Characterization of *Enterobacter cloacae* and *E. sakazakii* by Electrophoretic Polymorphism of Acid Phosphatase, Esterases, and Glutamate, Lactate 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 25 March 1986; revised 17 June 1986)

Acid phosphatase, esterases, and glutamate, lactate and malate dehydrogenases of 34 strains of *Enterobacter cloacae* and 22 strains of *Enterobacter sakazakii* were analysed by horizontal polyacrylamide agarose gel electrophoresis and by isoelectrofocusing in thin-layer polyacrylamide gel. The two species could be separated on the basis of distinct electrophoretic patterns of all enzymes analysed. Glutamate dehydrogenase and acid phosphatase were detected exclusively in *E. cloacae*, whereas esterase bands were more intensively stained in *E. sakazakii*. For each species, two zymotypes could be distinguished, on the basis of electrophoretic mobilities of malate dehydrogenase and banding patterns of esterase for *E. cloacae*, and by both isoelectric point and electrophoretic mobilities of an esterase and of lactate and malate dehydrogenases for *E. sakazakii*. The high degree of enzyme polymorphism within the two species permitted precise identification of strains. The variations in electrophoretic patterns might therefore provide useful epidemiological markers.

INTRODUCTION

Enterobacter cloacae, which may be isolated from human and animal faeces, is an opportunistic pathogen responsible for nosocomial infections. *Enterobacter sakazakii* (Farmer *et al.*, 1980), formerly known as yellow pigmented *E. cloacae*, has been isolated from sputum, faeces, spinal fluid and blood (Farmer *et al.*, 1980) and may cause neonatal meningitis (Muyltjens *et al.*, 1983). These two species, which form two distinct hybridization groups (Steigerwalt *et al.*, 1976; Farmer *et al.*, 1980; Izard *et al.*, 1983) were found to be phenotypically different in only a few biochemical tests (Farmer *et al.*, 1980).

Recently, using both conventional electrophoresis (CE) and isoelectrofocusing (IEF), enzyme polymorphism has been used for specific and subspecific differentiation of bacteria (Goullet & Picard, 1985*a, b*). Data obtained by the two procedures gave highly discriminative and reliable information, which reflected genetic divergence better than did biochemical tests (Picard & Goullet, 1985).

The purpose of this study was to evaluate phenotypic differences between and within *E. sakazakii* and *E. cloacae* on the basis of electrophoretic and IEF profiles of acid phosphatase (AP), esterases (EST), and glutamate, lactate and malate dehydrogenases (GDH, LDH and MDH).

METHODS

Strains and their cultivation. The names and sources of the test strains are given in Table 1. Bacteria were grown in Fernbach flasks containing 500 ml Tryptic Soy Broth (Difco). Flasks were vigorously shaken at 37 °C for 18 h.

Electrophoretic analysis. Preparation of extracts, inhibition by di-isopropyl fluorophosphate (DFP), CE and

Abbreviations: AP, acid phosphatase; CE, conventional electrophoresis; EST, esterase; GDC, genetic diversity coefficient; GDH, glutamate dehydrogenase; IEF, isoelectrofocusing; LDH, lactate dehydrogenase; MDH, malate dehydrogenase; M_R , relative mobility.

Table 1. *Strains examined*A. *Enterobacter cloacae*

Laboratory code	Strain designation*	Origin†	Laboratory code	Strain designation*	Origin†
1	11.76 ^a	Stool; Versailles	18	36.76 ^b	Urine; Marseille
2	8.76 ^a	Blood; Paris	19	HB 8 ^b	Blood; Clichy
3	HB 2 ^b	Lung; Clichy	20	HB 4 ^b	Urine; Clichy
4	7.76 ^a	Navel; Paris	21	HB 7 ^b	Pus; Clichy
5	34.76 ^a	Pus; Lagny	22	HB 11 ^b	Blood; Clichy
6	HB 9 ^b	Urine; Clichy	23	HB 1 ^b	Lung; Clichy
7	45.75 ^a	Frog; Turkey	24	60.80 ^a	Stool; Paris
8	HB 5 ^b	Lung; Clichy	25	HB 3 ^b	Urine; Clichy
9	HB 10 ^b	Urine; Clichy	26	32.72 ^a	Urine
10	41.76 ^a	Dog; Blois	27	44.80 ^a	Stool; Rabah, Morocco
11	9.76 ^a	Trachea; Paris	28	26.76 ^a	Spinal fluid; Niamey, Niger
12	10.76 ^a	Stool; Dakkar, Senegal	29	34.75 ^a	Blood; Bangui, Central African Republic
13	61.80 ^a	Bile; Paris	30	39.80 ^a	Urine; Bagdad, Iraq
14	77.80 ^a	Blood; Paris	31	50.75 ^a	Stool; Montpellier
15	78.80 ^a	Spinal fluid; Paris	32	29.76 ^a	Goat; Loir et Cher
16	79.80 ^a	Catheter; Paris	33	58.76 ^a	Stool; Le Mans
17	47.75 ^a	Starch	34	29.75 ^a	Field mouse; Indre

B. *Enterobacter sakazakii*

Laboratory code	Strain designation*	Origin†	Laboratory code	Strain designation*	Origin†
35	3.85 ^a	Water; Charenton	46	2.85 ^a	Urine; Clermont Ferrand
36	47.81 ^a	Pus; Spain	47	38.76 ^a	Blood; Paris
37	20.77 ^a	Stool; Paris	48	4.85 ^a	Stool; Chateauroux
38	20.73 ^a	Sputum; Toulon	49	33.83 ^a	Pus; Brest
39	3.74 ^a	Stool; Drancy	50	31.81 ^a	Blood; Amiens
40	6.85 ^a	Bird; Paris	51	53.82 ^a	Sausage; Paris
41	4.74 ^a	Urine; Paris	52	81.84 ^a	Stool; Paris
42	12.82 ^a	Spinal fluid; Poissy	53	14.77 ^a	Pus; St Etienne
43	14.82 ^a	Nose; Poissy	54	36.77 ^a	Pus; Paris
44	19.82 ^a	Stool; Poissy	55	17.74 ^a	Water; Belgium
45	36.82 ^a	Stool; Poissy	56	7.75 ^a	Tooth; Paris

* *a*, Strains from the collection of Service des Enterobacteries (Professeur Le Minor), Institut Pasteur, Paris, France, and provided by Dr C. Richard; *b*, strains isolated at Beaujon Hospital, France.

† Country of origin France unless otherwise stated.

IEF, establishment of correspondence of bands characterized in the two electrophoretic techniques and esterase staining, were done as described by Goullet (1973) and Goullet & Picard (1985*a*). Staining methods for the other enzymes were as follows: GDH, Baptist *et al.* (1969); LDH and MDH, Siciliano & Shaw (1976); AP, Uriel (1963). The electrophoretic mobility of a given variant was measured as the relative mobility value (M_R) (Goullet & Picard, 1985*b*) which is the ratio between the distance measured from the slower band (GDH of *E. cloacae* strain 45.75) to the band of the variant and the distance measured from the slower band to the faster band (esterase ES₁ of *E. sakazakii* strain 20.77).

RESULTS

Characterization of enzyme bands

IEF. Satisfactory resolution with distinct banding was obtained for MDH and LDH of *E. cloacae* and *E. sakazakii*, for AP of *E. cloacae* and for an esterase of *E. sakazakii*. Esterases of *E. cloacae*, AP of *E. sakazakii* and GDH of *E. cloacae* and *E. sakazakii* were not detected. With a narrow pH gradient from 4 to 6.5, MDH and AP gave a sharp and well-stained main band (used for pI determination) and two or three anodic satellite bands. LDH generally gave three bands (the one nearest the cathode was used for pI determination), whereas esterase gave a single band (Figs 1 and 2).

Fig. 1. Zymograms of *E. cloacae*. (a) Strain 14 from zymotype C₁; (b) strain 33 from zymotype C₂. Esterase EC₅, which migrated towards the cathode, is not shown.

strain 39 the range and SD values were 5.13 ± 0.02 for MDH, 4.88 ± 0.003 for LDH and 5.3 ± 0.03 for esterase ES₄.

Polyacrylamide agarose gel electrophoresis. This method was more sensitive than IEF for detecting the enzymes, all of which gave reproducible banding patterns. MDH, LDH, GDH and AP gave a single, well-stained band, but GDH and AP were detected only in *E. cloacae* (Figs 1 and 2). Diverse esterase bands, all of which degraded α -naphthyl acetate (numbered in order of decreasing mobility towards the anode) could be distinguished on the basis of their hydrolytic activity towards other substrates (β -naphthyl and indoxyl acetates, and α - and β -naphthyl butyrates), and in their sensitivity or resistance to DFP (10^{-3} M) (Table 2). For *E. cloacae* strains, esterase band EC₁ hydrolysed indoxyl acetate; band EC₂ did not hydrolyse other substrates; band EC₃ hydrolysed β -naphthyl acetate; band EC₄ hydrolysed α -naphthyl butyrate and indoxyl acetate and reacted slightly with β -naphthyl acetate; and band EC₅ (migrating to the cathode) hydrolysed β -naphthyl acetate. Esterases EC₁ and EC₃ were resistant to DFP whereas esterases EC₂, EC₄ and EC₅ were sensitive. Except for esterase EC₄, the bands resolved from crude extracts of *E. cloacae* were faint. For *E. sakazakii*, esterase bands ES₁, which hydrolysed indoxyl acetate, and ES₂, which hydrolysed β -naphthyl acetate and α - and β -naphthyl butyrates, were resistant to DFP; band ES₃ hydrolysed indoxyl acetate and α -naphthyl butyrate; band ES₄, which was identified as the esterase obtained by IEF, strongly hydrolysed α - and β -naphthyl acetates and indoxyl acetate, and reacted weakly with α -naphthyl butyrate. Esterases ES₃ and ES₄ were sensitive to DFP.

Table 2. *Hydrolytic activity and sensitivity to DFP (10^{-3} M) of esterase bands of E. cloacae and E. sakazakii*

	Band	Substrates hydrolysed*					Sensitivity to DFP†
		α -NA	α -NB	IA	β -NA	β -NB	
<i>E. cloacae</i>	EC ₁	+	—	+	—	—	R
	EC ₂	+	—	—	—	—	S
	EC ₃	+	—	—	+	—	R
	EC ₄	++	+	+	±	—	S
	EC ₅	+	—	—	+	—	S
<i>E. sakazakii</i>	ES ₁	+	—	+	—	—	R
	ES ₂	++	++	—	++	++	R
	ES ₃	++	++	±	—	—	S
	ES ₄	+++	+	+++	+++	—	S

* α -NA, α -naphthyl acetate; α -NB, α -naphthyl butyrate; IA, indoxyl acetate; β -NA, β -naphthyl acetate; β -NB, β -naphthyl butyrate. + + +, Intensively stained band; + +, moderately stained band; +, faint band; ±, very weak activity; —, no activity.

† R, resistant; S, sensitive.

Table 3. *pI and M_R values of enzyme bands in strains of E. cloacae*

For enzyme abbreviations see text.

	Strain code	pI/M _R values			M _R values					
		MDH	LDH	AP	GDH	EC ₁	EC ₂	EC ₃	EC ₄	EC ₅
Zymotype C ₁	1	5.3/35	5.35/26	5.9/60	5	89	—	48	—	—
	2	5.3/35	5.35/29	5.9/55	5	89	—	48	—	—
	3	5.3/35	5.35/29	5.85/55	3	96	—	—	—	—34
	4	5.3/35	5.35/29	5.85/59	4	89	—	48	—	—
	5	5.3/35	5.35/29	5.8/59	4	96	83	—	—	—34
	6	5.3/35	5.35/29	5.6/55	7	96	—	85	—	—34
	7	5.3/35	5.35/29	5.6/59	0	92	82	—	—	—34
	8	5.3/35	5.35/29	5.6/60	3	96	—	—	—	—
	9	5.3/35	5.35/29	5.6/60	5	96	—	—	—	—34
	10	5.3/35	5.35/30	5.6/60	4	96	83	—	—	—34
	11	5.3/35	5.30/26	5.9/60	0	89	—	48	—	—
	12	5.3/35	5.25/23	6.1/55	7	96	—	75	—	—34
	13	5.3/35	5.25/23	5.6/60	5	94	—	—	—	—34
	14	5.3/35	5.25/23	5.6/60	7	92	83	75	—	—34
	15	5.3/35	5.25/23	5.6/60	7	92	83	75	—	—34
	16	5.3/35	5.25/26	5.95/55	5	—	93	—	—	—
	17	5.3/35	5.25/26	5.6/49	5	96	77	—	—	—
	18	5.3/35	5.2/23	5.6/59	4	96	—	75	—	—34
	19	5.3/35	5.2/23	5.6/59	7	96	—	85	—	—34
	20	5.3/35	5.2/23	5.6/60	3	88	—	71	—	—34
	21	5.3/35	5.2/23	5.6/60	4	92	83	75	—	—34
	22	5.3/35	5.2/23	5.6/60	5	92	—	68	—	—34
	23	5.3/35	5.2/23	5.4/67	7	96	—	85	—	—34
	24	5.3/35	5.2/29	5.85/59	4	96	85	—	—	—34
	25	5.3/35	5.2/29	5.8/56	5	96	—	85	—	—34
	26	5.3/35	5.2/29	5.6/60	4	96	—	85	—	—41
	27	5.3/35	5.2/29	5.6/60	4	96	—	85	—	—34
	28	5.3/35	5.05/29	5.6/59	4	90	85	—	—	—34
	29	5.3/35	5.05/29	5.6/63	4	88	85	—	—	—34
Zymotype C ₂	30	5.3/42	5.35/29	5.8/59	3	83	—	—	70	—
	31	5.3/42	5.35/29	5.75/63	3	—	—	—	81	—
	32	5.3/42	5.35/29	5.7/63	3	—	—	—	81	—
	33	5.3/42	5.35/29	5.7/63	3	—	—	—	81	—
	34	5.3/42	5.35/29	5.5/67	3	—	—	—	81	—

Table 4. *pI* and M_R values of enzyme bands in strains of *E. sakazakii*

For enzyme abbreviations see text.

	Strain code	<i>pI</i> / M_R values			M_R values		
		MDH	LDH	ES ₄	ES ₁	ES ₂	ES ₃
Zymotype S ₁	35	5.15/41	4.9/34	5.35/53	96	92	—
	36	5.15/41	4.9/34	5.2/57	—	81	—
	37	5.15/41	4.9/48	5.35/56	100	79	—
	38	5.15/41	4.9/48	5.3/44	92	—	56
	39	5.15/41	4.9/48	5.3/44	—	92	56
	40	5.15/41	4.9/48	5.3/56	—	—	—
	41	5.15/41	4.9/48	5.2/56	—	92	—
	42	5.15/41	4.9/48	5.2/56	—	92	—
	43	5.15/41	4.9/48	5.2/56	—	92	—
	44	5.15/41	4.9/48	5.2/56	—	92	—
	45	5.15/41	4.9/48	5.2/56	—	92	—
	46	5.15/41	4.9/48	5.2/67	98	92	56
	47	5.15/41	4.9/48	5.1/62	—	96	66
	48	5.15/41	4.9/48	4.85/56	96	92	66
	49	5.15/41	4.9/48	—	—	92	—
	50	5.15/41	4.9/49	5.3/44	—	92	56
	51	5.15/41	4.9/49	5.2/44	—	92	56
	52	5.15/41	4.9/49	5.2/44	—	92	56
Zymotype S ₂	53	5.05/49	4.9/42	4.75/62	93	91	—
	54	5.05/49	4.9/42	4.75/62	93	91	—
	55	5.05/49	4.9/42	4.7/64	—	90	—
	56	5.05/49	4.9/42	4.7/64	96	85	—

Range of pI and mobilities of enzyme bands among the strains

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

E. cloacae. *pI* and M_R values of enzymes detected in all 34 strains are shown in Table 3. MDH, which had the same *pI* value (5.3) for all strains, had two mobility values ($M_R \approx 35$ and $M_R \approx 42$). These served to divide strains into two zymotypes: C₁ (strains 1 to 29) and C₂ (strains 30 to 34). LDH had five distinct *pI* values ranging from 5.05 to 5.35 and four mobility values from $M_R \approx 23$ to $M_R \approx 30$. For zymotype C₂, LDH had identical *pI* and M_R values. AP had ten distinct *pI* values ranging from 5.4 to 6.1, and seven mobility values ranging from $M_R \approx 49$ to $M_R \approx 67$. GDH had five mobility values ranging from $M_R \approx 0$ to $M_R \approx 7$. For zymotype C₂, GDH had identical M_R values. Esterases gave two distinct banding patterns. For zymotype C₁, esterase EC₁ was the most frequently detected among the strains, esterase EC₅ had the same mobility for 21 strains and esterase EC₄ was not detected; zymotype C₂ possessed esterase EC₄ but not esterases EC₂, EC₃ and EC₅. Thus, strains of zymotype C₂ could be distinguished from those of zymotype C₁ by differences in electrophoretic patterns of at least four enzymes.

E. sakazakii. *pI* and M_R values of enzymes detected in all 22 strains are shown in Table 4. Two zymotypes could be clearly distinguished among the strains by *pI* and/or mobilities of MDH, LDH and esterase ES₄. Zymotype S₁ (strains 35 to 52) was characterized by a *pI* of 5.15 and $M_R \approx 41$ for MDH, $M_R \approx 34$, $M_R \approx 48$ and $M_R \approx 49$ for LDH and by *pI* values ranging from 4.85 to 5.35 for esterase ES₄. Zymotype S₂ (strains 53 to 56) was characterized by a *pI* of 5.05 and $M_R \approx 49$ for MDH, by $M_R \approx 42$ for LDH and by *pI* values of 4.7 and 4.75 for esterase ES₄. Bands ES₂ and ES₄ were found in 20 and 21 strains, respectively, whereas ES₁ and ES₃ were each detected in only eight strains.

Fig. 3

Fig. 4

Fig. 3. Two-dimensional electrophoretic profiles of LDH (□) and MDH (○) from *E. cloacae* and of LDH (■) and MDH (●) from *E. sakazakii*. Each allozyme is defined by pI and M_R .

Fig. 4. Two-dimensional electrophoretic profiles of LDH (□), MDH (○) and esterase ES_4 (△) from *E. sakazakii* strains of zymotype S_1 and of LDH (■), MDH (●) and esterase ES_4 (▲) from *E. sakazakii* strains of zymotype S_2 . Each allozyme is defined by pI and M_R .

DISCUSSION

Using the zymogram procedure, LDH and MDH were resolved from crude extracts of both *E. cloacae* and *E. sakazakii*, whereas GDH and AP were detected only in *E. cloacae*. Several esterases were present in each of the two species. Except for esterases EC_1 and ES_1 , which were similar in their hydrolytic activity and in their resistance to DFP, the esterases of *E. sakazakii* could be clearly distinguished from those of *E. cloacae*. Comparison of results obtained by IEF and CE for esterases ES_1 , ES_2 and ES_3 of *E. sakazakii* and for esterases and GDH of *E. cloacae*, showed that CE was more sensitive than IEF for detection of enzyme activity (Tables 3 and 4), as has been already shown for some enzymes produced by *Yersinia*, *Providencia* and *Aeromonas* strains (Goullet & Picard, 1984, 1985b; Picard & Goullet, 1985). The number of electrophoretic variants (allozymes) was higher with IEF (Table 5) for LDH and AP of *E. cloacae* and with CE for LDH of *E. sakazakii*. For LDH and AP of *E. cloacae* and for esterase ES_4 of *E. sakazakii*, CE and IEF complement one another in the detection of electrophoretic enzyme variability. Conjunction of data obtained by the two techniques increased values of genetic diversity coefficients (GDC) (Nei, 1978; Musser *et al.*, 1986) obtained by a single electrophoretic procedure (Table 5). The two hydrolases were more electrophoretically polymorphic than the two dehydrogenases. GDC values for esterase, AP and LDH were considerably higher than GDC values for MDH. As previously observed (Shah & Williams, 1982; Goullet & Picard, 1985b; Picard & Goullet, 1985) MDH appears to be a useful molecular marker for both inter- and intra-species differentiation of bacteria.

Phenotypic differentiation of *E. sakazakii* from *E. cloacae* is principally based on non-diffusible yellow pigment production, absence of fermentation of D-sorbitol (Farmer *et al.*, 1980; Richard, 1984) and detection of α -glucosidase activity (Muytjens *et al.*, 1984). By determining physico-chemical parameters of specific proteins the present work strongly consolidated this differentiation of the species. Thus, mobilities and pI values of either LDH or MDH were sufficient to identify to species level the 56 *Enterobacter* strains analysed. This is illustrated by two-dimensional electrophoretic profiles (Goullet & Picard, 1985a), which clearly show that enzymes of *E. sakazakii* had lower pI values and/or higher M_R values than *E. cloacae* (Fig. 3). Esterase patterns of the two species were distinct from those of *Enterobacter aerogenes* and *Enterobacter gergoviae* (Goullet, 1980).

As indicated by two-dimensional electrophoretic profiles of LDH, MDH and esterase ES_4 (Fig. 4), electrophoretic distribution of allozymes among *E. sakazakii* strains provided evidence

Table 5. Numbers of allozymes (a)* and GDC values† for MDH, LDH, AP and esterase ES₄ obtained by IEF, CE and by conjunction of the two techniques

		IEF		CE		Conjunction of IEF and CE	
		a	GDC	a	GDC	a	GDC
MDH	<i>E. cloacae</i>	1	0	2	0.25	2	0.25
	<i>E. sakazakii</i>	2	0.34	2	0.34	2	0.34
LDH	<i>E. cloacae</i>	5	0.70	4	0.59	9	0.80
	<i>E. sakazakii</i>	1	0	4	0.66	4	0.66
AP	<i>E. cloacae</i>	10	0.72	7	0.79	18	0.89
ES ₄	<i>E. sakazakii</i>	8	0.85	8	0.86	13	0.95

* Including null allozymes.

† GDC (H) = $1 - \sum x_i^2/[n(n-1)]$ where x_i is the frequency of each allozyme and n is the number of strains in the samples calculated after deletion of strain 15 of *E. cloacae* and strains 43 to 45 of *E. sakazakii* (see Discussion).

for two zymotypes, S₁ and S₂. Combined use of IEF and CE was necessary to differentiate zymotypes defined on the basis of LDH or esterase ES₄ patterns. Thus the LDH of the two zymotypes, which had the same pI value (4.9) for all strains, was resolved by CE into variants with M_R values of ≈ 34 , ≈ 48 and ≈ 49 for zymotype S₁ and $M_R \approx 42$ for zymotype S₂. Conversely, esterase ES₄ of strain 47 (zymotype S₁), and of strain 53 and 54 (zymotype S₂), which had the same mobility for all strains, was resolved by IEF into variants with pI values of 5.1 and 4.75.

The high degree of enzyme polymorphism within *E. cloacae* and *E. sakazakii* enables precise identification of strains which may be useful for epidemiological purposes. For *E. cloacae*, all strains analysed had distinct electrophoretic patterns, with the exception of strains 14 and 15 which were isolated from two distinct clinical specimens (blood and spinal fluid) from the same patient. For *E. sakazakii*, strains 42 to 45 isolated from diverse sources in the same hospital had identical electrophoretic patterns (Table 3). This is strong evidence that these strains are identical.

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

REFERENCES

- BAPTIST, J. N., SHAW, C. R. & MANDEL, M. (1969). Zone electrophoresis of enzymes in bacterial taxonomy. *Journal of Bacteriology* **99**, 180–188.
- FARMER, J. J., III, ASBURY, M. A., HICKMAN, F. W., BRENNER, D. J. & THE ENTEROBACTERIACEAE STUDY GROUP (1980). *Enterobacter sakazakii*: a new species of *Enterobacteriaceae* isolated from clinical specimens. *International Journal of Systematic Bacteriology* **30**, 569–584.
- GOULLET, PH. (1973). An esterase zymogram of *Escherichia coli*. *Journal of General Microbiology* **77**, 27–35.
- 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. (1984). 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. (1985a). A two-dimensional electrophoretic profile for bacterial esterases. *Electrophoresis* **6**, 132–135.
- GOULLET, PH. & PICARD, B. (1985b). Etude du polymorphisme électrophoretique des lactate-, malate- et glutamate-deshydrogenases, de la phosphatase acide et des esterases de *Providencia alcalifaciens*, *P. stuartii* et *P. rustigianii*. *Annales de l'Institut Pasteur/Microbiologie* **136A**, 347–358.
- IZARD, D., RICHARD, C. & LECLERC, H. (1983). DNA relatedness between *Enterobacter sakazakii* and other members of the genus *Enterobacter*. *Annales de Microbiologie (Institut Pasteur)* **134A**, 241–245.
- MUSSER, J. M., BARENKAMP, S. J., GRANOFF, D. M. & SELANDER, R. K. (1986). Genetic relationships of serologically nontypable and serotype b strains of *Haemophilus influenzae*. *Infection and Immunity* **52**, 183–191.
- MUYTJENS, H. L., ZANEN, H. C., SONDERKAMP, H. J., KOLLEE, L. A., KAYE WACHSMUTH, I. & FARMER, J. J., III (1983). Analysis of eight cases of neonatal

- meningitis and sepsis due to *Enterobacter sakazakii*. *Journal of Clinical Microbiology* **18**, 115–120.
- MUYTJENS, H. L., VAN DER ROS-VAN DE REPE, J. & VAN DRUTEN, H. A. M. (1984). Enzymatic profiles of *Enterobacter sakazakii* and related species with special reference to the α -glucosidase reaction and reproducibility of the test system. *Journal of Clinical Microbiology* **20**, 684–686.
- 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 dehydrogenases, from *Aeromonas hydrophila*, *A. caviae* and *A. sobria*. *Journal of General Microbiology* **131**, 3385–3391.
- RICHARD, C. (1984). *Enterobacter*. In *Bergey's Manual of Systematic Bacteriology*, 9th edn, vol. 1, pp. 465–469. Edited by N. R. Krieg. Baltimore: Williams & Wilkins.
- SHAH, H. N. & WILLIAMS, R. A. D. (1982). Dehydrogenase patterns in the taxonomy of *Bacteroides*. *Journal of General Microbiology* **128**, 2955–2965.
- 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.
- STEIGERWALT, A. G., FANNING, G. R., FIFE ASBURY, M. A. & BRENNER, D. J. (1976). DNA relatedness among species of *Enterobacter* and *Serratia*. *Canadian Journal of Microbiology* **22**, 121–137.
- URIEL, J. (1963). Caractérisation d'enzymes par électrophorèse en gélose. In *Technique de Laboratoire*, pp. 930–935. Edited by J. Loiseleur. Paris: Masson.