Characterization of enterobacteria by esterase specific-activity profiles

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The spectrum of specific activities and the electrophoretic mobilities of esterases produced by 550 strains of *Enterobacteriaceae* belonging to 36 species and subclassified into six groups (group 1, *Escherichia coli*, *Shigella* and *Escherichia hermannii*; group 2, genus *Salmonella* and genus *Citrobacter*; group 3, genus *Klebsiella* and genus *Enterobacter*; group 4, genus *Serratia* and *Serratia fonticola*; group 5, genus *Proteus*, genus *Providencia* and genus *Morganella*; and group 6, genus *Yersinia*) were analysed by acrylamide/agarose gel electrophoresis using standardized methods for staining and mobility comparisons. Nineteen types of esterase were defined by their respective esterase specific-activity profile (ESAP). A multiple correspondence analysis (MCA) of the ESAP data enabled 82% of the strains in the 36 species to be correctly classified. In each group, the species were clearly delineated after MCA on both ESAP and electrophoretic mobility data. In addition, the smallest number of characters providing species identification of *Yersinia* strains by esterase polymorphism was identified by means of a binary segmentation tree technique.

Introduction

The classification of enterobacteria is based mainly on a combination of data provided by DNA–DNA hybridization studies and conventional phenotypic characters. However, enzyme electrophoresis has been shown to be a powerful tool for studying classification, population genetics and epidemiology. Oxidoreductases were the first enzymes studied (Bowman *et al.*, 1967; Baptist *et al.*, 1969), but esterases have been more widely used; 36 species belonging to *Escherichia* (Goullet, 1973; Goullet *et al.*, 1986), *Shigella* (Goullet, 1980b), *Proteus* and *Providencia* (Goullet, 1975; Goullet & Picard, 1985), *Salmonella* (Goullet, 1977), *Citrobacter* (Goullet & Richard, 1977), *Klebsiella*, *Enterobacter* (Goullet, 1980a; Goullet & Picard, 1986), *Serratia* (Goullet, 1978, 1981) and *Yersinia* (Goullet & Picard, 1984, 1988) have been investigated. More recently, multilocus enzyme electrophoresis, analysing in particular oxidoreductases and transferases, has been used for comparisons between and within *E. coli* and *Shigella* (Ochman *et al.*, 1983) and within *Salmonella* (Reeves *et al.*, 1989; Beltran *et al.*, 1989) and *Serratia marcescens* (Gargallo-Viola, 1989).

In the 36 enterobacterial species investigated, many

varieties of esterase were identified by the zymogram procedure on the basis of their spectrum of hydrolytic activity toward various substrates and their sensitivity or resistance to di-isopropyl fluorophosphate (DFP). Moreover these enzymes displayed great electrophoretic polymorphism. Thus, in contrast to most other enzymes, which differentiate bacteria only by their electrophoretic mobilities, the esterases can distinguish taxa both by their specific activity profiles and by their electrophoretic mobilities. The present work was done in order to determine the respective contributions of each of these two characteristics for inter- and intraspecies differentiation of the 36 species by means of standardized methods for esterase staining and electrophoretic mobility determination. Our findings demonstrate the considerable taxonomic value of esterase specific-activity profiles (ESAP) for characterizing genospecies, whereas the electrophoretic mobilities of the enzymes provide valuable information for delineating inter-related species.

Methods

Bacterial strains. The strains tested were those previously studied: *Shigella flexneri* (13 strains), *S. sonnei* (13 strains), *S. boydii* (14 strains) and *S. dysenteriae* (13 strains) (Goullet, 1980b); *Escherichia hermannii* (11 strains) (Goullet *et al.*, 1986); *Citrobacter freundii* (14 strains), *C. diversus* (*Levinea malonatica*) (14 strains) and *C. amalonaticus* (*L. amalonatica*) (14 strains) (Goullet & Richard, 1977); *Klebsiella pneumoniae* (14 strains), *K. oxytoca* (14 strains), *Enterobacter aerogenes*

Abbreviations: DFP, di-isopropyl fluorophosphate; ESAP, esterase specific-activity profile; MCA, multiple correspondence analysis; M_R , relative electrophoretic mobility; PCA, principal component analysis.

(16 strains) and *E. gergoviae* (16 strains) (Goullet, 1980a); *E. sakazakii* (22 strains) (Goullet & Picard, 1986); *Serratia marcescens* (16 strains), *S. liquefaciens* (16 strains), *S. plymuthica* (15 strains) and *S. marinorubra* (15 strains) (Goullet, 1978); *S. odorifera* (15 strains), *S. ficaria* (13 strains), *S. fonticola* (14 strains) (Goullet, 1981); *Proteus vulgaris* (12 strains), *P. mirabilis* (12 strains), *Providencia stuartii* (17 strains), *P. rettgeri* (11 strains) and *Morganella morganii* (12 strains) (Goullet, 1975); *P. rustigianii* (17 strains) (Goullet & Picard, 1985); *Yersinia pseudotuberculosis* (16 strains) (Goullet & Picard, 1984); and *Y. frederiksenii* (20 strains) (Goullet & Picard, 1988). For the other species, only strains showing a distinct esterase electrophoretic pattern were analysed: *E. coli* (12 strains) (Goullet, 1980b); *Salmonella enterica* (21 strains) (Goullet, 1977); *Enterobacter cloacae* (25 strains) (Goullet & Picard, 1986); *Providencia alcalifaciens* (16 strains) (Goullet & Picard, 1985); *Y. enterocolitica* (17 strains) (Goullet & Picard, 1984); and *Y. intermedia* (16 strains), *Y. kristensenii* (19 strains) and *Y. aldovae* (15 strains) (Goullet & Picard, 1988).

The 36 species were subclassified into six groups: group 1, *E. coli*, *Shigella* and *E. hermannii*; group 2, genus *Salmonella* and *Citrobacter*; group 3, genus *Klebsiella* and *Enterobacter*; group 4, genus *Serratia* and *S. fonticola*; group 5, genus *Proteus*, *Providencia* and *Morganella*; and group 6, genus *Yersinia*.

Preparation of bacterial extracts. Bacteria were grown in L-broth (Lennox, 1955) at 37 °C (except for *Yersinia* strains which were grown at 28 °C) for 18 h with agitation in a reciprocating water-bath shaker at about 70 oscillations min⁻¹. The cells were centrifuged, washed twice with 0.06 M-Tris/glycine buffer, pH 8.7, resuspended in the same buffer and disrupted by intermittent sonic oscillations (Sonifier cell disruptor B30; Branson) for 6 min at 4 °C. Cell debris was removed by centrifugation at 20000 g for 20 min at 4 °C. The crude extracts, containing 40 to 60 mg protein ml⁻¹, were stored at -20 °C until used.

Electrophoretic analysis. Horizontal slab acrylamide/agarose gel electrophoresis (7% w/v, acrylamide, 0.8% agarose, Tris/glycine buffer, pH 8.6) were performed as described previously (Uriel, 1966; Goullet, 1973).

Characterization of esterases. Esterases were stained as described previously (Uriel, 1963; Goullet, 1973) using α -naphthyl acetate, propionate and butyrate, β -naphthyl acetate, propionate and butyrate, and indoxyl acetate. Precise standardization of hydrolytic activity in gels towards naphthyl esters was obtained by staining for exactly 45 min at room temperature; the reaction was stopped by addition of 0.1 M-acetic acid. (An exception was indoxyl acetate staining, which was continued overnight.) Inhibition by DFP (10⁻³ M) was determined in crude extracts which were allowed to stand for 30 min at room temperature before electrophoresis.

Electrophoretic mobility determinations. The reproducibility of the electrophoretic mobility measurements was optimized by measuring the relative electrophoretic mobility values (M_R value) using a reference mobility scale of six esterases: esterase A and B from *Shigella sonnei* strain 2.77 (Goullet, 1980b); esterase B from *E. coli* strain FM2 (Goullet, 1973); esterase M from *Aeromonas hydrophila* strain 66 (Picard & Goullet, 1985); esterase E_{BA} from *Y. intermedia* strain CNY 87.70 (Goullet & Picard, 1988) and esterase P₄ from *Y. pseudotuberculosis* strain CNY 1833 (Goullet & Picard, 1984). The M_R value of a given variant was measured within the appropriate mobility range as the ratio between the distance measured from the slow reference esterase to the band of the variant and the distance measured from the slow reference esterase to the fast reference esterase.

Statistical analysis. A multiple correspondence analysis (MCA) (Greenacre, 1984; Lebart *et al.*, 1984; Tenenhaus & Young, 1985) was done by means of the SPAD.N program (Centre International de Statistique et d'Informatique Appliquées, Paris, France) from a table

containing 19 variables (the types of esterase) and 550 rows (the strains). MCA was then done for each of the six groups of species, including both the type of esterase and the esterase electrophoretic mobilities. For this purpose, three to five ranges of mobility were delineated according to the histograms of the M_R values of each esterase type within each group of species. A principal component analysis (PCA) was done with the M_R data for the 19 enzymes and compared to the results of the MCA. For each MCA, a hierarchical classification using Ward's criteria was done based on the eight first axes of the analysis and a dendrogram was obtained. The number of axes on which this analysis was done corresponded to the structural and stable portions of the variance whereas the rest of the variance corresponded to random noise and thus was discarded for the hierarchical classification. The number of classes corresponding to the number of species considered in each analysis was selected, and the strain compositions of the final classes were compared to those of the corresponding species. MCA is an eigenvector method of ordination similar to PCA. It differs from the latter in the use of a covariance matrix of χ^2 distances rather than a covariance matrix of Euclidean distances. Both techniques describe the dispersion or shape of a cloud of n objects or p variates in a multi-dimensional space by replacing the original data set by a new set of orthogonal linear coordinates in a space of significantly lower dimension. The explained variances of the elements of the data set are in decreasing order of magnitude with respect to these derived coordinates (Lebart *et al.*, 1984). Furthermore, the variables used for MCA are categories whereas those used for PCA are quantitative or continuous variables. Finally, a binary segmentation tree (Gueguen & Nakache, 1988) was obtained from the data of the MCA done on *Yersinia* strains (group 6).

Results and Discussion

Esterase characterization

The 146 distinct esterase bands previously described for the 36 species can be regrouped into 19 principal types (Table 1) on the basis of their hydrolytic activities on the seven synthetic substrates and of their sensitivity to DFP. Types 1, 3 to 7, 9 to 12, 14, 16 and 17 correspond to DFP-sensitive esterases and types 2, 8, 13, 15, 18 and 19 correspond to DFP-resistant esterases. Eight types of esterases showing comparable activity towards α - and β -naphthyl acetates were designated α - β esterases. Five of these hydrolysed the five other substrates and were distinguished from each other by their differential activity on acetyl (A), propionyl (P) and butyryl (B) esters as follows. Types 1 and 2, A > P > B; type 3, A < P $\approx$ B; type 4, A $\approx$ P $\approx$ B; type 5, A $\approx$ P > B. Type 6 was active on all substrates except β -naphthyl butyrate. Type 7 was active on the three acetyl esters and on α -naphthyl propionate. Type 8 was active on α - and β -naphthyl acetates. Seven types of esterases (types 9 to 15) hydrolysed mainly α -naphthyl esters and were designated α -esterases. Type 9 hydrolysed α - and β -naphthyl esters and indoxyl acetate. *Enterobacter gergoviae* esterase E₄ may be distinguished from other enzymes of this type by its greater activity towards β -naphthyl esters. Type 10 was distinguished from type 9 by its inactivity on β -

Table 1. Distribution of the 146 previously described esterase bands within the 19 types of esterase and the 36 species of enterobacteria

For each species, esterases are designated as in previous papers (see Methods for references). Consequently, the same designation can correspond to esterases belonging to distinct types, e.g. esterases E₁ of *Serratia fonticola*, *Citrobacter diversus*, *Serratia odorifera*, *Klebsiella pneumoniae*, *Salmonella enterica* and *Escherichia hermanii* correspond to types 3, 7, 8, 10, 13 and 17, respectively.

Species no.	α - β -Esterases								α -Esterases							β -Esterases				Total
	1	2*	3	4	5	6	7	8*	9	10	11	12	13*	14	15*	16	17	18*	19*	
Group 1																				
1	<i>Escherichia coli</i>								B	A					I	S	F	C		6
2	<i>Shigella flexneri</i>								B	A					I	S	F	C		6
3	<i>Shigella sonnei</i>								B	A					I	S	F	C		6
4	<i>Shigella boydii</i>								B	A					I	S	F	C		6
5	<i>Shigella dysenteriae</i>									A					I	S	F	C		5
6	<i>Escherichia hermanii</i>							E ₄		E ₆		E ₂				E ₅	E ₁			5
Group 2																				
7	<i>Salmonella enterica</i>								E ₃				E ₁	E ₄		S	F	E ₂		6
8	<i>Citrobacter freundii</i>								Major											1
9	<i>Citrobacter diversus</i>						E ₁		E ₃	E ₂							F			4
10	<i>Citrobacter amalonaticus</i>								Major											1
Group 3																				
11	<i>Klebsiella pneumoniae</i>							m ₃		E ₁							F	m ₂		4
12	<i>Klebsiella oxytoca</i>									E ₁		E ₃	E ₂							3
13	<i>Enterobacter aerogenes</i>							m ₃		E ₁							F	m ₂		4
14	<i>Enterobacter gergoviae</i>								E ₄	E ₁			m ₇				F	m ₆		5
15	<i>Enterobacter cloacae</i>							EC ₃		EC ₄			EC ₁	EC ₂						4
16	<i>Enterobacter sakazakii</i>	ES ₂				ES ₄							ES ₁	ES ₃						4
Group 4																				
17	<i>Serratia marcescens</i>			E ₃			E ₂			E ₁						E ₅			E ₄	5
18	<i>Serratia liquefaciens</i>			E ₃			E ₂			E ₁						E ₅			E ₄	5
19	<i>Serratia plymuthica</i>			E ₃						E ₁						E ₅			E ₄	4
20	<i>Serratia marinorubra</i>			E ₃			E ₂			E ₁							Em ₂			4
21	<i>Serratia odorifera</i>				E ₅		E ₁			E ₃							F		E ₄	5
22	<i>Serratia ficaria</i>						E ₁							E ₂		E ₅	F		E ₄	5
23	<i>Serratia fonticola</i>		E ₁	E ₃						E ₂									E ₄	4
Group 5																				
24	<i>Proteus vulgaris</i>								E α A								β B			2
25	<i>Proteus mirabilis</i>												E α				β B			2
26	<i>Providencia alcalifaciens</i>	$\alpha\beta$									E α AB						β A			3
27	<i>Providencia stuartii</i>													α a			β A			2
28	<i>Providencia rettgeri</i>										E α AB	E α					β A			3
29	<i>Providencia rustigianii</i>																β A			1
30	<i>Morganella morganii</i>	$\alpha\beta$										E α								2
Group 6																				
31	<i>Yersinia pseudotuberculosis</i>								P ₄				P _{aa}			P ₃	P ₁			4
32	<i>Yersinia enterocolitica</i>	E ₄					E ₂	E ₃ E ₅					E _{aa}			E ₆ E ₇	E ₁			6
33	<i>Yersinia intermedia</i>		E _{BA}				E ₂						E _{aa}				E ₁	E β R		5
34	<i>Yersinia frederiksenii</i>						E ₂						E _{aa}			E ₆ E ₇	E ₁	E β R		5
35	<i>Yersinia kristensenii</i>						E ₂						E _{aa}			E ₆ E ₇	E ₁			4
36	<i>Yersinia aldovae</i>						E ₂						E _{aa}			E ₆ E ₇	E ₁	E β R		5
Total		2	2	2	5	1	1	1	14	12	18	2	4	12	5	5	22	20	12	6

* DFP-resistant esterase.

naphthyl butyrate. Type 11 hydrolysed α -naphthyl acetate, propionate and butyrate, and indoxyl acetate, whereas types 12 and 13 were active only on α -naphthyl acetate and propionate, and indoxyl acetate. Of the type 12 esterases, *K. oxytoca* esterase E₃ and *P. rettgeri* esterase E α were more active on indoxyl acetate than on

α -naphthyl esters whereas *M. morganii* esterase E α and *E. hermanii* esterase E₂ were more active on α -naphthyl esters than indoxyl acetate. Types 14 and 15 hydrolysed α -naphthyl acetate and propionate. Four types of esterases (types 16 to 19) preferentially hydrolysed β -naphthyl esters and were designated β -esterases. Type 16

hydrolysed the three β -naphthyl esters and usually indoxyl acetate. Within this type, esterases βA and βB may be distinguished from each other (Goullet, 1975). Types 17 and 18 hydrolysed β -naphthyl acetate, whereas type 19 hydrolysed strongly indoxyl acetate, and weakly β -naphthyl acetate.

Distribution of the esterase types within the 36 species

Table 1 shows the distribution of the 19 types of esterase within the 36 species. Some types were detected in only one species, and were thus species specific, e.g. *Serratia odorifera* esterase E_5 (type 5), *Enterobacter sakazakii* esterase ES_4 (type 6) and *C. diversus* esterase E_1 (type 7). Some types were detected only in one group: types 4 and 19 were found in group 4 (*Serratia*), and types 1 and 11 in group 5 (*Proteus*, *Providencia* and *Morganella*). Esterase I (type 15) was found in group 1 only, and thus appeared to be specific to the *E. coli*-*Shigella* genospecies (Brenner *et al.*, 1972a, 1973). Some types were detected in distantly related species, e.g. type 3 in *Serratia fonticola* and *Y. intermedia*, while some types were more widespread, e.g. type 16 (22 species), type 10 (18 species) and type 17 (21 species). Most of this latter type of enzyme (esterases F and E_1) stained faintly. Type 9 encompasses esterases which are physicochemically and immunologically unrelated, e.g. *E. coli* esterase B and *Y. pseudotuberculosis* esterase P_4 (Goullet *et al.*, 1984, 1989).

Multivariate statistical analysis of the 550 strains tested

The diagram of the first two axes of the MCA done on the distribution of the 19 types of esterases in the 550 strains, which accounted for 25.53% of the variance, showed the projection of the centres of gravity of the 36 species (Fig. 1). The first axis distinguished *Proteus* and *Providencia* species and *Enterobacter sakazakii* species on their negative values and the *Escherichia*-*Shigella* species on their positive values. *Serratia* species were distinguished by the positive values of the second axis, whereas *Salmonella* and *Yersinia* species were distinguished on their negative values. A hierarchical classification was prepared from the first eight axes of the analysis which accounted for 70% of the variance. Thirty-six classes were selected from the dendrogram obtained and were correlated with the 36 species of enterobacteria. Some species were placed in the same class, e.g. *Shigella flexneri* and *S. sonnei*; *C. freundii* and *C. amalonaticus*; *K. pneumoniae* and *Enterobacter aerogenes*; *Serratia marcescens* and *S. liquefaciens*; *Y. kristensenii* and *Y. aldovae*. Some other species were divided into two classes, e.g. *Salmonella enterica*, *Enterobacter sakazakii*, *Serratia ficaria*, *Serratia odorifera* and *Y. enterocolitica*. Of the

Fig. 1. MCA of the 550 strains. Plot of species scores for the first two principal axes: ○, group 1; ●, group 2; □, group 3; ■, group 4; △, group 5; ▲, group 6. For definition of groups, see Methods. Within each group, the species are numbered as in Table 1.

strains of various species 82% were correctly classified within the corresponding classes (Table 2). These data varied according to the groups considered from 67% for group 1 (*Escherichia*-*Shigella*) to 97% for group 2 (*Salmonella* and *Citrobacter*) (Table 2). Most (91%) strains of the various groups of species were classified with the corresponding groups of classes. The percentage of accurately classified strains was higher after MCA than after PCA (76%). The strains producing all esterases indicated in Table 1 were accurately classified in their species; misclassified strains were restricted to those lacking one, two or three esterases. Thus, it is the absence of information which appears to be responsible for misclassification after MCA. However, these strains could all be easily distinguished from the species in which they were misclassified by at least one uncommon type of esterase and by the electrophoretic mobilities of the detected esterases.

Enzymes such as oxidoreductases and transferases, which are identified in gels by a single substrate, differentiate bacteria only on the basis of mobility variations (Bowman *et al.*, 1967; Baptist *et al.*, 1969; Ochman *et al.*, 1983; Beltran *et al.*, 1989; Reeves *et al.*, 1989; Gargallo-Viola, 1989) and require multiple comparative electrophoretic migrations, whereas esterases, which are identified from their activity on several substrates, allowed suitable genospecies characterization

Table 2. Accurately classified strains after hierarchical classification following MCA and PCA of the 550 strains

	No. of strains accurately classified:			
	Into their species after MCA	Into their species group after MCA	Into their species after PCA	Into their species group after PCA
<i>Escherichia-Shigella</i> (76 strains)	51 (67%)	67 (88%)	54 (71%)	67 (88%)
<i>Salmonella, Citrobacter</i> (63 strains)	61 (97%)	61 (97%)	59 (94%)	59 (94%)
<i>Klebsiella, Enterobacter</i> (108 strains)	90 (83%)	90 (83%)	71 (66%)	94 (87%)
<i>Serratia</i> (104 strains)	92 (88.5%)	102 (98%)	87 (84%)	104 (100%)
<i>Proteus, Providencia, Morganella</i> (96 strains)	84 (86.5%)	96 (100%)	80 (83%)	96 (100%)
<i>Yersinia</i> (103 strains)	75 (73%)	87 (84.5%)	66 (64%)	76 (74%)
All groups (550 strains)	453 (82.4%)	503 (91.5%)	417 (76%)	496 (90.2%)

Fig. 2. MCA of strains of group 1. Plot of strain scores for the first two principal axes: ○, *E. coli*; ●, *Shigella flexneri*; □, *S. sonnei*; ■, *S. boydii*; △, *S. dysenteriae*; ▲, *E. hermannii*.

of most of the strains by patterns resulting from combinations of the ESAP.

Multivariate statistical analysis of the six groups of species

The following analyses consider both the ESAP and the range of mobility of each esterase band.

Group 1. The distribution diagram of the first two axes of the MCA, which accounted for 49.5% of the variance, shows the projection of the strains (Fig. 2). The first axis

distinguished *Escherichia hermannii* strains on their negative values. Some *Shigella boydii* and *S. dysenteriae* strains were distinguished by the positive values of the second axis, whereas other *S. boydii* strains and *S. flexneri* serotype 6 were clustered. The hierarchical classification which correlated six classes with the six species of group 1 clearly distinguished *E. hermannii* strains. This species, which has only 40 to 46% DNA homology with *E. coli* K12 (Brenner *et al.*, 1982), is very distinct from *E. coli* on the basis of its esterases (Goullet *et al.*, 1986). A single class was formed by eight strains of *S. dysenteriae* whereas five other strains were grouped with most of the *S. boydii* strains. The remaining strains of this latter species constituted a class with the *S. flexneri* serotype 6 strains which were clearly separated from *S. flexneri* serotypes 1 to 5 (Dodd & Jones, 1982; Goullet, 1980b; Goullet *et al.*, 1983; Goullet & Picard, 1987; Petrovskaya & Bondarenko, 1977; Timakov *et al.*, 1972). *E. coli* strains were distributed in the classes containing *S. sonnei*, *S. flexneri* and *S. dysenteriae*. These findings agree with previous results showing the clusters of *Shigella*, as delineated by esterase polymorphism, closer to *E. coli* than to each other, and suggest that they are derived from distinct ancestral strains of *E. coli* (Goullet & Picard, 1987).

Group 2. The first axis of the MCA (45.2% of the variance) (Fig. 3) distinguished *Salmonella enterica* strains on their positive values and *C. diversus*, *C. amalonaticus* and *C. freundii* strains on their negative values, whereas *C. diversus* was distinguished from *C. amalonaticus* and *C. freundii* by the second axis (20.8% of the variance). The correlation of the four classes obtained after the hierarchical classification of the four species showed that all *S. enterica* and *C. diversus* strains were distinguished. *S. enterica* was distributed between two classes according to the mobility of esterase E_1 .

Fig. 3. MCA of strains of group 2. Plot of strain scores for the first two principal axes: ○, *Salmonella enterica*; ●, *Citrobacter freundii*; □, *C. diversus*; ■, *C. amalonaticus*.

Fig. 4. MCA of strains of group 3. Plot of strain scores for the first two principal axes: ○, *Klebsiella pneumoniae*; ●, *K. oxytoca*; □, *Enterobacter aerogenes*; ■, *E. gergoviae*; △, *E. cloacae*; ▲, *E. sakazakii*.

Interestingly, the two classes corresponded to (i) *S. enterica* subsp. *enterica* and *S. enterica* subsp. *salamae*, and (ii) *S. enterica* subsp. *arizonae*, *S. enterica* subsp. *diarizonae* and *S. enterica* subsp. *houtenae* (Le Minor *et al.*, 1982a; Le Minor & Popoff, 1987). All *S. enterica* strains displayed the same ESAP, corroborating the fact that this species is a unique DNA homology group (Crosa *et al.*, 1973) (except for *S. enterica* subsp. *bongori* strains (Le Minor *et al.*, 1982b; Reeves *et al.*, 1989), which are not included in this study). *C. amalonaticus* and *C. freundii* strains could not be separated in this analysis but can be distinguished because the major esterase of all *C. amalonaticus* strains migrates faster than that of all *C. freundii* strains (Goullet & Richard, 1977).

Group 3. The first axis of the MCA (25% of the variance) (Fig. 4) distinguished *Enterobacter sakazakii*, *E. cloacae* and *K. oxytoca* by their positive values. *E. gergoviae* (Richard *et al.*, 1976; Brenner *et al.*, 1980c) were distinguished from *E. aerogenes* and *K. pneumoniae* by the second axis (18.5% of the variance). The hierarchical classification allocated 90.7% of strains within their species. *E. sakazakii*, which is distinct from *E. cloacae* as shown by DNA reassociation studies (Steigerwalt *et al.*, 1976; Farmer *et al.*, 1980; Izard *et al.*, 1983), was also distinguished by its esterases. All strains of *E. gergoviae* were distinguished, whereas ten strains of *E. cloacae* appeared to be more poorly defined. *K. pneumoniae* and

Fig. 5. MCA of strains of group 4. Plot of strain scores for the first two principal axes: ○, *Serratia marcescens*; ●, *S. liquefaciens*; □, *S. plymuthica*; ■, *S. marinorubra*; △, *S. odorifera*; ▲, *S. ficaria*; ▽, *S. fonticola*.

Fig. 6. MCA of strains of group 5. Plot of strain scores for the first two principal axes: ○, *Proteus vulgaris*; ●, *Proteus mirabilis*; □, *Providencia alcalifaciens*; ■, *Providencia stuartii*; △, *Providencia rettgeri*; ▲, *Providencia rustigianii*; ▽, *Morganella morganii*.

E. aerogenes, which are genotypically related (Brenner *et al.*, 1972b), were not distinguished by their ESAP, but were delineated by the electrophoretic mobilities of their esterases (Goullet, 1980a).

Group 4. The distribution diagram of the first two axes of the analysis, which accounted for 31.9% of the variance (Fig. 5), distinguished *Serratia marinorubra* and *S. odorifera* by their positive values and *S. fonticola* and *S. marcescens* by their negative values. *S. ficaria* was distinguished by the negative values of the second axis and *S. liquefaciens* and *S. plymuthica* by the negative values of the first axis. Of the strains of all the *Serratia* species, 93.3% were distinguished by the hierarchical classification. One *S. liquefaciens* strain was misclassified in *S. marcescens* and three strains of the latter species were misclassified in the former. This result shows that the two species are closely related according to their esterase polymorphism. All strains of *S. plymuthica* and of *S. fonticola* were correctly classified. *S. fonticola* appeared to be related to other *Serratia* based on their esterase patterns, as was previously demonstrated by DNA reassociation studies (Crosa *et al.*, 1974; Gavini *et al.*, 1979).

Group 5. The first axis of the MCA (21.5% of the variance) (Fig. 6) separated *Providencia stuartii* from *P.*

Fig. 7. MCA of strains of group 6. Plot of strain scores for the first two principal axes: ○, *Yersinia pseudotuberculosis*; ●, *Y. enterocolitica*; □, *Y. intermedia*; ■, *Y. frederiksenii*; △, *Y. kristensenii*; ▲, *Y. aldovae*.

rettgeri, whereas the second axis (18% of the variance) distinguished *M. morganii*, *P. alcalifaciens*, *P. rustigianii*, *P. mirabilis* and *P. vulgaris*. The hierarchical classification allowed identification of 88.7% of the strains within their species. The least well classified species was *P. alcalifaciens* where seven strains were misclassified by the statistical computations. However, these strains, which included several null alleles, were distinguished from other species by other esterases (Goullet & Picard, 1985). The esterase data for *Providencia* were in agreement with the genetic divergence measured by DNA reassociation studies (Brenner *et al.*, 1978).

Group 6. The first axis of the MCA (21.2% of the variance) (Fig. 7) distinguished *Y. intermedia* by its positive values whereas the second axis (15.4% of the variance) separated *Y. enterocolitica* from *Y. frederiksenii*. The hierarchical classification allowed delineation of 93.2% of strains within the six *Yersinia* species. *Y. aldovae* was distinguished whereas *Y. frederiksenii* was the least clearly separated species with five misclassified strains. This species was very heterogeneous in terms of esterases with frequent null alleles and several other esterases detected in only a few strains (Goullet & Picard, 1988), and is, in fact, composed of several DNA homology groups (Brenner *et al.*, 1980a; Ursing *et al.*, 1980).

To summarize, the electrophoretic mobilities of esterases enabled the delineation of all interrelated species which

Fig. 8. Binary segmentation tree for the characterization of *Yersinia* strains. E₄, esterase E₄; E_{BA}, esterase E_{BA}; E₆, esterase E₆; E₇, esterase E₇; P₃, esterase P₃; E_{BR}, esterase E_{BR}; E_{2a}, esterase E_{2a}.

were placed in the same classes after MCA of the ESAP data.

Binary segmentation tree for the characterization of *Yersinia* strains

The smallest number of characters providing species identification of *Yersinia* strains by esterase data was identified by preparing a binary segmentation tree with the 96 *Yersinia* strains correctly classified by MCA. Six characters were sufficient (Fig. 8): the first two were the existence of esterase E₄ in *Y. enterocolitica* and esterase E_{BA} in *Y. intermedia*; the other four were particular electrophoretic variants. Thus the *Y. enterocolitica*-like species, which were in some cases distinguished from each other and from *Y. enterocolitica sensu stricto* by a few conventional phenotypic traits (Brenner *et al.*, 1980b; Ursing *et al.*, 1980; Bercovier *et al.*, 1980, 1984), were readily characterized by the electrophoretic polymorphism of their esterases.

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