 315–35.