Distinctive Electrophoretic Patterns of Esterases from *Levinea malonatica*, *Levinea amalonatica* and *Citrobacter*

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SUMMARY

Esterases of 42 strains of *Levinea malonatica*, *Levinea amalonatica* and *Citrobacter* were analysed by horizontal slab electrophoresis in polyacrylamide-agarose gel using several synthetic substrates. On the basis of esterase zymograms a distinctive pattern was established for each of the three species. *Levinea malonatica* was characterized by two major bands: one hydrolysing acetate esters but not butyrate esters; and the other hydrolysing α -naphthyl acetate and reacting weakly with α -naphthyl butyrate and β -naphthyl acetate. *Levinea amalonatica* showed one prominent band that hydrolysed α -naphthyl esters and reacted weakly with β -naphthyl esters. *Citrobacter* strains showed one major band that hydrolysed α -naphthyl esters and appeared slightly more active towards β -naphthyl esters than that of *L. amalonatica*. Considerable variations in electrophoretic mobility were observed among *Citrobacter* strains. *Levinea amalonatica* was less variable. In addition, one minor anodal band reacting with β -naphthyl acetate was observed in both *L. malonatica* and *Citrobacter*.

The relative molecular sizes of the major esterase bands were determined by disc electrophoresis with gels of different acrylamide concentrations. The molecular size of the major band of *Citrobacter* appeared to be smaller than that of the corresponding esterase band of *L. amalonatica*.

INTRODUCTION

Levinea is a new genus recently described by Young *et al.* (1971) and is composed of two species, *L. malonatica* and *L. amalonatica*. These differ from each other and from *Citrobacter* in biochemical characteristics (Young *et al.*, 1971; Richard, Brisou & Lioult, 1972) as well as polynucleotide sequences (Crosa *et al.*, 1974). The aim of this work was to establish whether these three species may be differentiated by their esterase (EC. 3.1.1.) patterns, as reported for other enterobacteria (Goullet, 1973, 1975).

METHODS

The bacteria used are listed in Table 1.

Biochemical and physiological tests were as previously described (Richard *et al.*, 1972). Growth conditions, preparation of extracts, heat denaturation, inhibition by di-iso-

Table 1. *Levinea* and *Citrobacter* strains examined

Code	Strain designation	Origin,* place and date	Carbohydrate fermentation†					Biotype‡
			Sorbose	Dulcitol	Sucrose	Raffinose	Melibiose	
<i>Levinea malonatica</i>								
1	M212.73	Urine, Brussels, 1973	—	—	—	—	—	a
2	M227.73	Urine, Strasbourg, 1973	—	—	—	—	—	a
3	M236.73	Urine, Paris, 1973	—	—	—	—	—	a
4	M303.74	Blood, Saint-Denis, 1974	—	—	—	—	—	a
5	M94.72	Urine, Paris, 1972	—	+	—	—	—	c
6	M33.73	Trachea, Toulon, 1973	—	+	—	—	—	c
7	M208.73	Urine, Strasbourg, 1973	—	+	+	—	—	d
8	M220.73	Blood, St Etienne, 1973	—	+	+	—	—	d
9	M229.73	Faeces, Strasbourg, 1973	—	+	+	—	—	d
10	M310.74	Environment, Strasbourg, 1974	—	+	+	—	—	d
11	M327.74	Urine, Paris, 1974	—	+	+	—	—	d
12	M235.73	Trachea, Paris, 1973	(+)	—	+	—	—	e
13	M312.74	Pus, Paris, 1974	(+)	—	+	—	—	e
14	M328.74	Urine, Paris, 1974	—	—	+	—	—	e
<i>Levinea amalonatica</i>								
15	A195.64	Faeces, Levallois-Perret, 1964	+	—	—	—	—	b
16	A196.65	Faeces, St Etienne, 1965	+	—	—	—	—	b
17	A30.70	Urine, Paris, 1970	+	—	—	—	—	b
18	A61.71	Faeces, Cairo, 1971	+	—	—	—	—	b
19	A95.72	Faeces, Lyon, 1972	+	—	—	—	—	b
20	A188.73	Faeces, South Korea, 1973	+	—	—	—	—	b
21	A211.73	Faeces, Brussels, 1973	+	—	—	—	—	b
22	A226.73	Faeces, Strasbourg, 1973	+	—	—	—	—	b
23	A231.73	Faeces, Prague, 1973	+	—	—	—	—	b
24	A273.74	Faeces, Strasbourg, 1974	+	—	—	—	—	b
25	A279.74	Faeces, Madrid, 1974	+	—	—	—	—	b
26	A305.74	Faeces, St Etienne, 1974	+	—	—	—	—	b
27	A317.74	Faeces, Upper Volta, 1974	+	—	—	—	—	b
28	A325.74	Faeces, Annecy, 1974	+	—	—	—	—	b
<i>Citrobacter</i> sp.								
29	FM.14	Urine, Paris, 1970	(+)	—	—	—	—	
30	47.71	Faeces, Rouen, 1971	+	—	—	—	—	
31	7.72	Urine, St Germain-en-Laye, 1972	+	+	+	+	+	
32	12.72	Faeces, Baghdad, 1972	+	—	+	—	—	
33	14.72	Faeces, Baghdad, 1972	+	+	—	—	+	
34	66.72	Faeces, Perigueux, 1972	+	+	—	—	+	
35	13.74	Faeces, Colmar, 1974	+	+	—	—	—	
36	25.74	Throat, Tangier, 1974	+	—	+	+	+	
37	26.74	Faeces, Orly, 1974	(+)	+	—	—	+	
38	30.74	Faeces, Montmorency, 1974	+	+	+	—	—	
39	75.74	Faeces, Forminy, 1974	+	+	—	—	—	
40	76.74	Faeces, St Cyr, 1974	+	+	+	(+)	+	
41	77.74	Faeces, Provins, 1974	+	+	+	+	+	
42	81.74	Faeces, Le Mans, 1974	—	+	—	—	+	

* All except no. 10 were of human origin.

† +, Positive test; (+), late positive; —, negative test.

‡ According to Richard *et al.* (1972).

fluoropropyl phosphate (DFP), protein estimation, acrylamide-agarose gel electrophoresis and esterase staining were as described previously (Goullet, 1973, 1975), with the following modifications. Bacteria were grown in L broth (Lennox, 1955) without glucose and harvested during the exponential phase. They were washed twice in 0.06 M-Tris/glycine buffer, pH 8.7, before ultrasonic disruption. Esterase bands of *E. coli* K12 (Goullet, 1973) were used as a parallel electrophoretic control. DFP (Calbiochem) was used at 10^{-6} to 10^{-3} M.

Disc electrophoresis. The buffer and gel solutions were as described by Davis (1964). Gel columns were formed in glass tubes (80 × 4 mm). For small pore gel, the bisacrylamide/acrylamide weight ratio was kept constant at 1:30 and the acrylamide was varied from 5 to 12 % (w/v). The same small pore gel concentration was used for each run. The tracking dye was bromophenol blue. Electrophoresis was at room temperature in a Canalco model 1200 apparatus using 2 mA per tube until the dye front had migrated into the small pore gel; thereafter 5 mA per tube was maintained until the dye front had migrated to 40 mm.

Fig. 1. *Levinea malonatica*. (a) Schematic representation of mobility and activity of esterase bands (strain 11). Horizontal slab acrylamide-agarose gel electrophoresis was performed using 7 % (w/v) acrylamide and discontinuous Tris/glycine buffer, pH 8.7 (Uriel, 1966). Esterase activity was revealed by the method of Uriel (1961). Substrates used were: 1, α -naphthyl acetate; 2, α -naphthyl butyrate; 3, β -naphthyl acetate; 4, β -naphthyl butyrate. Relative intensity of staining: $\text{XXXX} > \text{XXXX} > \text{XXXX} > \text{XXXX}$. (b) Esterase patterns of the 14 strains arranged in order of increasing mobility of E_2 esterase bands. $\blacksquare$, E_1 band; XXXX , E_2 band; XXXX , E_3 band. The M_F values were only used comparatively.

The esterases were stained by α -naphthyl acetate (Sigma) using naphthanil diazo blue B (Dajac Laboratories, Philadelphia, Pennsylvania, U.S.A.) as dye coupler.

Electrophoretic mobility. The M_F value, i.e. the distance moved by the esterase band as a percentage of the distance moved by the dye front, was the average obtained from four to six runs.

RESULTS

Reproducible esterase patterns of *L. malonatica*, *L. amalonatica* and *Citrobacter* were obtained with the four synthetic substrates. All M_F values were approximate because of variations between different runs. However, electrophoretic relationships could be established between the strains by numerous replicate assays comparing esterase bands in adjacent positions on the same gel.

Levinea malonatica

In general, two major bands were observed (Fig. 1a). The faster moving esterase band (E_1) hydrolysed α - and β -naphthyl acetates but not butyrate esters; it was inhibited by 10^{-5} M-DFP. The second band (E_2) hydrolysed α -naphthyl acetate and reacted weakly with α -naphthyl butyrate and β -naphthyl acetate; it was inhibited by 5×10^{-5} M-DFP. These two esterase bands were inactivated by 10 min treatment at 60 °C. They were accompanied by a slower moving band (E_3) which was less deeply stained by α -naphthyl acetate than the major bands, but reacted with the other three substrates. One minor fast migrating band (F), $M_F \approx 94$, was detected by β -naphthyl acetate. Bands E_2 and E_3 were seen in all 14 strains (Fig. 1b). The E_3 band showed several types of electrophoretic mobility. In

Fig. 2. Plot of logarithm esterase mobility versus gel concentration. ●—●, Esterase band E_1 of *L. malonatica* (strain 11); $10tg\alpha = 7.3$. ○—○, Esterase band E_2 of *L. malonatica* (strain 11); $10tg\alpha = 6.1$. □---□, Major esterase band of *L. amalonatica* (strain 27); $10tg\alpha = 7.4$. ■---■, Major esterase band of *Citrobacter* sp. (strain 40); $10tg\alpha = 6.0$.

strain 10, the E_1 band migrated more slowly than the E_2 band; in strains 7, 12 and 14 it was not detected. No biotype pattern was noted.

According to Hedrick & Smith (1968), the negative slope ($10tg\alpha$) of the straight line obtained by plotting $100 \log M_r$ against gel concentration is directly related to molecular size. The effect of varying the acrylamide concentration on the relative mobility of bands E_1 and E_2 (strain 11) indicated that the molecular size of esterase band E_1 was greater than that of esterase band E_2 (Fig. 2).

Levinea amalonatica

One prominent esterase band hydrolysed α -naphthyl esters and reacted weakly with β -naphthyl esters (Fig. 3a). It was inactivated by 10 min treatment at 60 °C and strongly inhibited by 10^{-6} M-DFP (Fig. 5). In addition, one minor band was often detected by α -naphthyl acetate. The major band was observed in all 14 strains (Fig. 3b). Despite their different geographical origins, strains 18, 20, 21, 23, 25 and 27 appeared identical in terms of major band mobility.

The influence of acrylamide concentration on the relative mobility of the major band of strain 27 is shown in Fig. 2.

Citrobacter

Strains of *Citrobacter* showed one major esterase band that hydrolysed α -naphthyl esters and appeared to be slightly more active towards β -naphthyl esters than that of *L. amalonatica* (Fig. 4a). This band was inactivated by heat and inhibited by 5×10^{-6} M-DFP (see Fig. 5). One minor fast migrating band (F), $M_r \approx 95$, was detected by β -naphthyl acetate. Considerable variations were observed among the 14 strains in the electrophoretic mobility of the major band (Fig. 4b). In some strains an esterase band that hydrolysed α -naphthyl acetate migrated faster than the major band. In addition, some faint bands were detected by α - or β -naphthyl acetates.

The molecular sieving effect in polyacrylamide disc electrophoresis indicated that the molecular size of the major esterase band of strain 40 was smaller than that of the major band of *L. amalonatica* strain 27 (Fig. 2).

Fig. 3. *Levinea amalonatica*. (a) Schematic representation of mobility and activity of esterase bands (strain 27). Experimental conditions, key and relative intensity of staining as in Fig. 1 legend. (b) Electrophoretic distribution of the major band, , among the 14 strains.

Fig. 4. *Citrobacter*. (a) Schematic representation of mobility and activity of esterase bands (strain 39). Experimental conditions, key and relative intensity of staining as in Fig. 1 legend. (b) Electrophoretic distribution of the major band, . The 14 strains are arranged in order of increasing mobility of this band. , Esterase band hydrolysing α -naphthyl acetate.

Fig. 5. DFP inhibition of the major esterase bands. Enzyme activity demonstrated with α -naphthyl acetate as substrate. 1, Untreated preparation; 2, with 10^{-6} M-DFP. (a) *Levinea malonatica* (strain 11); (b) *L. amalonatica* (strain 27); (c) *Citrobacter* sp. (strain 40).

Table 2. Characteristics of major bands of *L. malonatica*, *L. amalonatica* and *Citrobacter*

Organism and esterase band	Substrates* hydrolysed†				Inhibitory concn of DFP (M)	Electrophoretic mobility (M_F value)‡	Molecular sieving effect (10tg α)
	α NA	α NB	β NA	β NB			
<i>L. malonatica</i> E ₁	++++	—	+++	—	10^{-5}	66.90 ± 2.98	7.3
<i>L. malonatica</i> E ₂	++++	+	+	—	5×10^{-6}	62.42 ± 1.08	6.1
<i>L. amalonatica</i>	++++	++++	+	+	10^{-6}	70.57 ± 0.93	7.4
<i>Citrobacter</i>	++++	++++	++	++	5×10^{-6}	63.42 ± 4.12	6.0

* α NA, α -naphthyl acetate; α NB, α -naphthyl butyrate; β NA, β -naphthyl acetate; β NB, β -naphthyl butyrate.

† +++++, +++, ++, +, Intensities of esterase band; —, no activity.

‡ Mean values $\pm$ S.D.

DISCUSSION

Extracts of strains of *Levinea* and *Citrobacter* have different esterases. The individual characteristics of the four major bands are summarized in Table 2. According to the values of the negative slope from plots of $100 \log M_F$ against acrylamide concentration, the molecular sizes of the E₁ esterase band of *L. malonatica* and the only esterase band of *L. amalonatica* were similar. The same is true for the smaller E₂ esterase band of *L. malonatica* and the major esterase band of *Citrobacter*. Further studies including other enterobacteria and using reference proteins are in progress. In addition to the above four esterases, one minor band migrating near the marker dye front and reacting with β -naphthyl acetate was observed in strains of *L. malonatica* and *Citrobacter*. The mobility of this fast band is distinct for each species and different from that of *Escherichia coli* (Goullet, 1973).

On the basis of the esterase data, distinctive patterns may be established for *L. malonatica*, *L. amalonatica* and *Citrobacter*. This differentiation is consistent with the genetic divergences detected by DNA hybridization (Crosa *et al.*, 1974). The differences in the esterase patterns of *L. malonatica* and *L. amalonatica* are striking but the differences are less marked between *L. amalonatica* and *Citrobacter*, which seems to agree with the common biochemical characteristics of the last two species (Young *et al.*, 1971; Richard *et al.*, 1972).

Considerable electrophoretic variations were observed with the *L. malonatica* E₃ band and to a lesser degree with the *Citrobacter* major band. The major band of *L. amalonatica*

is less variable. Thus, with respect to carbohydrate fermentation (see Table 1) as well as esterase mobilities, *L. amalonatica* appears more homogeneous than *Citrobacter*.

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