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Relationships between Electrophoretic Patterns of Esterases from Salmonella

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SUMMARY

Esterases of 85 strains of the four biochemically-defined subgenera of *Salmonella*, when analysed by the acrylamide–agarose zymogram technique using several synthetic substrates, gave four principal bands (E_1, E_2, E_3, E_4) and two minor ones. The E_1 esterase band hydrolysed α-naphthyl acetate, whereas the E_2 band hydrolysed β-naphthyl acetate. These bands were resistant to di-isofluoropropyl phosphate (DFP) and their electrophoretic distribution among the strains occurred within a relatively small M_F range, M_F being the distance moved by the esterase band as a percentage of the distance moved by the dye front. The E_3 band hydrolysed α-naphthyl acetate and α-naphthyl butyrate and, to a lesser degree, β-naphthyl esters, whereas the E_4 band hydrolysed α-naphthyl acetate. These bands were sensitive to DFP and their electrophoretic distribution among the strains occurred in a wide M_F range. All Salmonella strains were closely related in terms of their esterase profiles. However, the divergences in electrophoretic distribution of bands E_3 and E_4 were sufficient to recognize the subgenera of most of the Salmonella strains analysed.

INTRODUCTION

Several esterases that differed in their activity towards naphthyl esters, sensitivity to heat, sensitivity to di-isofluoropropyl phosphate (DFP) and electrophoretic mobility were found in *Escherichia coli* (Goullet, 1973). Differential esterase patterns were subsequently reported for species of Proteus and Providencia (Goullet, 1975) and for *Levinea amalonatica*, *L. malonatica* and Citrobacter (Goullet & Richard, 1977). For further investigations into the esterases of enterobacteria, the genus *Salmonella* was chosen because, despite much bacteriological data, the speciation within this composite group remains debatable (Kauffmann, 1963, 1965, 1975; Le Minor, Rhode & Taylor, 1970; Ewing, 1972; Bascomb *et al.*, 1973; Le Minor & Rhode, 1974; Johnson *et al.*, 1975; Véron & Le Minor, 1975).

This paper reports the characterization and distribution of esterase bands (EC. 3.1.1.) in the four biochemical clusters of *Salmonella*, designated as subgenera by Kauffmann (1963, 1965) and proposed as species by Le Minor *et al.* (1970).

METHODS

The strains used, kindly provided by C. Richard (Institut Pasteur, Paris, France), are listed in Table 1.

Growth conditions, preparation of extracts, heat denaturation, inhibition by DFP,

Table 1. *Salmonella species examined*

Code	Organism	Serotype	Strain designation*	Origin†, place and date	Growth‡ conditions
	Subgenus I (*S. kauffmannii* group)§				
1	*S. typhimurium*	4,5,12:i:1,2	W LT2	—	E, S
2	*S. typhimurium*	1,4,5,12:i:1,2	W K9	—	S
3	*S. typhimurium*	1,4,5,12:i:1,2	C934.70	H. faeces, Toulon, 1970	E
4	*S. typhimurium*	1,4,5,12:i:1,2	C940.70	H. blood, Algiers, 1970	E
5	*S. typhimurium*	1,4,5,12:i:1,2	C941.70	H. faeces, Mulhouse, 1970	E
6	*S. tsevié*	4.12:i:e,n,z_{15}	C1141.72	H., Togo, 1972	E
7	*S. cholerae suis*	6,7:c:1,5	W1348-K34	—	S
8	*S. cholerae suis*	6,7:—:1,5	W1350-K36	—	S
9	*S. cholerae suis*	6,7:c:1,5	C16.67	H. blood, S. Vietnam, 1967	E, S
10	*S. cholerae suis*	6,7:c:1,5	C7.69	Pus, S. Vietnam, 1969	E
11	*S. cholerae suis*	6,7:c:1,5	C10.72	H. blood, S. Vietnam, 1972	E
12	*S. cholerae suis*	6,7:c:1,5	C9.74	H. blood, S. Vietnam, 1974	E
13	*S. oranienburg*	6,7:m,t:—	C107.69	H. faeces, Lyon, 1969	E
14	*S. oranienburg*	6,7:m,t:—	C1.70	Flour, Algiers, 1970	E
15	*S. oranienburg*	6,7:m,t:—	C10.70	H. faeces, Montpellier, 1970	E
16	*S. oranienburg*	6,7:m,t:—	C18.70	H. faeces, Nantes, 1970	E
17	*S. somone*	6,7:z_4,z_{24}:—	C532.69	Lizard, Dakar, 1969	E, S
18	*S. nanergou*	6,8:g,s,t:—	C1140.72	H., Togo, 1972	E
19	*S. enteritidis*	1,9,12:g,m:—	W K64	—	S
20	*S. enteritidis*	1,9,12:g,m:1,7	C191.74	H. faeces, Abidjan, 1974	E
21	*S. enteritidis*	1,9,12:g,m:1,7	C224.74	H. faeces, Montpellier, 1974	E
22	*S. enteritidis*	1,9,12:g,m:1,7	C243.74	H. faeces, Lilles, 1974	E
23	*S. enteritidis*	1,9,12:g,m:1,7	C244.74	H. faeces, La Rochelle, 1974	E, S
24	*S. dublin*	1,9,12,Vi:g,p:—	C154.70	H. faeces, Besançon, 1970	E
25	*S. dublin*	1,9,12,Vi:g,p:—	C156.70	H. faeces, St Nazaire, 1970	E
26	*S. dublin*	1,9,12,Vi:g,p:—	C157.70	H. blood, Nice, 1970	E, S
27	*S. gallinarum-pullorum*	1,9,12:—:—	C5.72	Hen, Dakar, 1972	E
28	*S. gallinarum-pullorum*	1,9,12:—:—	C9.72	Hen, Lyon, 1972	E
29	*S. gallinarum-pullorum*	1,9,12:—:—	C13.72	Hen, Chateau-Thierry, 1972	E
30	*S. gallinarum-pullorum*	1,9,12:—:—	C1.73	Guinea, Chateau-Thierry, 1973	E
31	*S. gallinarum-pullorum*	1,9,12:—:—	C4.73	Guinea, Bourges, 1973	E, S
32	*S. gallinarum-pullorum*	1,9,12:—:—	C4bis.73	Bird, Lyon, 1973	E
33	*S. gallinarum-pullorum*	1,9,12:—:—	C5.73	Bird, Paris, 1973	E
34	*S. anatum*	3,10:e,h:1,6	C4.70	H. faeces, Lille, 1970	E
35	*S. anatum*	3,10:e,h:1,6	C13.70	H. faeces, Tunis, 1970	E
36	*S. anatum*	3.10:e,h:1,6	C139.70	H. faeces, Bordeaux, 1970	E
37	*S. anatum*	3,10:e,h:1,6	C141.70	H. faeces, Paris, 1970	E
38	*S. kande*	1,3,19:b:e,n,z_{15}	W1134.72	H., Togo, 1972	E
39	*S. kibi*	16:z_4,z_{23}:—	C479.68	Lizard, Ghana, 1968	E, S
40	*S. mango*	38:k:1,5	W1135.72	H., Togo, 1972	E
41	*S. waycross*	41:z_4,z_{23}:—	C237	Urine, USA, —	E, S
42	*S. dapango*	47:r:1,2	W1136.72	—, 1972	E
	Subgenus II (*S. salamae* group)§				
43	*Salmonella* sp.	4,12:g,m,t:z_{39}	C1308.73	Meat, S. Africa, 1973	E
44	*Salmonella* sp.	9,12:l,z_{28}:e,n,x	C1411.74	Meat, S. Africa, 1974	E, S
45	*Salmonella* sp.	9,46:m,t:e,n,x	C1158.72	Monkey, Africa, 1972	E
46	*Salmonella* sp.	3,10:g,m,s,t:—	C1177.72	Meat, Africa, 1972	E, S
47	*Salmonella* sp.	1,13,23:z_{29}:—	C1076.72	Lizard, Africa, 1972	E, S
48	*Salmonella* sp.	16:b:z_{39}	C1316.73	Tortoise, Great Britain, 1973	E
49	*Salmonella* sp.	16:z:z_{42}	C1381.73	Urine, South Africa, 1973	E, S
50	*Salmonella* sp.	17:b:z_6	C1062.72	Snake, Holland, 1972	E
51	*Salmonella* sp.	1,40:g,t:1,5	C1457.74	Meat, Great Britain, 1974	E
52	*Salmonella* sp.	1,44:e,n,x:1,6	C1312.73	Meat, Botswana, 1973	E
53	*Salmonella* sp.	45:z_{29}:1,5	C1409.74	Meat, South Africa, 1974	E
54	*Salmonella* sp.	57:d:1,5	W1061.72	Lizard, Mulhouse, 1972	E
55	*Salmonella* sp.	58:z_{10}:z_6	C1128.72	Lizard, Holland, 1972	E, S
56	*Salmonella* sp.	58:z_{10}:1,6	W1449.74	Reptile, Switzerland, 1974	E
	Subgenus III (*S. arizonae*)§				
57	*S. arizonae*	16:k:z_{53}	C2078.64	—, 1964	E, S
58	*S. arizonae*	16:z_{10}:e,n,x,z_{15}	C110.68	—, 1968	E
59	*S. arizonae*	21:l,v:z_{57}	W1306.73	Snake, West Germany, 1973	E
60	*S. arizonae*	21:i:1,5,7	C1483.74	Reptile, Israel, 1974	E, S
61	*S. arizonae*	35:z_{29}:—	C6680.60.227	—	E
62	*S. arizonae*	38:z_{52}:z_{53}	W1305.73	Snake, South Africa, 1973	E, S
63	*S. arizonae*	47:k:1,5,7	C1045.72	Salamander, West Germany, 1972	E
64	*S. arizonae*	51:k:z_{35}	C1484.74	Reptile, Israel, 1974	E, S
65	*S. arizonae*	53:k:z	W1285.73	Viper, Switzerland, 1973	E

* W, Collection of the World Health Organization Collaborating Centre for Reference and Research on Salmonella (L. Le Minor, Institut Pasteur, Paris, France); C, Collection du Centre National des Salmonelles (S. Le Minor, Institut Pasteur, Paris, France).

† H, Human origin.

‡ E, Bacteria harvested during the exponential phase; S, bacteria harvested during the stationary phase.

§ Species designation according to Le Minor *et al.* (1970).

Table 1 (*cont.*)

Code	Organism	Serotype	Strain designation*	Origin†, place and date	Growth‡ conditions
66	*S. arizonae*	60:l,v:z	W5716.59	—, 1959	E
67	*S. arizonae*	60:k:z_{35}	C2076.64	—, 1964	E, S
68	*S. arizonae*	60:z_{52}:1,5	W1014.72	Snake, West Germany, 1972	E
69	*S. arizonae*	61:r:z_{35}	W1347.73	—, Atlanta, 1973	E, S
70	*S. arizonae*	61:z_{10}:z_{35}	W1489.74	Reptile, West Germany, 1974	E
71	*S. arizonae*	65:a:1,5,7	W1501.74	H., Zaire, 1974	E, S
	Subgenus IV (*S. houtenae* group)§				
72	*S. mundsburg*	11:g,z_{51}:—	C1636	Gecko, Madagascar, —	E
73	*Salmonella* sp.	11:z_4,z_{32}:—	W457.68	—, Atlanta, 1968	E, S
74	*S. ochsenzoll*	16:z_4,z_{23}:—	C1550	Chameleon, West Germany, —	E, S
75	*Salmonella* sp.	18:z_{36},z_{38}:—	C1256.72	Snake, Switzerland, 1972	E, S
76	*Salmonella* sp.	21:z_4,z_{23}:—	W1403	Reptile, Holland, —	E
77	*Salmonella* sp.	38:g,z_{51}:—	W1205.72	Snake, Switzerland, 1972	E
78	*Salmonella* sp.	38:z_4,z_{23}:—	C577.69	Reptile, West Germany, 1969	E, S
79	*Salmonella* sp.	41:z_4,z_{23}:—	C513.69	Monkey, U.S.A., 1969	E
80	*Salmonella* sp.	43:g,z_{51}:—	C1361.73	Python, Zurich, 1973	E
81	*Salmonella* sp.	45:g,z_{51}:—	C264.66	—, Atlanta, 1966	E, S
82	*Salmonella* sp.	48:z_4,z_{32}:—	C296.67	—, 1967	E
83	*Salmonella* sp.	50:g,z_{51}:—	W K1322	—, Atlanta, —	E
84	*Salmonella* sp.	53:g,z_{51}:—	C1186.72	H. faeces, Manchester, 1972	E, S
85	*Salmonella* sp.	53:z_4,z_{23}:—	W1068.72	Reptile, U.S.A., 1972	E, S

protein estimation, acrylamide–agarose gel electrophoresis and esterase staining were as described previously (Goullet, 1973, 1975), with the following modifications. Bacteria were grown in L broth (Lennox, 1955) without glucose and harvested during the exponential phase or during the stationary phase (see Table 1). They were washed twice in 0·06 M-Tris/glycine buffer, pH 8·7, before ultrasonic disruption. The M_F value, i.e. the distance moved by the esterase band as a percentage of the distance moved by the dye front, was the average obtained from four to six runs. Esterase bands of *E. coli* K12 (Goullet, 1973) were used as a parallel electrophoretic control. DFP (Calbiochem) was used at 10^{-6} to 10^{-3} M.

RESULTS

Esterase band differentiation by naphthyl esters

Reproducible esterase patterns were obtained with the four synthetic substrates. All bacterial strains analysed gave between three and six anodal esterase bands which varied in sharpness and colour intensity. Four principal esterase bands, designated as E_1, E_2, E_3 and E_4 in order of decreasing electrophoretic mobility, were defined (Fig. 1 *a*). Bands E_1 and E_4 hydrolysed α-naphthyl acetate. The E_1 band remained active in the presence of 10^{-4} M-DFP whereas the E_4 band was inhibited by 10^{-5} M-DFP. The E_2 band hydrolysed β-naphthyl acetate but was not affected by 10^{-3} M-DFP. The E_3 band hydrolysed both α- and β-naphthyl esters but produced a darker stain with the α-forms; it was inhibited by 10^{-6} M-DFP. These four esterase bands were inactivated by 10 min treatment at 60 °C. The E_2 band and, in some cases, the E_3 band were more pronounced in bacteria harvested during the stationary phase. In addition, two minor bands reacting with β-naphthyl acetate occupied the extreme positions of the zymogram: the faster band (F), $M_F \approx 95$, was very faint and was inactivated by treatment at 60 °C, the slower one (S), $M_F \approx 11$, was diffuse and remained active at this temperature.

All M_F values were approximate because of variations between different runs. However, electrophoretic relationships could be established between the strains by numerous replicate assays comparing esterase bands in adjacent positions on the same gel.

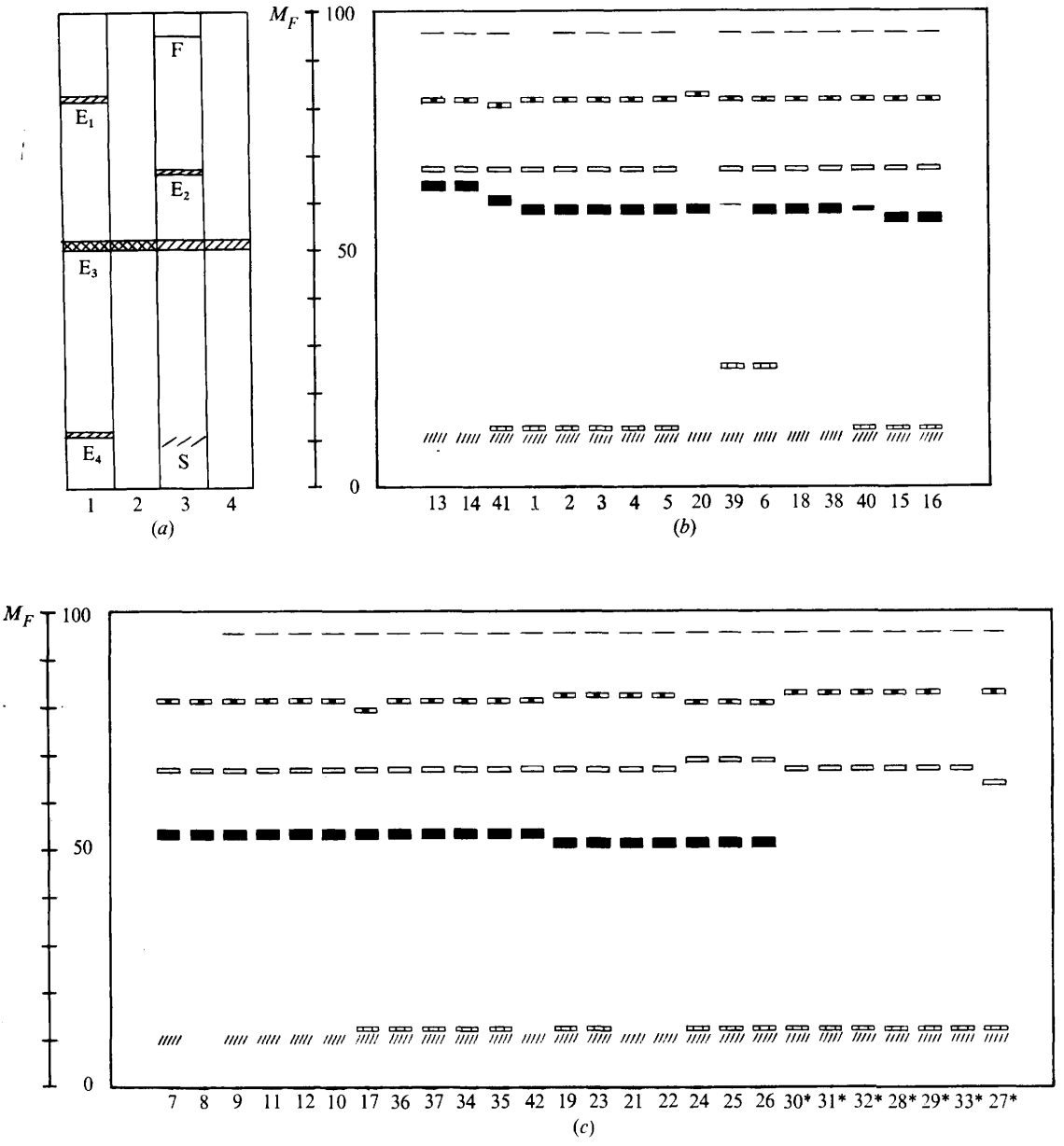

Fig. 1. *Salmonella* subgenus I. (*a*) Schematic representation of mobility and activity of esterase bands (strain 23). Horizontal slab acrylamide–agarose gel electrophoresis was performed using 7 % (w/v) acrylamide and discontinuous Tris/glycine buffer, pH 8·7 (Uriel, 1966). Esterase activity was revealed by the method of Uriel (1961). Substrates used were: 1, α-naphthyl acetate; 2, α-naphthyl butyrate; 3, β-naphthyl acetate; 4, β-naphthyl butyrate. Relative intensity of staining: ⊠ > ▨ > ▨ > ▨. (*b*), (*c*) Esterase patterns of the 42 strains arranged in order of decreasing mobility of E_3 esterase bands. ◧, E_1 band; ▭, E_2 band; ▬, E_3 band; ▭, E_4 band; ——, fast band; /////, slow band. Asterisks indicate *S. gallinarum-pullorum* strains.

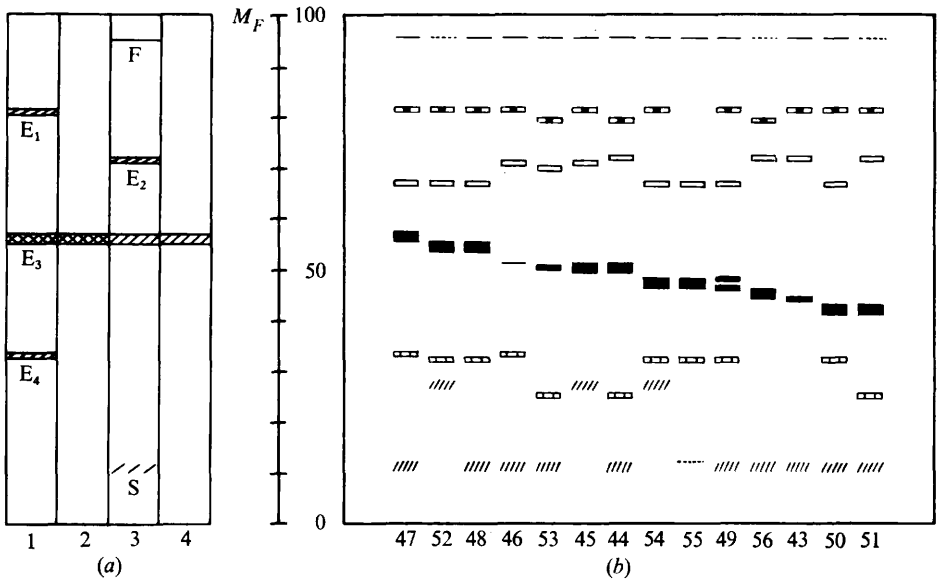

Fig. 2. *Salmonella* subgenus II. (*a*) Schematic representation of mobility and activity of esterase bands (strain 47). (*b*) Esterase patterns of the 14 strains arranged in order of decreasing mobility of E_3 esterase bands. Experimental conditions, relative intensity of staining and key as in legend to Fig. 1.

Salmonella subgenus I (*S. kauffmannii*)

The E_3 band of strains of subgenus I was clearly visible in most of the strains but very faint in strain 39 and undetectable in *S. gallinarum-pullorum* strains (Fig. 1*b*, *c*). Its mobility ranged from $M_F \approx 62$ to $M_F \approx 52$. The E_4 band was very close to the S band when 7% acrylamide was used but became distinct at 5% acrylamide. In some strains, it was not detected.

Salmonella subgenus II (*S. salamae*)

The esterase bands from strains of this subgenus had essentially the same hydrolysing activity, and DFP and heat sensitivity as those of subgenus I (Fig. 2*a*). The E_2 and E_3 bands exhibited electrophoretic heterogeneity (Fig. 2*b*). The E_3 band was seen in all strains though it was very faint in strain 46. The E_4 band migrated faster than the corresponding band of subgenus I. The differences in esterase band mobilities enabled each of the 14 strains analysed to be distinguished.

Salmonella subgenus III (*S. arizonae*)

The strains of this subgenus also gave six bands but the E_4 band was located between bands E_2 and E_3 (Fig. 3*a*). The E_3 band was generally less stained and migrated slower than the corresponding bands of subgenera I and II (see Figs 1*a*, 2*a*). In some strains, the E_3 band or the E_4 band was not detected.

Salmonella subgenus IV (*S. houtenae*)

Bands E_1, E_2 and E_3 of subgenus IV (Fig. 4*a*) had similar characteristics to those of subgenus III. The E_4 band was not detected. The E_3 band mobility varied from $M_F \approx 40$ to

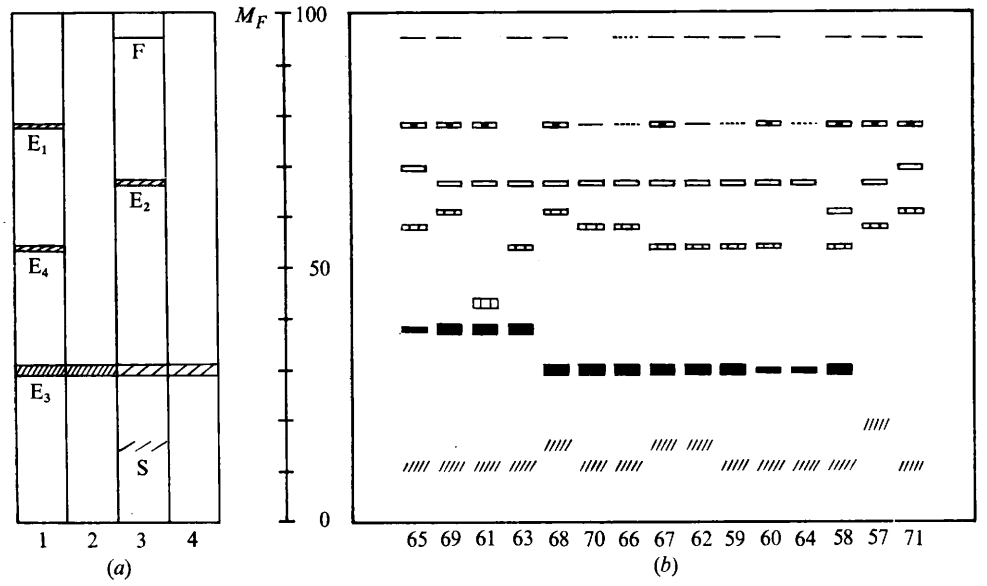

Fig. 3. *Salmonella* subgenus III. (*a*) Schematic representation of mobility and activity of esterase bands (strain 67). (*b*) Esterase patterns of the 15 strains arranged in order of decreasing mobility of E_3 esterase bands. Experimental conditions, relative intensity of staining and key as in legend to Fig. 1.

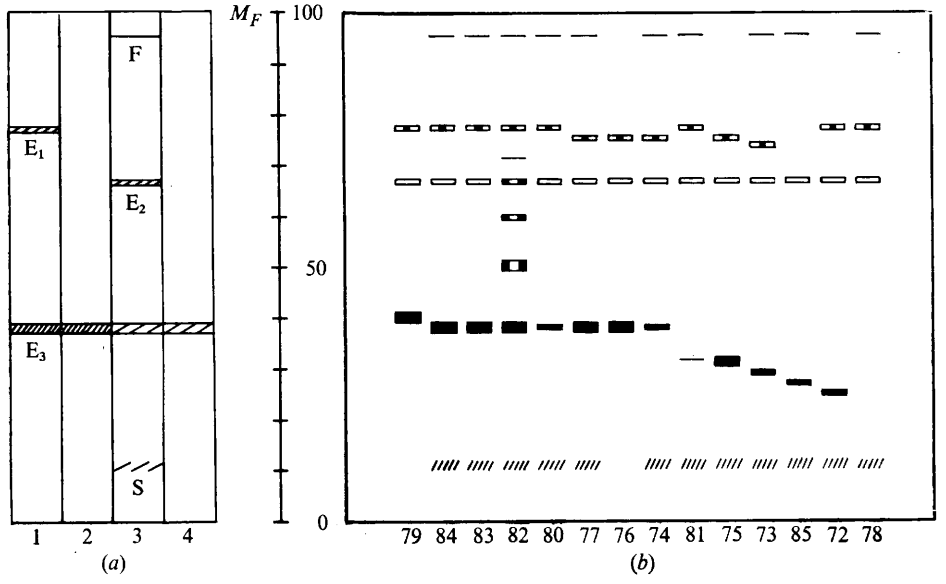

Fig. 4. *Salmonella* subgenus IV. (*a*) Schematic representation of mobility and activity of esterase bands (strain 84). (*b*) Esterase patterns of the 14 strains arranged in order of decreasing mobility of E_3 esterase bands. Experimental conditions, relative intensity of staining and key as in legend to Fig. 1. Unusual bands hydrolysing α- and β-naphthyl acetates are indicated by ▮▮.

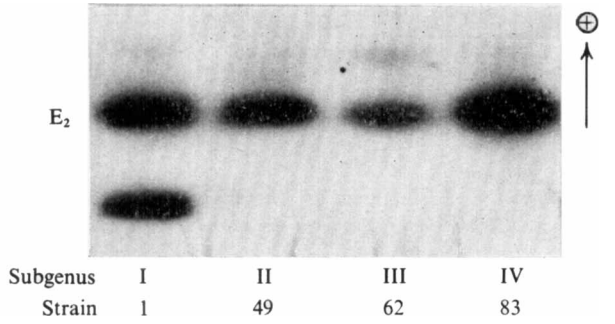

Fig. 5. Migration of E_2 esterase bands from strains of different subgenera. Enzyme activity was demonstrated with β-naphthyl acetate as substrate.

Table 2. *M_F values of esterase bands in the four subgenera of Salmonella*

Mean M_F values are given together with the standard deviation. Sample sizes are given in parentheses

Band	Subgenus I	Subgenus II	Subgenus III	Subgenus IV
E_1	81·34±0·85 (41)	80·54±0·87 (13)	78·0 ±0·0 (11)	76·15±1·14 (13)
E_2	67·07±0·72 (41)	69·21±2·35 (14)	67·0 ±1·96 (15)	67·0 ±0·0 (13)
E_3	55·14±3·51 (35)	48·50±4·41 (14)	32·46±3·84 (13)	34·61±5·09 (13)
E_4	12·92±3·40 (28)	30·27±3·40 (11)	55·85±4·67 (14)	—

$M_F \approx 25$ (Fig. 4*b*). It was very faint in strain 81 and not detected in strain 78. Bands E_2 and S showed electrophoretic uniformity with respect to one another. The E_2 band mobility was identical to that of numerous strains of subgenera I, II and III. Figure 5 illustrates this electrophoretic relatedness between the four subgenera. Strain 82 exhibited a set of unusual bands hydrolysing acetate esters but not butyrate esters. Strains 74, 76, 78, 79 and 85 showed no special esterase relationships with strains 39 and 41 of subgenus I though all have the same flagellar antigens: z_4, z_{23} (see Table 1).

DISCUSSION

Six esterase bands differing in their activity towards naphthyl esters, sensitivity to DFP and electrophoretic mobility were found in extracts of Salmonella. The M_F values of the E_1, E_2, E_3 and E_4 bands in the four subgenera are compared in Table 2. Bands E_1 and E_2 showed little variation in mobility. In contrast bands E_3 and E_4 showed considerable electrophoretic variability. The mobility of E_3 decreased from subgenus I to subgenera III and IV whereas the mobility of E_4 increased from subgenus I to subgenus III. The two minor bands F and S observed in the four subgenera are electrophoretically distinct from the corresponding bands of *E. coli* (Goullet, 1973).

With respect to the characteristics of the various esterase bands, all Salmonella strains appeared to be closely related, which is consistent with the 70 to 87 % of DNA reassociation between strains belonging to different subgenera (Crosa *et al.*, 1973; Stoleru, Le Minor & Lhéritier, 1976). However, the divergences in the electrophoretic distribution of bands E_3 and E_4 observed between the four biochemically-defined clusters, although far less striking than those observed between the Proteus species (Goullet, 1975) and between *L. malonatica* and *L. amalonatica* (Goullet & Richard, 1977), are sufficient to recognize the subgenera of most of the Salmonella strains analysed.

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