Differentiation of *Shigella* by Esterase Electrophoretic Polymorphism

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The electrophoretic mobilities of four esterases (A, B, C and I) of 182 strains of *Shigella dysenteriae*, *S. flexneri*, *S. boydii* and *S. sonnei* were compared to those of 636 strains of *Escherichia coli* from various origins, including the Alkalescens Dispar group and enteroinvasive strains. Discriminant analysis of the distribution of esterases among the strains revealed that *Shigella* could be distinguished from *E. coli* by differences in the distribution of allozymes of esterases C and I. Principal components analysis distinguished four major clusters of *Shigella* strains corresponding to the following: *S. dysenteriae* serotype 1; *S. flexneri* serotypes 1 to 5; *S. flexneri* serotype 6 and *S. boydii* serotypes 2 and 4; and *S. sonnei*. The last three were characterized by distinct electrophoretic variants of carboxylesterase B, as judged by the two-dimensional electrophoretic profile and titration curves. The distinct esterase pattern obtained for the strains of *S. boydii* serotype 13 substantiates the view that this serotype may constitute a new species.

INTRODUCTION

DNA hybridization studies have shown that *Escherichia coli* and *Shigella* (except *S. boydii* serotype 13) are part of the same genetic species (Brenner *et al.*, 1972, 1973). However, numerical taxonomy, nutritional characterization and enzyme electrophoresis indicate that there are differences between these organisms (Johnson *et al.*, 1975; Veron & Le Minor, 1975; Ochman *et al.*, 1983) and between the four taxospecies of *Shigella* [*S. dysenteriae*, *S. flexneri*, *S. boydii* and *S. sonnei* (Goullet, 1980; Dodd & Jones, 1982)].

Previous work has demonstrated that four electrophoretically variable esterases – bands A, B, C and I – are produced both by *E. coli* and *Shigella* strains, and that variations in the number and mobility of these bands between *Shigella* strains define different esterase patterns which appear to be distinct for each species (Goullet, 1980). By comparing esterase mobilities of *E. coli* isolates obtained from three distinct sources (animal and human gastro-intestinal tracts and extra-intestinal infections in man) we have shown that these three bacterial populations differ in their esterase electrophoretic polymorphism (Goullet & Picard, 1986a), and that the frequency of strains showing slow mobilities of esterase B (pattern B₂) is considerably higher when they originate from extra-intestinal infections than when they are obtained from the intestinal tract of healthy individuals (Goullet & Picard, 1986a, b).

The aim of this work was to analyse esterase polymorphism in *Shigella* species and to describe the relationships of the esterase patterns of these organisms and those of *E. coli*. The study is composed of two major parts: (i) a comparison of the conventional electrophoresis (CE) data obtained from *Shigella* with those obtained from the three populations of *E. coli* previously

Abbreviations: AIS, animal intestinal strains; CE, conventional electrophoresis; DFP, di-isopropyl fluorophosphate; E_t, electrophoretic type; GDC, genetic diversity coefficient; HES, human extra-intestinal infection strains; HIS, human intestinal strains; IEF, isoelectrofocusing; M_F, electrophoretic mobility; WE_t, weighted electrophoretic type.

Table 1. *Strains of Shigella examined*

The strain abbreviations are as follows: CNS, Centre National des *Shigella* (strains provided by Dr Toucas, Institut Pasteur, Paris, France); NCTC, National Collection of Type Cultures; CIP, Collection de l'Institut Pasteur, Paris, France; NCDC, National Centers for Disease Control, Atlanta, USA; PHLL, Public Health Laboratory, Leicester, UK.

S. dysenteriae

Serotype	Strain	Serotype	Strain	Serotype	Strain
1	CNS 12.77	1	CIP 53.136	5	CIP 57.42
1	CNS 15.77	2	CNS 2.64	6	NCTC 9762*
1	NCTC 4837*	2	CNS 3.76	6	NCTC 9956*
1	NCTC 8571*	2	CNS 4.77	7	CNS 31.73
1	Sakazakii 4379-60*	2	CNS 5.77	7	CIP 52.123
1	CIP 56.33	3	CIP 53.127	8	NCTC 8599*
1	CIP 62.17	3	CNS 17.77	8	NCTC 9346*
1	CIP 58.1	4	CIP 59.2	9	CNS 5.76
1	CIP 52.27	4	CNS 6.77	9	CIP 58.25
1	CIP 55.90	5	CNS 30.73	10	NCTC 9351
1	CIP 54.95	5	CIP 52.120	10	CIP 58.27

S. flexneri

Serotype	Strain	Serotype	Strain	Serotype	Strain
1	CNS 9.76	2	CNS 464.80	6	CNS 14.79
1	CNS 8.77	2	CNS 468.80	6	CNS 66.79
1	CNS 401.77	3	CNS 6.77	6	NCDC 2924.71
1	NCDC 1921.71	3	CNS 418.77	6	CNS 7590.75
1	CNS 316.80	3	NCDC 2783.71	6	CNS 190.77
1	CNS 317.80	3	CNS 178.80	6	CNS 238.78
1	CNS 367.80	3	CNS 250.80	6	CNS 347.78
1	CNS 431.80	3	CNS 319.80	6	CNS 371.78
1	CNS 449.80	3	CNS 400.80	6	CNS 415.78
1	CNS 451.80	3	CNS 422.80	6	CNS 462.78
1	CNS 452.80	3	CNS 437.80	6	CNS 74.79
1	CNS 471.80	4	CNS 3.75	6	CNS 99.79
2	CNS 18.70	4	NCDC 6603.63	6	CNS 185.79
2	CNS 20.76	4	CNS 441.78	6	CNS 294.79
2	CNS 416.77	4	CNS 109.79	6	CNS 10.80
2	CNS 446.77	4	CNS 157.80	6	CNS 91.80
2	NCDC 2747.71	4	CNS 399.80	6	CNS 222.80
2	CNS 270.80	4	CNS 461.80	6	CNS 233.80
2	CNS 293.80	4	CNS 465.80	6	CNS 246.80
2	CNS 318.80	5	NCDC 6154.61	6	CNS 254.80
2	CNS 362.80	5	CNS 236.78	6	CNS 285.80
2	CNS 389.80	5	CNS 237.78	6	CNS 340.80
2	CNS 390.80	5	CNS 288.78	6	CNS 358.80
2	CNS 401.80	5	CNS 86.80	6	CNS 360.80
2	CNS 430.80	6	CNS 6.74	6	CNS 364.80
2	CNS 432.80	6	CNS 3.77	6	CNS 463.80
2	CNS 441.80	6	CNS 445.77	6	CNS 50.78
2	CNS 450.80	6	CNS 302.78	6	CNS 388.78
2	CNS 454.80	6	CNS 354.78		

Table 1. (continued)

<i>S. boydii</i>					
Serotype	Strain	Serotype	Strain	Serotype	Strain
1	CIP 54.73	5	CNS 19.80	11	NCTC 9360*
1	CNS 32.70	6	NCTC 9771*	12	NCTC 9772*
1	CNS 42.80	7	NCTC 9333*	13	NCTC 9361*
2	CIP 54.74	8	CIP 56.35	13	CIP 58.21
2	CNS 16.76	9	CIP 57.43	13	CIP 58.22
3	PHLL 37746/75*	9	CIP 2.77	13	CIP 76.114
3	NCTC 9329*	10	CIP 599	13	CIP 76.115
4	CIP 54.76	10	CNS 12.77	14	NCTC 9766*
4	CNS 5.77	10	CNS 12.80	14	NCTC 8592*
5	CIP 56.36	11	CNS 41.80	15	NCTC 8590*
5	CNS 7.77	11	NCTC 9321	15	NCTC 9365*
5	CNS 21.77	11	CIP 56.18		
<i>S. sonnei</i>					
	Strain		Strain		Strain
	CNS P.273		CNS 4.76		CNS 191.77
	CNS 46.70		CNS 2.77		CNS 217.77
	CNS 350.73		CNS 11.77		CNS 241.77
	CNS 413.73		CNS 13.77		CNS 586.77
	CNS 43.74		CNS 15.77		CNS 1.78
	CNS 5.75		CNS 16.77		CNS 471.78
	CNS Tri 7.75		CNS 18.77		CNS 489.78
	CNS 20.75		CNS 110.77		CNS 570.78
	CNS NAJ. 76		CNS 130.77		CNS 423.79
					CNS 424.79

* Derivative of culture provided by Dr Jones, Department of Microbiology, The University, Leicester, UK.

investigated and with those from the Alkalescens Dispar group and entero-invasive *E. coli* strains; and (ii) a refined analysis of esterase B variants by use of two-dimensional electrophoretic profiles (Goullet & Picard, 1985) and titration curves (Rhigetti *et al.*, 1978).

METHODS

Shigella strains. The test strains are shown in Table 1.

E. coli strains. Two hundred and eleven animal intestinal strains (AIS), 142 human intestinal strains (HIS), 257 extraintestinal infection strains (HES) and 14 Alkalescens Dispar group strains were analysed by Goullet (1980) and Goullet & Picard (1986a); 12 entero-invasive *E. coli* strains from the serotypes 0136, 0115, 028, 0143 (two strains including strain 4608-58, Sansonetti *et al.*, 1983), 0112, 042, 0159, 029, 0124, 025, 0152 were obtained from Dr Sansonetti (Service des Enterobacteries de l'Institut Pasteur de Paris, Professeur Le Minor).

Culture conditions. All isolates were cultured in Fernbach flasks containing 500 ml L broth (Lennox, 1955) without glucose. The flasks were shaken vigorously for 18 h at 37 °C in a reciprocating water bath shaker set at about 70 oscillations min⁻¹.

Electrophoretic analysis. The preparation of extracts, inhibition by di-isopropyl fluorophosphate (DFP), heat inactivation, horizontal slab PAGE (7%, w/v, acrylamide; Tris/glycine buffer, pH 8.6), estimation of electrophoretic mobility (M_r), polyacrylamide gel isoelectrofocusing (IEF), determination of isoelectric points and esterase staining were described by Goullet (1973) and Goullet & Picard (1985).

Each variant was designated as an allozyme. When an esterase was not detected in a strain, it was recorded as 'null allozyme' (and scored 0). Each distinctive combination of allozymes for the four varieties of esterases was designated as an electrophoretic type (E_i). In principal components analyses, the weighted electrophoretic type (WE_i) designated the number of strains showing the same E_i (Goullet & Picard, 1986a).

Combined IEF-CE titration curves. Details of the technique used are described in Rhigetti *et al.* (1978). The essential features are that, in the first step, a pH gradient (pH 3.5 to 10) is formed across the gel; the crude extract is then applied in a groove running across the gradient, and electrophoresis is done perpendicular to this gradient. Sigmoidal pH mobility curves approximating to the titration curves of esterase are visualized by the specific staining procedure.

Statistical analyses. Principal components analyses and discriminant analysis were done using the Biomedical Programs of the University of California (Dixon, 1981). The electrophoretic data were converted into binary code by considering each allozyme as a variable, occurring in either of two states, i.e. present = 1 or absent = 0 [for full details see Ochman *et al.* (1983) and Goullet & Picard (1986a)].

RESULTS

Comparative distribution of esterase mobilities in Shigella and E. coli strains

A comparison of the distribution of allozymes of esterase A (Table 2) showed that a greater proportion of *Shigella* strains than *E. coli* strains possessed allozyme A-81; the opposite was true for allozyme A-75 and for allozyme A-78. The most frequently observed allozymes of esterase B produced by *Shigella* had the same mobilities as the three most frequent allozymes of pattern B₁ of *E. coli* (Goullet & Picard, 1986a, b). All *Shigella* strains had fast-migrating esterase B except for two strains of serotypes 2 and 3 of *S. flexneri*, where esterase B was slow-migrating. Considerably higher proportions of allozymes C-57, C-59 and C-61 were found for *Shigella* esterase C than for esterase C from the *E. coli* strains. Contrasting with the Gaussian distribution of 20 allozymes from *E. coli*, esterase I of *Shigella* had only two allozymes: I-68 (detected in less than 10% of *E. coli* strains) was encountered in more than 50% of *Shigella* strains; I-65 (detected in only one *E. coli* strain) was observed in 37 (17%) *Shigella* strains. Thus, some allozymes of esterases C and I appeared significantly more frequently in *Shigella* than in *E. coli* strains. The number of null allozymes scored for esterases A, B and I was higher for *Shigella* than for *E. coli*.

Genetic diversity of Shigella

The genetic diversity coefficients (GDCs) (Nei, 1978, Selander *et al.*, 1986) determined for the four taxospecies of *Shigella* distinguished among isolates are shown in Table 3. The mean genetic diversity was lower for *S. sonnei* than for the other taxospecies.

Electrophoretic types in the four taxospecies of Shigella

The 182 *Shigella* strains contained 23 distinct combinations of allozymes for the four esterases (electrophoretic types B, A, C, I: E_{IBACI}) (Table 4). All strains of *S. sonnei* and of *S. flexneri* had at least three esterases whereas some strains of *S. boydii* and of *S. dysenteriae* had only one or two esterases. Each taxospecies was characterized by distinct electrophoretic types with the exception of 15 strains of *S. boydii* (serotypes 3, 5, 7, 9, 11 and 15) and nine strains of *S. dysenteriae* (serotypes 2, 5 and 8) which produced only esterase C. However, several strains of these serotypes were differentiated by an additional esterase band. *S. sonnei* appeared to be relatively electrophoretically homogeneous, while *S. flexneri* was clearly separated into two groups: one for serotypes 1 to 5 and another for serotype 6 (Goullet, 1980; Goullet *et al.*, 1983). This latter group resembled strains of *S. boydii* serotypes 2 and 4. *S. boydii* serotype 13 did not produce esterases B, A and I but five strains of this serotype had one esterase band at $M_F \simeq 70$ that hydrolysed α - and β -naphthyl acetates, α -naphthyl butyrate and indoxyl acetate, and three strains had one esterase band at $M_F \simeq 72$ that hydrolysed β -naphthyl acetate and butyrate and indoxyl acetate. The activity of these two bands was resistant to DFP (10^{-3} M) but sensitive to heat (10 min, 60 °C). The strains of this serotype did not produce esterase F (Goullet, 1973, 1980). The strains of *S. dysenteriae* serotype 10 and a strain of *S. dysenteriae* serotype 7 produced esterase B which was not observed in the other serotypes (Goullet, 1980).

Distinctive electrophoretic types of Shigella and E. coli

The 12 entero-invasive *E. coli* strains exhibited 12 electrophoretic types (Table 5) which were distinct from the *Shigella* electrophoretic types. Of the 636 *E. coli* strains, there were 328 distinct electrophoretic types, including the 316 reported by Goullet & Picard (1986a), plus five additional electrophoretic types of the Alkalescens Dispar group and seven of entero-invasive *E. coli*.

The electrophoretic relationships between *Shigella* and *E. coli* were analysed by comparing the 23 E_{IBACI} of *Shigella* with strains of *E. coli* showing the corresponding E_i (Table 4). As the

Table 2. *Electrophoretic distribution of esterases A, B, C and I in E. coli and Shigella populations*

The values given are the percentage of strains with each allozyme; particularly significant values are shown in **bold type**. NA, Null allozyme.

M_F	Esterase A		Esterase B		Esterase C		Esterase I	
	<i>E. coli</i>	<i>Shigella</i>	<i>E. coli</i>	<i>Shigella</i>	<i>E. coli</i>	<i>Shigella</i>	<i>E. coli</i>	<i>Shigella</i>
44					0.94			
45								
46					1.41			
47					0.15			
48					9.27	1.09		
49								
50					1.41			
51								
52					1.41			
53					5.34		0.15	
54					1.41			
55					21.06		0.15	
56					0.31			
57			12.89		10.84	48.90	0.62	
58					0.15		0.31	
59					3.61	19.78	0.62	
60			5.34		19.65		4.71	
61					0.62	18.13	0.47	
62			2.35	0.54	2.51	0.54	3.30	
63			0.31		0.47		2.98	
64				0.54			0.47	
65							0.62	17.58
66			1.88		0.31		3.77	
67			0.15				0.47	
68			9.59	17.03			9.90	51.64
69								
70			48.89	17.03			31.28	
71	0.47							
72	0.31		8.80	25.27			7.23	
73			2.35				4.08	
74	0.15		2.51				1.57	
75	48.74	29.12	0.15				2.98	
76								
77								
78	28.45	6.59					1.10	
79								
80								
81	13.20	42.85						
82								
83	0.94							
NA	7.70	21.44	4.71	39.59	19.13	11.56	23.11	30.78

Table 3. *Genetic diversity (H) of esterases A, B, C and I in each of the four taxospecies of Shigella*

$H = 1 - \sum x_i^2(n/n - 1)$ where x_i is the frequency for each allozyme and n the number of strains in each taxospecies.

	A	B	C	I	Mean
<i>S. sonnei</i>	0	0	0.51	0	0.127
<i>S. flexneri</i>	0.468	0.665	0.498	0	0.407
<i>S. boydii</i>	0.321	0.469	0.594	0.298	0.420
<i>S. dysenteriae</i>	0.667	0.165	0.597	0.298	0.431

Table 4. *Distribution of the esterase electrophoretic types among the strains of the four taxospecies of Shigella and their correspondence with those of the 636 E. coli strains*

Taxospecies	Serotype	No. of strains	E_{IBACI} of <i>Shigella</i>	Number and percentage of strains of <i>E. coli</i> having the same E_i as <i>Shigella</i> *			
				E_{IBA}	E_{IBAC}	E_{IBAI}	E_{IBACI}
<i>S. sonnei</i>		16	70 81 61 65	35 (5.5%)	0	0	0
		1	70 81 62 65		1 (0.15%)	0	0
		11	70 81 0 65		2 (0.30%)	0	0
<i>S. flexneri</i>	1-5	30	68 75 57 68	38 (5.97%)	4	0	0
	1	1	68 75 0 68		5 (0.78%)	1 (0.15%)	1 (0.15%)
	1	1	64 75 57 68		0	0	0
	2	1	62 75 57 68		4 (0.63%)	0	0
	2, 3, 5	20	0 75 57 68		19 (3%)	1 (0.15%)	4 (0.62%)
<i>S. flexneri</i>	6	31	72 81 59 68	27 (4.25%)	0	6 (0.94%)	0
	6	2	72 81 0 68		1 (0.15%)	0	0
<i>S. boydii</i>	2, 4	4	72 81 59 65	27 (4.25%)	0	0	0
	12	1	72 81 55 0		19 (3%)	5 (0.78%)	3 (0.45%)
	6, 10	4	72 0 57 0		0	0	0
	1, 3, 8	4	72 0 0 0	7 (1.1%)	1 (0.15%)	1 (0.15%)	1 (0.15%)
	14†	2	0 81 57 68		0	0	0
	3, 5, 7, 9, 11, 15‡	15	0 0 57 0	0	0	0	0
	13§	5	0 0 61 0		0	0	0
<i>S. dysenteriae</i>	1	12	0 78 61 0	11 (1.7%)	0	4 (0.62%)	0
	10	2	70 0 48 0	27 (4.25%)	1 (0.15%)	8 (1.25%)	0
	7	1	70 81 0 68	35 (5.5%)	2 (0.30%)	3 (0.45%)	0
	3, 6, 7, 9	4	0 81 57 68	0	0	0	0
	9	1	0 81 0 68		0	0	0
	3, 4, 6	4	0 81 57 0		0	0	0
	2, 5, 8¶	9	0 0 57 0		0	0	0

* The 27 strains with an identical E_{IBA} to *S. boydii* strains of serotypes 2, 4 and 12 were the same as those with an identical E_{IBA} to *S. flexneri* strains serotype 6; the 35 strains with an identical E_{IBA} to *S. dysenteriae* strains of serotype 7 were the same as those with an identical E_{IBA} to strains of *S. sonnei*; the two strains with an identical E_{IBAC} to strains of *S. dysenteriae* serotype 7 were the same as those with an identical E_{IBAC} to strains of *S. sonnei*.

† Strains of *S. boydii* serotype 14 also exhibited a band at $M_F \approx 72$ that hydrolysed α -naphthyl butyrate and weakly hydrolysed α -naphthyl and indoxyl acetates, and which was resistant to DFP (10^{-3} M).

‡ Strains of *S. boydii* serotypes 3 and 5, and strains of serotypes 9 and 15, exhibited a faint band, at $M_F \approx 76$ and $M_F \approx 74$ respectively, that hydrolysed α -naphthyl acetate.

§ Strains of *S. boydii* serotype 13 exhibited two additional bands (see results).

|| Strains of *S. dysenteriae* serotype 10 did not produce esterase F.

¶ Three additional bands, resistant to DFP, were detected in strains of *S. dysenteriae* serotype 8: one band at $M_F \approx 72$ from strain NCTC 9346 hydrolysed α - and β -naphthyl acetates; one band at $M_F \approx 72$ from strain NCTC 8599 hydrolysed α - and β -naphthyl acetates, α -naphthyl butyrate and indoxyl acetate (this band was resistant to heat); a third band at $M_F \approx 70$ from the two strains hydrolysed α - and β -naphthyl butyrates.

Table 5. *Electrophoretic types of esterases produced by the 12 entero-invasive E. coli strains*

Strain	B	A	C	I
0136	74	81	57	61
0115	73	78	63	68
028	72	81	57	60
0143 (4608-58)	70	78	57	60
0143	70	78	57	59
0112	70	78	0	0
042	70	75	60	68
0159	70	75	60	65
059	70	75	48	63
0124	68	75	50	0
025	66	81	60	0
0152	0	75	48	0

Fig. 1. Principal components analysis of the 175 *Shigella* strains and of the 636 *E. coli* strains. (a) Plot of score of allozymes of the four esterases for the first two principal components: ■, esterase A; ●, esterase B; ○, esterase C; □, esterase I. (b) Plot of score of weighted electrophoretic types for the first two principal components: ●, *S. dysenteriae* serotypes 2 to 10; ▲, *S. flexneri* serotypes 1 to 5; ▼, *S. flexneri* serotype 6; ◆, *S. boydii*; ■, *S. sonnei*; ◇, Alkalescens Dispar group; ▽, entero-invasive *E. coli*; ○, AIS; □, HIS; △, HES. For clarity, some of the allozymes of the various esterases which were projected near the origin of the axes have been omitted from the Figure.

electrophoretic distribution of esterases A and B appeared to be less discriminative than those of esterases C and I for differentiation of *Shigella* from *E. coli*, we analysed the identical E_{tBA} , E_{tBAC} , E_{tBAI} and E_{tBACI} observed in the two organisms. The results were as follows: (i) 168 strains (26.5%) of *E. coli* had identical E_{tBA} with those of *Shigella*; (ii) 35 (5.50%) and 32 (5%) strains of *E. coli* had identical E_{tBAC} and E_{tBAI} , respectively; and (iii) five strains (0.78%) had identical E_{tBACI} . These five strains had one or three null allozymes which reduced the discriminative power of the enzyme analysis.

Principal components analyses

Three analyses were made: one for *Shigella* strains and the 636 *E. coli* strains, one with only *Shigella* strains, and one with *Shigella*, the Alkalescens Dispar group and entero-invasive *E. coli* strains. The strains of *Shigella boydii* serotype 13 and of *S. dysenteriae* serotype 8 that had atypical esterase patterns were not included in these analyses.

***Shigella* and *E. coli* strains.** The first two principal components accounted for 8.14% of the total variance. Projection of different allozymes (Fig. 1a) on the first two axes revealed that allozymes A-81, B-72, I-65, C-59, C-61 and C-55 were distinguished by the first component and that the second component opposed allozymes A-78, B-57 and C-60 to allozymes A-75, B-68, B-0, I-68 and C-57. Projection of WE_t (Fig. 1b) distinguished one group (I) displayed by component 1, consisting of electrophoretic types recovered from all strains of *S. flexneri* serotype 6, some strains of *S. boydii*, the majority of strains of *S. sonnei* and certain *E. coli* strains, principally those from animal sources; a second group (II), composed of strains of *S. flexneri* serotypes 1 to 5 and some strains of *S. dysenteriae* was opposed by component 2 to a third group (III) composed of HES of *E. coli* exhibiting allozymes B-57, A-78 and C-60. The remaining strains of *S. sonnei*, *S. boydii* and *S. dysenteriae* were not distinguished by this analysis.

***Shigella* strains.** The first two principal components accounted for 13% of the total variance. Projections of the allozymes (Fig. 2a) from the first two components revealed that the first component opposed allozymes A-75, B-68 and C-57 to allozymes A-81, B-70 and I-65, and that

Fig. 2. Principal components analysis of the *Shigella* strains. (a) Plot of score of allozymes for the first two principal components: symbols as in legend to Fig. 1. (b) Plot of score of weighted electrophoretic types for the first two principal components: ●, *S. dysenteriae* serotype 1; other symbols as in legend to Fig. 1. For clarity, some of the allozymes of the various esterases which were projected near the origin of the axes have been omitted from the Figure.

Fig. 3. Principal components analysis of the *Shigella* strains and of the *Alkalescens Dispar* group and entero-invasive *E. coli* strains. (a) Plot of score of allozymes for the first two principal components: symbols as in legend to Fig. 1. (b) Plot of score of weighted electrophoretic types for the first two principal components: symbols as in legends to Figs. 1 and 2. For clarity, some of the allozymes of the various esterases which were projected near the origin of the axes have been omitted from the Figure.

the second component opposed allozymes A-0, A-78, B-0, I-0 and C-61 to allozymes I-68, C-59, B-72 and A-81. Projection of *WE*_i (Fig. 2b) showed that the first component opposed strains of *S. flexneri* serotypes 1 to 5 to strains of *S. sonnei*, and that the second component opposed strains of *S. flexneri* serotype 6, strains of *S. boydii* serotypes 2 and 4, and a strain of *S. dysenteriae* serotype 7, to strains of *S. dysenteriae* serotype 1, and to the remaining strains of *S. dysenteriae* and of *S. boydii*.

Shigella and *Alkalescens Dispar* group and entero-invasive strains of *E. coli*. The first two principal components accounted for 11.6% of the total variance. The projections of allozymes

Table 6. Discriminant functions (*F*) used by discriminant analysis to classify the 792 observations within the population of *Shigella* and the three populations of *E. coli*

Allozyme used	<i>F</i>	Selection order	Coefficient			
			<i>Shigella</i>	<i>E. coli</i> HES	<i>E. coli</i> HIS	<i>E. coli</i> AIS
I-68	50.25	1	13.37	6.51	5.33	6.60
I-65	47.70	2	15.42	3.22	1.43	2.64
C-57	44.77	7	11.07	4.48	3.41	5.97
C-55	33.87	5	7.02	6.16	5.50	9.51
C-61	25.33	9	15.67	6.21	6.01	7.15
C-59	25.10	10	14.25	7.02	7.84	8.32
B-0	20.28	3	12.05	5.96	7.31	7.45
A-81	18.93	8	8.94	4.31	5.63	5.76
A-0	16.22	11	4.68	5.21	4.59	9.24
B-70	13.75	6	5.68	6.88	8.63	8.38
B-57	10.43	4	13.11	14.38	11.29	12.52
B-68	9.64	12	8.52	5.91	8.37	7.69
B-74	9.00	14	3.53	4.60	6.42	8.65
C-54	8.14	15	9.04	7.70	13.28	8.35
C-44	8.01	19	11.57	6.96	6.36	12.61
I-60	7.52	16	1.02	1.30	4.50	1.65
C-48	6.23	20	5.78	4.35	4.70	6.44
I-70	6.14	13	5.42	6.25	4.87	6.26
A-75	6.10	17	3.48	2.80	4.09	2.74
C-46	5.25	21	6.57	3.11	4.86	7.64
I-0	5.61	23	7.46	5.50	5.13	5.42
B-60	5.48	18	6.08	8.77	7.55	6.18
C-56	4.62	22	3.20	7.56	5.87	9.75
C-0	4.57	24	7.37	6.32	5.22	5.95

Table 7. Results of the classification according to discriminant analysis calculations from the 785 observations

Strains of entero-invasive *E. coli* and of the Alkalescens Dispar group which could not be classified as HES or HIS were not included in the discriminant analysis.

	Percentage of strains correctly classified	Calculated <i>Shigella</i>	Calculated HES	Calculated HIS	Calculated AIS
<i>Shigella</i> strains (175)	96.0	168	4	2	1
<i>E. coli</i> strains					
HES (257)	58.4	12	150	61	34
HIS (142)	69.7	6	16	99	21
AIS (211)	61.1	15	17	50	129
Total strains		201	187	212	185

and of WE_1 of *Shigella* strains (Fig. 3) were similar to those of the preceding analysis. Projection of WE_1 did not distinguish any groups corresponding to the Alkalescens Dispar group and entero-invasive *E. coli* strains which displayed a wide distribution throughout the plan. Thus, for the two latter principal component analyses, four major clusters of *Shigella* strains were distinguished: (i) *S. dysenteriae* serotype 1; (ii) *S. flexneri* serotypes 1 to 5; (iii) *S. flexneri* serotype 6 and *S. boydii* serotypes 2 and 4 and one strain of *S. dysenteriae* serotype 7; and (iv) *S. sonnei*.

Discriminant analysis

The 785 strains and the 59 allozymes were used in stepwise discriminant analyses to distinguish *Shigella* strains from AIS, HES and HIS (Tables 6 and 7). Among the 24 allozymes selected for discriminant function (Table 6) the most significant were I-68, I-65, C-57, C-55, C-61

Fig. 4. Two-dimensional electrophoretic profile of esterase B variants of 182 *Shigella* strains and of 610 *E. coli* strains with the number of isolates of *E. coli* in which each variant is detected. ○, Variant produced by *E. coli* strains; ●, variant produced by *Shigella* strains; ⊙, variant produced both by *E. coli* and *Shigella* strains.

and C-59. As can be seen in the distribution of esterase mobilities, allozymes of esterases I and C appeared to be more significant for distinguishing between *Shigella* strains and *E. coli* strains than allozymes of esterases A and B. Application of the discriminant functions (corresponding to the coefficients presented in Table 6) to the 785 strains allowed the correct classification of 96% of the *Shigella* strains and of 58.4%, 69.7% and 61.1% of HES, HIS and AIS of *E. coli*, respectively (Table 7). Discriminant analysis demonstrated that the *Shigella* strains were clearly distinguished from the three populations of *E. coli* strains by esterase electrophoretic polymorphism.

Two-dimensional electrophoretic profile of esterase B produced by Shigella strains

The isoelectric points of esterase B determined by thin-layer IEF were grouped around two points: (i) pI 4.6 for strains of *S. sonnei* and *S. flexneri* serotypes 1 to 5, one strain of *S. dysenteriae* serotype 10 and three strains of *S. boydii* serotypes 3, 6 and 12; and (ii) pI 4.75 for strains of *S. flexneri* serotype 6, two strains of *S. flexneri* serotypes 1 and 3, strains of *S. boydii* serotypes 2 and 4 and one strain of *S. dysenteriae* serotype 7. Fig. 4 shows the two-dimensional electrophoretic profile (Goullet & Picard, 1985) established by plotting isoelectric points against electrophoretic mobilities of the esterase B produced by the 182 strains of *Shigella* and the 610 strains of *E. coli*. Forty-one and seven distinct electrophoretic variants were defined, both by pI and M_F , for *E. coli* and *Shigella* strains, respectively. Five variants (v_1 to v_5) of *Shigella* were identical both in pI and M_F values to five variants of pattern B_1 of *E. coli* (Goullet & Picard, 1986b), whereas two variants (v_6 and v_7) were only observed in *Shigella* strains. The most frequently observed variants in *Shigella* strains were v_2 (pI 4.6, $M_F \approx 70$) for *S. sonnei* strains, v_3 (pI 4.6, $M_F \approx 68$) for *S. flexneri* serotypes 1 to 5 strains and v_4 (pI 4.75, $M_F \approx 72$) for *S. flexneri* serotype 6 and for some *S. boydii* strains. Variant v_2 was the most frequently encountered variant in *E. coli* strains. Variant v_3 had the same pI as v_2 , but differed in its M_F . Variant v_4 was distinct from the two latter variants according to both electrophoretic parameters (Fig. 4).

Titration curves produced by combined IEF-CE

Titration curves were used to improve electrophoretic discrimination within each of the three major electrophoretic variants of esterase B (v_2 , v_3 and v_4) and between variants v_2 and v_3 . A single titration curve demonstrating a close molecular relationship between the proteins was

Fig. 5. Comparative titration curves obtained by combined IEF-CE for the most frequent esterase B variant of *E. coli* and for variant v_3 of *S. flexneri* strains of serotypes 1 to 5. S, variant v_3 of *S. flexneri* serotype 1 (pI 4.6, $M_F \approx 68$); E, most frequent variant of *E. coli* (pI 4.6, $M_F \approx 70$). The arrow indicates the groove in which the crude enzyme extract was placed (see Methods).

obtained when this method was applied to five strains chosen at random from each of the three variants of *Shigella*. Similarly, a single titration curve was obtained when variant v_2 of *S. flexneri* serotype 6 and variant v_2 of *S. boydii* serotypes 2 and 4 were titrated together, and another single curve was obtained when each of the three *Shigella* variants were titrated with the homologous *E. coli* variants. A typical 'fork' form (Fig. 5) was produced when the mobility curves from v_3 (pI 4.6, $M_F \approx 68$) were compared with those of the most frequently occurring *E. coli* variant (pI 4.6, $M_F \approx 70$). This may be interpreted as the substitution of a histidine in the *E. coli* variant for an arginine in the v_3 variant of *S. flexneri* (Rhigetti *et al.*, 1978).

DISCUSSION

The phenotypic differences between *Shigella* and *E. coli* strains have been substantiated at the molecular level by comparative analyses of the esterase polymorphism found in 182 strains of *Shigella* and 636 strains of *E. coli*. The individual distributions of esterase mobilities (Table 2), comparisons between electrophoretic type (Table 4) and discriminant analysis all indicated that this distinction is principally determined by allozymes of esterases C and I. *Shigella* variants v_2 , v_3 and v_4 of esterase B showed a close molecular relationship with the three corresponding *E. coli* variants of pattern B₁, as judged by the single titration curves obtained in each case, while *Shigella* variants v_6 and v_7 were distinguished by their pI from *E. coli* variants of pattern B₂ (Goulet & Picard, 1986b). The absence of esterase pattern B₂ in *Shigella* strains is in agreement with the low proportion of extra-intestinal infections usually observed with these organisms. All the strains of *Shigella* were differentiated from all those of *E. coli* by distinct electrophoretic profiles. Thus, the six *Shigella* strains, indistinguishable from *E. coli* strains on the basis of their E_{IBACI} , were distinguished from them by the pI of esterase B: the strain of *S. flexneri* serotype 1 with the electrophoretic type B-68, A-75, C-0, and I-68 had an esterase pI value of 4.6 while the corresponding strain of *E. coli* had an esterase pI of 4.75. Among strains with electrophoretic type B-72, A-81, C-55 and I-0, esterase B of the strain of *S. boydii* serotype 12 had a pI of 4.6 and the strains of *E. coli* had an esterase pI of 4.75; the four strains of *S. boydii* serotypes 1, 3, and 8, with electrophoretic type B-72, A-0, C-0 and I-0 had esterase pI values of 4.75, and the strain of *E. coli* with the same E_i had an esterase pI of 4.5. Principal components analyses showed that *S. flexneri* and some *S. boydii* strains were more distantly related to *E. coli* than is *S. sonnei*. This

Table 8. Comparison of genetic diversity (H) in *Shigella* and *E. coli* obtained with esterases and enzymes analysed by Ochman *et al.* (1983)

$H = 1 - \sum x_i^2$ where x_i is the frequency for each allozyme. The numbers of each allozyme are indicated in parentheses.

	Esterases (present study)		Other enzymes (Ochman <i>et al.</i> , 1983)	
	<i>Shigella</i>	<i>E. coli</i>	<i>Shigella</i>	<i>E. coli</i>
No. of strains	182	636	123	1 608
No. of electrophoretic types	23	328	23	279
Mean GDC	0.687	0.822	0.588*	0.620*
GDC esterase B	0.752 (6)	0.820 (13)	MPI† 0.646 (5)	0.710 (9)
GDC esterase C	0.748 (6)	0.885 (19)	6PG† 0.627 (6)	0.715 (20)
GDC esterase A	0.631 (4)	0.723 (8)	ACO† 0.556 (4)	0.616 (9)
GDC esterase I	0.620 (3)	0.860 (21)	IDH† 0.526 (3)	0.440 (6)

* Calculated with 12 enzymes.

† The four most polymorphic enzymes for *Shigella* strains. (Abbreviations: MPI, mannose phosphate isomerase; 6PG, 6-phosphogluconate dehydrogenase; ACO, aconitase; IDH, isocitrate dehydrogenase.)

observation agrees with the molecular similarity of v_2 from *S. sonnei* with the most frequent variant of esterase B produced by *E. coli* strains and confirms the relationship already observed between these organisms. The 12 entero-invasive *E. coli* strains were distinguished from each of the 182 *Shigella* strains, suggesting that esterase electrophoretic polymorphism is not correlated with the presence of a virulence plasmid in these bacteria (Sansone *et al.*, 1983). Table 8 compares the GDCs (Nei, 1975), obtained with *Shigella* and *E. coli* strains, of the four esterases and of other enzymes analysed by Ochman *et al.* (1983). In both studies, the mean GDC is lower for *Shigella* than for the *E. coli* strains but the esterases appeared to be more polymorphic than other enzymes in the two organisms.

Principal component analyses of esterase mobilities distinguished four major clusters of *Shigella* strains corresponding to the following: *S. dysenteriae* serotype 1; *S. flexneri* serotypes 1 to 5; *S. flexneri* serotype 6 and *S. boydii* serotypes 2 and 4; and *S. sonnei*. Our results are in agreement with those of Dodd & Jones (1982) who showed that it is possible to distinguish phenotypically at least four taxospecies of *Shigella*. The distinct esterase pattern of *S. dysenteriae* serotype 1 (Goullet, 1980) is confirmed by the present work showing electrophoretic homogeneity of the 12 strains tested. *S. flexneri* serotype 6 differs from *S. flexneri* serotypes 1 to 5 by three distinct mobilities and resembles strains of *S. boydii* serotypes 2 and 4 (Goullet, 1980; Goullet *et al.*, 1983). The individual nature of *S. flexneri* serotype 6 as compared to *S. flexneri* serotypes 1 to 5 is supported by the two-dimensional electrophoretic profile of esterase B which showed two distantly related variants v_3 and v_4 , probably separated by at least three successive mutations for the two clusters of strains (Picard *et al.*, 1987). These findings support the division of strains of serotypes 1 to 5 and serotype 6 into two distinct subphenons (Dodd & Jones, 1982), and suggest a revision of taxonomic status of strains designated *S. flexneri* serotype 6 (see, e.g., Timakov *et al.*, 1972; Petrovskaya & Bondarenko, 1977; Dodd & Jones, 1982; Goullet *et al.*, 1983). *S. sonnei* appears to be electrophoretically the most homogeneous species and is clearly separated from the other taxospecies of *Shigella*. Our findings show that the four clusters of *Shigella* strains as delineated by esterase polymorphism appear to be closer to *E. coli* than to each other and suggest that they are derived from distinct ancestral strains of *E. coli*.

A fifth cluster was represented by strains of *S. boydii* serotype 13 which was clearly distinguished from other *Shigella* strains both by absence of bands A, B, I and F and by the presence of two additional esterase bands. This correlates well with the work of Brenner *et al.* (1973, 1982), who showed that *S. boydii* serotype 13 showed low DNA homology with other *S. boydii* serotypes, other *Shigella* species and *E. coli*. Esterase patterns substantiate the view that this serotype may constitute a new species. The existence of other clusters remains possible but their identification was hampered by the absence of two or three esterase bands or by a limited number of strains.

REFERENCES

- BRENNER, D. J., FANNING, G. R., SKERMAN, F. J. & FALKOW, S. (1972). Polynucleotide sequence divergence among strains of *Escherichia coli* and closely related organisms. *Journal of Bacteriology* **109**, 953–965.
- BRENNER, D. J., FANNING, G. R., MIKLOS, G. V. & STEIGERWALT, A. G. (1973). Polynucleotide sequence relatedness among *Shigella* species. *International Journal of Systematic Bacteriology* **23**, 1–7.
- BRENNER, D. J., STEIGERWALT, A. G., GAIL WATHEN, H., GROSS, R. J. & ROWE, B. (1982). Confirmation of aerogenic strains of *Shigella boydii* 13 and further study of *Shigella* serotypes by DNA relatedness. *Journal of Clinical Microbiology* **16**, 432–436.
- DIXON, W. (1981). BMDP statistical software. Berkeley, California: University of California Press.
- DODD, C. E. R. & JONES, D. (1982). A numerical taxonomic study of the genus *Shigella*. *Journal of General Microbiology* **128**, 1933–1957.
- GOULLET, PH. (1973). An esterase zymogram of *Escherichia coli*. *Journal of General Microbiology* **77**, 27–35.
- GOULLET, PH. (1980). Esterase electrophoretic pattern relatedness between *Shigella* species and *Escherichia coli*. *Journal of General Microbiology* **117**, 493–500.
- GOULLET, PH. & PICARD, B. (1985). A two-dimensional electrophoretic profile for bacterial esterases. *Electrophoresis* **6**, 132–135.
- GOULLET, PH. & PICARD, B. (1986a). Comparative esterase electrophoretic polymorphism of *Escherichia coli* isolates obtained from animal and human sources. *Journal of General Microbiology* **132**, 1843–1851.
- GOULLET, PH. & PICARD, B. (1986b). Highly pathogenic strains of *Escherichia coli* revealed by distinct electrophoretic pattern of carboxylesterase B. *Journal of General Microbiology* **132**, 1853–1858.
- GOULLET, PH., PICARD, B. & TOUCAS, M. (1983). Différenciation du serotype 6 des sérotypes 1–5 de *Shigella flexneri* par électrophorèse et focalisation isoélectrique de leurs estérases. *Annales de microbiologie* **134B**, 269–276.
- JOHNSON, R., COLWELL, R. R., SAKAZAKI, R. & TAMURA, K. (1975). Numerical taxonomy, studies of the Enterobacteriaceae. *International Journal of Systematic Bacteriology* **25**, 12–37.
- LENNOX, E. S. (1955). Transduction of linked genetic characters of the host by bacteriophage P1. *Virology* **1**, 190–206.
- NEI, M. (1975). *Molecular Population Genetics and Evolution*. New York: Elsevier.
- NEI, M. (1978). Estimation of average heterozygosity and genetic distance from a small sample of individuals. *Genetics* **89**, 583–590.
- OCHMAN, H., WHITTAM, T. S., CAUGANT, D. A. & SELANDER, R. K. (1983). Enzyme polymorphism and genetic population structure in *Escherichia coli* and *Shigella*. *Journal of General Microbiology* **129**, 2715–2726.
- PETROVSKAYA, V. G. & BONDARENKO, V. M. (1977). Recommended corrections to the classification of *Shigella flexneri* on a genetic basis. *International Journal of Systematic Bacteriology* **27**, 171–175.
- PICARD, B., GOULLET, PH. & KRISHNAMOORTHY, R. (1987). A novel approach to study the structural basis of enzyme polymorphism: analysis of carboxylesterase B of *E. coli* as model. *Biochemical Journal* **241**, 877–881.
- RIGHETTI, P. G., KRISHNAMOORTHY, R., GIANAZZA, E. & LABIE, D. (1978). Protein titration curves by combined isoelectric focusing-electrophoresis with hemoglobin mutants as models. *Journal of Chromatography* **166**, 455–460.
- SANSONETTI, P. J., D'HAUTEVILLE, H., ECOBICHON, C. & POURCEL, C. (1983). Molecular comparison of virulence plasmids in *Shigella* and enteroinvasive *Escherichia coli*. *Annales de microbiologie* **134A**, 295–318.
- SELANDER, R. K., CAUGANT, D. A., OCHMAN, H., MUSSER, J. M., GILMOUR, M. N. & WHITTAM, T. S. (1986). Methods of multilocus enzyme electrophoresis for bacterial population genetics and systematics. *Applied and Environmental Microbiology* **51**, 873–884.
- TIMAKOV, V. D., PETROVSKAYA, V. G., BONDARENKO, V. M. & KHOMENKO, N. A. (1972). Genetic data concerning *Shigella flexneri* serotypes 5 and 6. *International Journal of Systematic Bacteriology* **22**, 149–154.
- VERON, M. & LE MINOR, L. (1975). Nutrition and taxonomy of 'Enterobacteriaceae' and related bacteria. II. General results and classification. *Annales de microbiologie* **126B**, 111–124.