Characterization of *Yersinia enterocolitica*, *Y. intermedia*, *Y. aldovae*, *Y. frederiksenii*, *Y. kristensenii* and *Y. pseudotuberculosis* by Electrophoretic Polymorphism of Acid Phosphatase, Esterases, and Glutamate and Malate Dehydrogenases

By PH. GOULLET*† AND B. PICARD

Laboratoire de Microbiologie, Faculté de Médecine Xavier Bichat (Université Paris VII),
16 rue Henri Huchard, 75018 Paris, France

(Received 8 April 1987; revised 24 August 1987)

Acid phosphatase, esterases, and glutamate and malate dehydrogenases of 192 strains of *Yersinia enterocolitica*, *Y. intermedia*, *Y. aldovae*, *Y. frederiksenii*, *Y. kristensenii* and *Y. pseudotuberculosis* were analysed by horizontal polyacrylamide agarose gel electrophoresis and by isoelectrofocusing in thin-layer polyacrylamide gels. The six species were clearly separated from each other by their distinct enzyme electrophoretic polymorphism. For *Y. enterocolitica*, the strains of biotype 5 were differentiated from the other biotypes by the mobility of glutamate dehydrogenase. For *Y. frederiksenii*, six zymotypes were delineated by pI and by the mobility of the enzymes. Variation in number or mobility of esterases within each species could represent a marker for epidemiological and ecological analyses. A linear relationship was obtained between the mean genetic diversity coefficient of enzymes and the mean percentage DNA–DNA relatedness of *Y. intermedia*, *Y. aldovae*, *Y. enterocolitica* and *Y. frederiksenii*.

INTRODUCTION

The genus *Yersinia* consists of seven different species: *Y. pseudotuberculosis*, *Y. enterocolitica*, *Y. intermedia*, *Y. frederiksenii*, *Y. kristensenii*, *Y. ruckeri* (Bercovier & Mollaret, 1984) and *Y. aldovae* (Bercovier *et al.*, 1984). However, *Y. ruckeri* may constitute a new genus.

Y. enterocolitica and *Y. pseudotuberculosis* have been clearly characterized by the distinctive electrophoretic patterns of their esterases. In *Y. enterocolitica*, five principal zymotypes were delineated: zymotypes A and B for strains of biotype 1, zymotypes C and D for strains of biotypes 2 and 3, respectively, and zymotype E for strains of biotype 4 or biotype 5 (Goullet & Picard, 1984a). *Y. intermedia* (Brenner *et al.*, 1980b), *Y. frederiksenii* (Ursing *et al.*, 1980), *Y. kristensenii* (Bercovier *et al.*, 1980) and *Y. aldovae* (Bercovier *et al.*, 1984), previously described as *Y. enterocolitica*-like organisms, isolated mainly from various environmental sources (water, fish, foods), were clearly distinguished from *Y. enterocolitica sensu stricto* by DNA–DNA hybridization (Brenner *et al.*, 1980a; Bercovier *et al.*, 1984). However, the phenotypic characterization based, for some species, on a limited number of conventional biochemical tests required the analysis of primary gene products.

By analysis of electrophoretic polymorphism of acid phosphatase (AP), esterases, and malate and glutamate dehydrogenases (MDH and GDH), the present work establishes specific and intra-specific differentiation of the six *Yersinia* species.

† Address for correspondence: Laboratoire de Microbiologie, Hôpital Beaujon, 100 Boulevard de General Leclerc, 92118 Clichy, France.

Abbreviations: AP, acid phosphatase; DFP, di-isopropyl fluorophosphate; GDH, glutamate dehydrogenase; IEF, isoelectrofocusing; M_f , electrophoretic mobility; MDH, malate dehydrogenase.

Table 1. *Strains examined*

CNY, Collection de Centre National et Centre Collaborateur OMS pour les Yersinia (Professor H. H. Mollaret), Institut Pasteur, Paris, France; CIP, Collection Institut Pasteur; ATCC, American Type Culture Collection, USA; CDC, Center for Diseases Control, USA.

<i>Yersinia enterocolitica</i>				<i>Yersinia intermedia</i>		
Laboratory code	Strain designation	Bio-type	Source	Laboratory code	Strain designation	Source
1, 3, 7, 8	CNY 6168, 6155, 6554, 6166	1	Sewage, Denmark	1	CNY 8301	Drinking water, Norway
2, 5, 6	CNY 5919, 6551, 6552	1	Wild rodent, France	2, 5, 6, 10, 16, 25	CNY 6237, 6187, 5983, 6194, 6191, 6151	Sewage, Denmark
4	CNY 5429	1	Hen, Poland	3, 15	CNY 8870, 8871	Drinking water, Italy
9	CNY 7333	1	Milk, Czechoslovakia	4	CNY 5630	Hen stool, France
10	CNY 842	1	Human throat, USA	7	CNY 6300	Fish, Denmark
11	CNY 7309	1	Czechoslovakia	8, 9, 11, 17	CNY, 8771, 8772, 8770, 8773	Drinking water, Czechoslovakia
12, 13, 17	CNY 2, 3, 517	5	Hare, France	12, 13	CNY 8575, 8576	Water, France
14	CNY 1144	5	Hare, Belgium	14	CNY 8713	Human stool, USA
15, 16	CNY 2879, 2880	5	Goat, Norway	18	CNY 955	Water, Norway
				19, 20	CNY 6249, 6250	Pig, Denmark
				21	CNY 8877	Human blood, South Africa
				22, 23	CNY 5797, 6030	Human blood, France
				24	CNY 3953 (CIP 80.28, ATCC 29909)	Human, USA

METHODS

Bacterial strains. The test strains were the 53 strains of *Y. enterocolitica* and the 30 strains of *Y. pseudotuberculosis* previously analysed for their esterase patterns (Goullet & Picard, 1984a) together with 109 new strains listed in Table 1. Bacteria were grown in Fernbach flasks containing 500 ml L broth (Lennox, 1955). Flasks were shaken vigorously at 28 °C for 18 h.

Electrophoresis. Protein extracts were prepared and analysed as described previously (Goullet & Picard, 1984a, 1985a). Electrophoretic mobility (M_f value), isoelectrofocusing (IEF), esterase staining and the determination of correspondence between the two electrophoretic techniques were estimated using published procedures (Goullet & Picard, 1984a, 1985a).

Enzyme tests and substrates. For esterases, the substrates used were α - and β -naphthyl acetates, α - and β -naphthyl butyrates and indoxyl acetate. Inhibition by di-isopropyl fluorophosphate (DFP) was estimated as previously described (Goullet, 1980). GDH activity was detected by the method of Baptist *et al.* (1969), MDH activity by the method of Siciliano & Shaw (1976) and AP activity according to Uriel (1963).

RESULTS

Characterization of enzyme bands

Polyacrylamide agarose gel electrophoresis. MDH and GDH each gave a single, well-stained band whereas AP was not detected. Nine principal kinds of esterase bands were defined by their activities toward synthetic substrates and their sensitivity or resistance to DFP. Six of these (E_1 , E_2 , E_6 , E_7 , E_{aa} and E_{1ab}) were similar to the corresponding bands of *Y. enterocolitica* (Goullet & Picard, 1984a). Esterase E_1 was faint for all strains of *Y. intermedia* and for some strains of *Y. frederiksenii* and *Y. kristensenii*. Three other esterases, designated as bands $E_{\beta R}$, E_{ac} and E_{BA} ,

Table 1 (continued)

<i>Yersinia frederiksenii</i>			<i>Yersinia kristensenii</i>		
Laboratory code	Strain designation	Source	Laboratory code	Strain designation	Source
1, 2, 3, 8, 9, 10, 11, 14	CNY 5975, 5960, 5966, 5982, 6150, 5981, 6175 (CIP 80.29), 5976	Sewage, Denmark	1, 3, 4, 5, 6, 17	CNY 5874, 5878, 5873, 6072, 5894, 5520	Wild rodent stool, France
4, 6	CNY 8718, 8721	Human stool, USA	2, 20	CNY 1474, 1475	Water, Norway
5	CNY 8684	Human stool, FRG	7	CNY 103	Sheep, Faroe Island
7	CNY 6334	Human blood, France	8	CNY 8782	Drinking water, Czechoslovakia
12, 13	CNY 8801, 8797	Rat, Czechoslovakia	9	CNY 6048	Human stool, Australia
15	CNY 867	Human stool, Belgium	10, 14	CNY 8346, 8347	Human stool, France
16, 17	CNY 8824, 8826	Dog stool, Brazil	11	CNY 8680	Human stool, FRG
18	CNY 8849	Human, France	12	CNY 490	Hare spleen, France
19, 20	CNY 8786, 8787	Drinking water, Czechoslovakia	13	CNY 3605	Monkey stool, Japan
			15	CNY 105	Human urine, Denmark
			16	CNY 5553	Water, Czechoslovakia
			18, 19	CNY 8711, 8712	Human stool, USA
			21	CNY 5591	Wild rodent spleen, France

<i>Yersinia aldovae</i>		
Laboratory code	Strain designation	Source
1, 2, 12, 13, 20, 21, 22, 23, 24, 26	CNY 7927, 7928, 7971, 7970, 8286, 8288, 8285, 8290, 8287, 7898	Water, Norway
3, 4, 5, 7, 9, 18	CNY 6005 (CDC 669.83, ATCC 35236), 6679, 7092, 7328, 7090, 7091	Drinking water, Czechoslovakia
6, 8, 10, 19	CNY 7331, 7089, 7111, 7112	Surface water, Czechoslovakia
11, 16	CNY 7683, 7109	Water, Czechoslovakia
14, 15, 25	CNY 7618, 7622, 7632	Fish, Norway
17	CNY 7096	River, Czechoslovakia

were clearly distinguished. Band $E_{\beta R}$ hydrolysed only β -naphthyl acetate. Band E_{ac} degraded α -naphthyl acetate and butyrate. Band E_{BA} hydrolysed butyryl esters and, to a lesser degree, acetyl esters. Esterase E_{BA} was sensitive to 10^{-3} M-DFP whereas esterases $E_{\beta R}$ and E_{ac} were resistant to this agent.

IEF. Satisfactory resolution with distinct banding was obtained for MDH and AP. The latter enzyme could not be detected in *Y. pseudotuberculosis*. GDH was not detected. MDH gave a well-stained major band (used for pI determination) and two or three anodic satellite bands when a narrow pH range from 4 to 6.5 was used. AP gave a major band when a narrow pH range from 8 to 10.5 was used. For esterases, the best results were obtained with β -naphthyl acetate as substrate. Only four esterases (bands E_1 , E_{BA} , $E_{\beta R}$ and E_7) were detected using two narrow pH gradients (4 to 6.5 and 5 to 8). The four enzymes gave sharp, faintly stained bands which were used for pI determination.

Range of pI and mobilities of enzyme bands among the strains

The electrophoretic relationships between the strains were established by numerous replicate runs comparing enzyme bands side by side on the same gel. The reproducibility of the results was confirmed as described by Picard & Goullet (1985).

Y. enterocolitica. (Tables 2 and 3.) MDH had the same pI and mobility values in all strains. GDH had an identical M_F value in strains of biotypes 1 to 4 but a distinct value in biotype 5.

Table 2. *pI* and M_F values of enzyme bands in *Y. enterocolitica* and *Y. pseudotuberculosis*

The 53 strains of *Y. enterocolitica* and the 30 strains of *Y. pseudotuberculosis* were those previously analysed for their esterase patterns (Goullet & Picard, 1984*a*).

Species	MDH (pI/M_F)	GDH (M_F)	AP (pI)
<i>Y. enterocolitica</i>			
Biotype 1	5.75/36	16	8.5
	5.75/36	16	8.7
Biotype 2	5.75/36	16	8.7
Biotype 3	5.75/36	16	8.7
Biotype 4	5.75/36	16	8.7
Biotype 5	5.75/36	17	8.7
<i>Y. pseudotuberculosis</i>	5.8/42	17	—

Table 3. *pI* and M_F values of enzyme bands in the 17 new strains of *Y. enterocolitica*

The pI/M_F for MDH was 5.75/36 for all strains.

Strain code	GDH (M_F)	AP (pI)	E ₁ (M_F)	E ₂ (M_F)	E ₃ (M_F)	E ₄ (M_F)	E ₅ (M_F)	E ₆ (M_F)	E ₇ (M_F)
1	16	8.7	82	65	55	47	20	—7	73
2	16	8.7	82	65	55	47	20	—7	—
3	16	8.7	78	65	55	47	20	—7	75
4	16	8.7	78	65	55	47	20	—7	73
5, 6, 7	16	8.7	78	65	55	47	20	—7	70
8	16	8.7	78	65	55	47	20	—7	—
9	16	8.5	82	65	60	47	22	—7	78
10	16	8.5	82	65	60	47	22	—7	75
11	16	8.5	78	65	60	47	22	—7	75
12, 13, 14, 15, 16	17	8.7	80	63	—	46	—	—20	70
17	17	8.7	80	63	—	46	—	—5	70

Acid phosphatase was monomorphic with the exception of strains from zymotype B (Goullet & Picard, 1984*a*), in which it focused at pH 8.5. The 11 new strains of biotype 1 and the six new strains of biotype 5 exhibited the same esterase electrophoretic pattern as the strains analysed previously (Goullet & Picard, 1984*a*).

Y. pseudotuberculosis. (Table 2.) MDH has identical pI and M_F values and GDH an identical M_F value for all strains. AP was not detected.

Y. intermedia. (Table 4.) MDH and GDH were monomorphic in all strains. AP focused at pH 9.75 except for strains 6 and 22 where it focused at pH 8.8 and pH 9.8, respectively. Esterases E₁, E _{β R} and E_{BA} were detected in all strains. Bands E₂ and E _{α a} were detected in the majority of strains and band E₇ was detected in only seven strains. Esterase E₂ showed two bands. Band B₂ was replaced by band E _{α b} in strain 22. Esterases E₁ and E₇ showed one type of mobility, esterases E₂ and E _{β R} showed two types and esterases E_{BA} and E _{α a} showed three types of mobility. Strain 1 had an additional band at $M_F \approx 76$ for esterase E_{BA}. The pI of esterases E _{β R} and E_{BA} were respectively 5.35 and 6.4 for all strains.

Y. aldovae. (Table 5.) MDH and GDH were monomorphic in all strains whereas AP was polymorphic with four distinct pI values ranging from 9.1 to 9.7. Esterase bands E₁ and E _{β R} were detected in all strains (except esterase E _{β R} for strain 1). Esterase E₁ had a pI value of 5 but showed four types of mobility. Most strains (22) had esterase E₁ at $M_F \approx 82$. In strain 24, band E _{α a} was replaced by esterase E _{α b}. Esterase E _{β R} had the same mobility in strains 2 to 25 but showed several pI values ranging from 6.1 to 6.35. Strain 2 possessed an additional band at $M_F \approx 26$. In strain 26 the mobility of E _{β R} was $M_F \approx 65$ and the pI was 5.7. Band E₆ was observed in strains 16 to 25 and band E₇ in strains 1 to 15. Strain 1 showed two bands for esterase E₇.

Table 4. *pI* and M_F values of enzyme bands in strains of *Y. intermedia*

For all strains the pI/M_F for MDH was 5.75/36, the M_F of GDH was 18, and the M_F of E_1 was 82.

Strain code	AP (pI)	E_2 (M_F)	E_7 (M_F)	$E_{\alpha\alpha}$ (M_F)	$E_{\beta R}$ (M_F)	E_{BA} (M_F)
1	9.75	63/66	16	—	48	30/76
2, 3	9.75	63/66	16	—	48	30
4, 5	9.75	63/66	16	77	48	30
6	8.8	63/66	16	77	48	30
7	9.75	64/68	16	77	48	30
8, 9	9.75	64/68	—	77	48	30
10, 11, 12, 13, 14	9.75	63/66	—	77	48	30
15, 16, 17, 18, 19, 20	9.75	63/66	—	76	48	30
21	9.75	63/66	—	76	46	30
22	9.8	63*	—	75	48	30
23, 24	9.75	63/66	—	75	48	32
25	9.75	—	—	75	48	32

* In strain 22, esterase E_2 was replaced by esterase $E_{\alpha\beta}$.

Table 5. *pI* and M_F values of enzyme bands in strains of *Y. aldovae*

For all strains the pI/M_F for MDH was 5.75/36, and the M_F of GDH was 14.

Strain code	AP (pI)	E_1 (M_F)	E_2 (M_F)	E_6 (M_F)	E_7 (M_F)	$E_{\alpha\alpha}$ (M_F)	$E_{\beta R}$ (M_F)
1	9.5	78	—	—	16/26	—	—
2	9.35	79	64	—	16	—	26/44
3, 4, 5, 6, 7	9.5	82	—	—	16	78	44
8, 9, 10, 11	9.5	82	79	—	16	—	44
12	9.5	82	—	—	16	79	44
13	9.5	82	—	—	16	82	44
14	9.5	82	64/70	—	10	81	44
15	9.5	82	—	—	10	79	44
16, 17, 18	9.5	82	—	—7	—	78	44
19	9.7	82	—	—7	—	78	44
20, 21	9.5	82	75	—7	—	78	44
22	9.7	82	75	—7	—	78	44
23	9.5	82	64	—7	—	78	44
24	9.5	82	75	—7	—	78*	44
25	9.35	84	64/58	—7	—	80	44
26	9.1	84	—	—	—	81	65

* In strain 24, esterase $E_{\alpha\alpha}$ was replaced by esterase $E_{\alpha\beta}$.

Y. frederiksenii. (Table 6.) MDH, GDH and AP were polymorphic and could serve to delineate six zymotypes. MDH had the same pI and M_F values in strains of zymotypes F_1 to F_4 and was distinct in strains of zymotype F_5 and F_6 . GDH was identical for strains of zymotypes F_2 and F_3 but distinct for each of the other zymotypes. AP distinguished zymotype F_2 from zymotype F_3 . This delineation of six zymotypes was confirmed by the M_F values of esterase $E_{\alpha\alpha}$, by the M_F and pI values of esterases E_1 and E_7 and by the occurrence in some strains of additional esterases. Zymotype F_1 was distinguished from zymotype F_2 by a difference in pI and esterase E_1 mobility (pI 5/ M_F $\approx$ 84 and pI 4.9/ M_F $\approx$ 87, respectively) and the presence of bands $E_{\beta R}$ and $E_{\alpha\beta}$ (M_F $\approx$ 34). Zymotypes F_3 and F_4 had the same pI and mobility for esterase E_1 (pI 4.85/ M_F $\approx$ 90) but differed in the pI and mobility of esterase E_7 (pI 6.5/ M_F $\approx$ 8 and pI 6.4/ M_F $\approx$ 16, respectively). Moreover, strains of zymotype 5 showed esterase $E_{\alpha\alpha}$ at M_F $\approx$ 80 and band E_7 at pI 5.9 and M_F $\approx$ 42 and produced three additional esterases (M_F $\approx$ 67, 64 and 53), which hydrolysed α -naphthyl, β -naphthyl and indoxyl acetates, respectively. The first two were faint and sensitive to DFP and the third was resistant to DFP. Strains of zymotype 6 did not show esterases $E_{\alpha\alpha}$ and E_7 but produced an additional esterase designated as band E_{AB} which hydrolysed the five substrates, was sensitive to DFP and migrated at M_F $\approx$ 64.

Table 6. pI and M_F values of enzyme bands in strains of *Y. frederiksenii*

	Strain code	MDH (pI/M_F)	GDH (M_F)	AP (pI)	E_1 (M_F)	E_2 (M_F)	E_7 (M_F)	$E_{\alpha\alpha}$ (M_F)	$E_{\alpha\beta}$ (M_F)	$E_{\beta R}$ (M_F)
Zymotype F ₁	1	5.8/36	17	9.5	84	59	16	—	69	48
	2	5.8/36	17	9.5	84	—	15	74	69	48
	3	5.8/36	17	9.5	84	59	16	74	69	48
	4	5.8/36	17	9.5	84	—	16	74	69	48
	5	5.8/36	17	9.45	84	59	—	74	69	—
	6	5.8/36	17	9.5	84	59	16	74	69	—
	7, 8, 9, 10	5.8/36	17	9.5	84	64	8	74	—	48
Zymotype F ₂	11	5.8/36	16	8.8	87	65	8	74	—	—
	12, 13	5.8/36	16	8.8	87	64	8	74	—	—
Zymotype F ₃	14	5.8/36	16	9.35	90	64	8	78	—	—
Zymotype F ₄	15	5.8/36	14	8.4	90	64	16	78	—	—
Zymotype F ₅	16, 17, 18	5.3/42	19	8.8	90	—	42	80	—	—
Zymotype F ₆	19, 20	5.6/36	18	9.75	84	—	—	—	59	—

Table 7. pI and M_F values of enzyme bands in strains of *Y. kristensenii*

Strain code	MDH (pI/M_F)	GDH (M_F)	AP (pI)	E_1 (M_F)	E_2 (M_F)	E_6 (M_F)	E_7 (M_F)	$E_{\alpha\alpha}$ (M_F)	$E_{\alpha\beta}$ (M_F)
1	5.65/40	21	9.5	82	68	—	16	77	—
2	5.65/36	20	9.5	82	68	—	16	77	—
3, 4	5.65/40	20	9.5	82	68	—	16	77	—
5	5.65/40	20	9.5	82	68	—	15	77	—
6	5.65/40	20	9.5	82	68	—5	—	77	—
7	5.65/40	20	8.95	82	63*	—5	—	—	63
8	5.65/40	20	9.25	82	68*	—5	—	—	63†
9	5.65/40	20	8.4	82	68*	—5	—	79	63†
10	5.65/40	20	8.95	82	68*	—5	—	79	63†
11	5.65/40	20	8.95	82	70*	—5	—	79	68†
12	5.65/40	20	8.95	82	70*	—5	—	79†	—
13	5.65/40	20	8.95	82	70*	—5	—	—	—
14	5.65/40	21	8.95	82	60*	—	16	68†	—
15	5.65/36	20	8.95	82	60*	—	16	68†	—
16	5.65/40	—	8.95	—	62*	—	16	68†	—
17	5.65/40	17	8.95	84	62*	—	16	68†	—
18, 19	5.65/40	20	8.95	84	63*	—	16	—	72†
20	5.65/40	20	8.4	84	—	—	16	—	70†
21	5.65/36	20	8.95	—	60*	—	16	70†	—

* Band E'_2 (see text).† Band $E'_{\alpha\alpha}$ (see text).‡ Band $E'_{\alpha\beta}$ (see text).

Y. kristensenii. (Table 7.) MDH which had the same pI value for all strains, had two mobility values. GDH and AP were polymorphic with three mobility values and four pI values, respectively. Esterase bands may be divided according to their mobilities. The slow moving esterases corresponded to band E_7 or E_6 which migrated at $M_F \approx 16$ or $M_F \approx -5$, respectively and were detected in all strains. In strains 1 to 6, the fast moving bands consisted of esterases E_1 , $E_{\alpha\alpha}$ and E_2 , and an esterase designated band E_B ; this esterase hydrolysed butyryl ester and to a lesser degree the other substrates, was resistant to DFP and migrated at $M_F \approx 60$. These fast bands all showed identical mobilities in the six strains. In the other strains (7 to 21) the fast bands varied considerably in number and in the type of esterase activity. Some bands were unambiguously identified as esterases E_1 and $E_{\alpha\alpha}$, whereas atypical bands, designated as $E'_{\alpha\alpha}$, $E'_{\alpha\beta}$ and E'_2 , differed from esterases $E_{\alpha\alpha}$, $E_{\alpha\beta}$ and E_2 , respectively, in their hydrolysing properties and/or their sensitivity to DFP (results not shown). Additionally, an esterase band similar to

esterase E_{AB} of *Y. frederiksenii* was detected in strains 15 to 19 and was polymorphic with three mobility values ranging from $M_F \approx 77$ to $M_F \approx 82$.

DISCUSSION

Polyacrylamide agarose gel electrophoresis and IEF were complementary for the detection of GDH and AP activities. Only AP, MDH and esterases E_1 , E_{AB} , E_{BR} and E_7 were resolved by IEF. The lower resolving power of IEF for detecting enzyme activity, which had previously been observed for some esterases and other enzymes (Goullet & Picard, 1985*b*, 1986; Picard & Goullet, 1985), may be caused by interaction between carrier ampholytes and proteins or by denaturation of these enzymes at acidic pI values. Thus, titration curves of GDH (Picard *et al.*, 1987) demonstrated the pH sensitivity of this enzyme at its isoelectric point, as has been previously observed for other bacterial species. The fact that AP was not resolved in polyacrylamide agarose gel electrophoresis at pH 8.6 could be explained by its alkaline pI values (from 8.4 to 9.75). The two electrophoretic procedures were complementary in their resolution of enzyme polymorphism. Thus, the number of MDH variants detected by polyacrylamide agarose gel electrophoresis, IEF and a combination of the two techniques was three, five and seven, respectively. Twelve distinct esterase bands were resolved in cellular extracts of *Y. intermedia*, *Y. aldovae*, *Y. frederiksenii* and *Y. kristensenii*. Six esterase bands were similar to those of *Y. enterocolitica* (Goullet & Picard, 1984*a*). Two major kinds of esterases not detected in *Y. enterocolitica* and *Y. pseudotuberculosis* were found in other *Yersinia* species: esterase E_{BA} in *Y. intermedia* and esterase E_{BR} in *Y. aldovae*, *Y. intermedia* and *Y. frederiksenii*. Esterases E_{AC} , E_{AI} , E_B and E_{AB} were detected in some strains of *Y. frederiksenii* and *Y. kristensenii*. Band E_{AB} resembled esterases E_3 and E_5 of *Y. enterocolitica* in its hydrolysing activity and its sensitivity to DFP.

This work demonstrates that *Y. enterocolitica*, *Y. intermedia*, *Y. aldovae*, *Y. frederiksenii*, *Y. kristensenii* and *Y. pseudotuberculosis* may be characterized by the distinctive distribution of the electrophoretic bands of AP, MDH, GDH and esterases. These enzyme data appeared to be sufficient to identify to species level 192 recent isolates. Analysis of primary gene products therefore strongly supports the phenotypic characterization of the six species based on conventional biochemical tests (Bercovier & Mollaret, 1984; Bercovier *et al.*, 1984). The limited number of electrophoretic types characterized by AP, MDH and GDH within each species (Table 8) suggests that electrophoretic analysis of these enzymes can be used as a preliminary

Table 8. Number of enzyme electrophoretic types detected within the six species of *Yersinia*

	AP, MDH and GDH electrophoretic types*	Esterase electrophoretic types†	Enzyme electrophoretic types‡
<i>Y. enterocolitica</i> (70 strains)	3	20	22
<i>Y. pseudotuberculosis</i> (30 strains)	1	5	5
<i>Y. intermedia</i> (25 strains)	3	11	12
<i>Y. aldovae</i> (26 strains)	4	14	18
<i>Y. frederiksenii</i> (20 strains)	7	13	14
<i>Y. kristensenii</i> (21 strains)	10	17	19

* Distinct combination of electrophoretic variants for AP, MDH and GDH.

† Distinct combination of electrophoretic variants for all esterases.

‡ Distinct combination of electrophoretic variants for all enzymes.

Fig. 1. Relationship between the mean genetic diversity coefficient of AP, MDH, GDH and esterases E_{2a} , E_1 and E_6 – E_7 and the mean percentage DNA–DNA relatedness in *Y. intermedia* (●), *Y. aldovae* (■), *Y. enterocolitica* (▲) and *Y. frederiksenii* (▼). For the four species, the mean relative binding ratio at 60 °C was calculated from the data obtained by Brenner *et al.* (1980a) and Bercovier *et al.* (1984).

screening for species identification. Biochemical tests, O antigen determination, antibiotic and bacteriophage susceptibilities have been proposed for epidemiological or ecological analyses (Bercovier *et al.*, 1978; Baker & Farmer, 1982). However, the higher degree of electrophoretic polymorphism of esterases allows more precise identification of strains. Similar results have been obtained for *Escherichia coli* (Goullet & Picard, 1984b), *Aeromonas hydrophila* (Picard & Goullet, 1985) and *Enterobacter sakazakii* (Goullet & Picard, 1986). The separation of zymotypes A and B of *Y. enterocolitica* (Goullet & Picard, 1984a) was confirmed by differences in the pI values of their APs whereas biotypes 4 and 5 (unresolved by esterase mobility) were differentiated by GDH mobility. Strains of *Y. kristensenii*, which in some cases were hardly distinguishable by esterase electrophoretic patterns because of considerable variation in the number and kind of bands, were clearly characterized by the pI of their MDH and also by a combination of the mobility of GDH and the pI value of AP. Identification of the six zymotypes of *Y. frederiksenii* by MDH, GDH and AP patterns was substantiated by esterase patterns. Strains of zymotypes F_5 and F_6 appeared to be dramatically different from each other and from other strains of *Y. frederiksenii*. These considerable molecular divergences suggest that the strains corresponding to these zymotypes are genetically very different from other *Y. frederiksenii* strains. This requires further studies including DNA–DNA hybridization. The strains of *Y. intermedia* (rhamnose, melibiose and α -methyl-D-glycoside positive) (Brenner *et al.*, 1980b) and *Y. aldovae* (rhamnose positive, sucrose, cellobiose and sorbose negative) (Bercovier *et al.*, 1984), which are most biochemically distinct from *Y. enterocolitica*, appeared to have the most homogeneous enzyme electrophoretic patterns. In the species which are less biochemically distinct from *Y. enterocolitica*, i.e. *Y. frederiksenii* (rhamnose positive) (Ursing *et al.*, 1980) and *Y. kristensenii* (Voges–Proskauer and sucrose negative) (Bercovier *et al.*, 1980), the strains appeared to be heterogeneous in their electrophoretic pattern.

The mean genetic diversity coefficient of enzymes (Nei, 1978; Selander *et al.*, 1986) calculated from the electrophoretic data for MDH, GDH, AP and for esterase E_1 , esterase E_{2a} and esterases E_6 and E_7 increased from *Y. intermedia* (0.221) to *Y. aldovae* (0.351), *Y. enterocolitica* (0.392) and *Y. frederiksenii* (0.494). Good correlation between enzyme polymorphism and DNA heterogeneity for the four species was demonstrated by the existence of a linear relationship between the mean genetic diversity coefficient of the six enzymes and the average percentage DNA–DNA relatedness obtained by Brenner *et al.* (1980a) and Bercovier *et al.* (1984) (Fig. 1).

The authors thank Professor H. H. Mollaret for strains and Madame C. Gaillard and Madame N. Hautier for technical assistance. This work was supported by grant from the Conseil Scientifique de la Faculté Xavier Bichat (Université Paris VII).

REFERENCES

- BAKER, P. M. & FARMER, J. J., III (1982). New bacteriophage typing system for *Yersinia enterocolitica*, *Yersinia kristensenii*, *Yersinia frederiksenii* and *Yersinia intermedia*. Correlation with serotyping, biotyping and antibiotic susceptibility. *Journal of Clinical Microbiology* **15**, 491–502.
- BAPTIST, J. N., SHAW, C. R. & MANDEL, M. (1969). Zone electrophoresis of enzymes in bacterial taxonomy. *Journal of Bacteriology* **99**, 180–188.
- BERCOVIER, H. & MOLLARET, H. H. (1984). *Yersinia*. In *Bergey's Manual of Systematic Bacteriology*, vol. 1, pp. 498–506. Edited by N. R. Krieg. Baltimore: Williams & Wilkins.
- BERCOVIER, H., BRAULT, J., BARRE, N., TREIGNIER, M., ALONSO, J. M. & MOLLARET, H. H. (1978). Biochemical, serological and phage typing characteristics of 459 *Yersinia* strains isolated from a terrestrial ecosystem. *Current Microbiology* **1**, 353–357.
- BERCOVIER, H., URSING, J., BRENNER, D. J., STEIGERWALT, A. G., FANNING, G. R., CARTER, G. P. & MOLLARET, H. H. (1980). *Yersinia kristensenii*: a new species of Enterobacteriaceae composed of sucrose-negative strains (formerly called atypical *Yersinia enterocolitica* or *Yersinia enterocolitica*-like). *Current Microbiology* **4**, 219–224.
- BERCOVIER, H., STEIGERWALT, A. G., GUIYOULE, A., HUNTLEY-CARTER, G. & BRENNER, D. J. (1984). *Yersinia aldovae* (formerly *Yersinia enterocolitica*-like group X2): a new species of Enterobacteriaceae isolated from aquatic ecosystems. *International Journal of Systematic Bacteriology* **34**, 166–172.
- BRENNER, D. J., URSING, J., BERCOVIER, H., STEIGERWALT, A. G., FANNING, G. R., ALONSO, J. M. & MOLLARET, H. H. (1980a). Deoxyribonucleic acid relatedness in *Yersinia enterocolitica* and *Yersinia enterocolitica*-like organisms. *Current Microbiology* **4**, 195–200.
- BRENNER, D. J., BERCOVIER, H., URSING, J., ALONSO, J. M., STEIGERWALT, A. G., FANNING, G. R., CARTER, G. P. & MOLLARET, H. H. (1980b). *Yersinia intermedia*: a new species of Enterobacteriaceae composed of rhamnose-positive, melibiose positive, raffinose-positive strains (formerly called *Yersinia enterocolitica* or *Yersinia enterocolitica*-like). *Current Microbiology* **4**, 207–212.
- GOULLET, PH. (1980). Distinctive electrophoretic patterns of esterases from *Klebsiella pneumoniae*, *K. oxytoca*, *Enterobacter aerogenes* and *E. gergoviae*. *Journal of General Microbiology* **117**, 483–491.
- GOULLET, PH. & PICARD, B. (1984a). Distinctive electrophoretic and isoelectric focusing patterns of esterases from *Yersinia enterocolitica* and *Yersinia pseudotuberculosis*. *Journal of General Microbiology* **130**, 1471–1480.
- GOULLET, PH. & PICARD, B. (1984b). Typage électrophorétique des esterases d'*Escherichia coli* au cours de septicémies. *Presse médicale* **13**, 1079–1081.
- GOULLET, PH. & PICARD, B. (1985a). A two-dimensional electrophoretic profile for bacterial esterases. *Electrophoresis* **6**, 132–135.
- GOULLET, PH. & PICARD, B. (1985b). Etude du polymorphisme électrophorétique des lactate-, malate- et glutamate-deshydrogénases, de la phosphatase acide et des esterases de *Providencia alcalifaciens*, *P. stuartii* et *P. rustigianii*. *Annales de l'Institut Pasteur/Microbiologie* **136A**, 347–358.
- GOULLET, PH. & PICARD, B. (1986). Characterization of *Enterobacter cloacae* and *E. sakazakii* by electrophoretic polymorphism of acid phosphatase, esterases, and glutamate, lactate and malate dehydrogénases. *Journal of General Microbiology* **132**, 3105–3112.
- LENNOX, E. S. (1955). Transduction of linked characters of the host by bacteriophage PI. *Virology* **1**, 190–206.
- NEI, M. (1978). Estimation of average heterozygosity and genetic distance from a small sample of individuals. *Genetics* **89**, 583–590.
- PICARD, B. & GOULLET, PH. (1985). Comparative electrophoretic profiles of esterases, and of glutamate, lactate and malate dehydrogénases, from *Aeromonas hydrophila*, *A. caviae* and *A. sobria*. *Journal of General Microbiology* **131**, 3385–3391.
- PICARD, B., KRISHNAMOORTHY, R. & GOULLET, PH. (1987). Enzyme polymorphism in bacteria: study of molecular relatedness by combined isoelectric focusing-electrophoresis. *Electrophoresis* **8**, 149–152.
- 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.
- SICILIANO, M. & SHAW, C. R. (1976). Separation and visualization of enzymes on gels. In *Chromatographic and Electrophoretic Techniques*, 4th edn, vol. 2, pp. 185–209. Edited by I. Smith. Chicago: Yearbook Medical Publishers.
- URIEL, J. (1963). Caractérisation d'enzymes par électrophorèse en gélose. In *Technique de Laboratoire*, pp. 930–935. Edited by J. Loiseleur. Paris: Masson.
- URSING, J., BRENNER, D. J., BERCOVIER, H., FANNING, R., STEIGERWALT, A. G., BRAULT, J. & MOLLARET, H. H. (1980). *Yersinia frederiksenii*: a new species of Enterobacteriaceae composed of rhamnose-positive strains (formerly called atypical *Yersinia enterocolitica* or *Yersinia enterocolitica*-like). *Current Microbiology* **4**, 213–217.