Esterase Zymograms of *Proteus* and *Providencia*

By PH. GOULLET

*Laboratoire de Microbiologie, Faculté de Médecine
Xavier-Bichat, Université Paris VII
U.E.R. Biomédicale des Cordeliers, 75 270 P C 06, France*

(Received 20 August 1974; revised 28 September 1974)

SUMMARY

The intracellular esterases of 80 strains of *Proteus* and *Providencia* were analysed by the acrylamide-agarose zymogram technique using several synthetic substrates.

The esterase bands were classified in five main groups. The α A-esterase bands hydrolysed α -naphthyl acetate and were resistant or relatively insensitive to di-isofluoropropyl phosphate (DFP). The α B-esterase band hydrolysed both α -naphthyl acetate and α -naphthyl butyrate and were very sensitive to DFP. Both groups of esterase bands were inactivated by heat. The β A- and β B-esterase bands hydrolysed β -naphthyl acetate and were sensitive to DFP; these were distinguishable by the difference in their relative activity towards β -naphthyl butyrate and in their relative stability to heat. The $\alpha\beta$ -esterase bands hydrolysed α - and β -naphthyl acetates and α - and β -naphthyl butyrates; they were inactivated by heat and were sensitive to DFP.

The distribution of these esterase bands among the strains of *Proteus* and *Providencia* and their electrophoretic patterns established esterase profile types which correlate with the classification based on traditional bacteriological tests. The degree of inter-strain similarity in esterase pattern varied highly among species. The homogeneity of *Proteus mirabilis* and especially of *Providencia stuartii* contrasted with the heterogeneity of other species. This disparity suggests that the bacteria of the tribe Proteae have not the same degree of intra-specific differentiation in physico-chemical properties of esterases.

INTRODUCTION

Comparative studies of enzymes at the molecular level in enterobacteria were designed both to clarify the relationships between these organisms and to provide insight into the processes of molecular evolution and protein differentiation in bacteria (Bowman, Brubaker, Frischer & Carson, 1967; Baptist, Shaw & Mandel, 1969; Murphy & Mills, 1969; Cocks & Wilson, 1972).

The esterases (carboxylic ester hydrolases, EC. 3. 1. 1.), which represent a broad spectrum of enzymes with common esterolytic activity but with distinct substrate specificity and electrophoretic properties, seemed suitable material for these investigations. Preliminary research revealed that representative species of Enterobacteriaceae had differences in the specificity and electrophoretic mobility of their esterases (Goullet, 1971). The *Escherichia coli* esterases were recently characterized by the zymogram procedure (Goullet, 1973). This report is concerned with the characterization and distribution of esterase bands in *Proteus* and *Providencia*.

Table 1. *Strains used*

Organism	Strain‡	Date of Isolation	Code	Organism	Strain‡	Date of Isolation	Code		
<i>Proteus vulgaris</i>	LM3.70	1970	1	Biotype 2	LM4.70	1970	44		
	LM2.72	1972	2		LM2.71	1971	45		
	LM3.72	1972	3	Biotype 3	LM6.71	1971	46		
	LM4.72	1972	4		CR5.70	1970	47		
	FM36	1971	5		LM1.73	1973	48		
	HB70.99	1970	6	<i>Providencia alcalifaciens</i> †	Biotype 1	CR6.69	1969	49	
	HB71.29	1971	7			LM4.69	1969	50	
	HB71.35	1971	8			LM5.70	1970	51	
	HB71.63	1971	9			LM7.70	1970	52	
	HB71.64	1971	10			LM11.71	1971	53	
	HB72.67	1972	11			LM6.72	1972	54	
	HB72.68	1972	12			LM8.72	1972	55	
<i>P. mirabilis</i>	CR8.70	1970	13			Biotype 2	LM9.72	1972	56
	LM1.72	1972	14	LM7.69	1969		57		
	LM4.72	1972	15	LM8.69	1969		58		
	LM10.72	1972	16	LM6.70	1970		59		
	LM17.72	1972	17	Biotype 4	LM7.72	1972	60		
	FM30	1970	18		LM10.72	1972	61		
	HB70.38	1970	19		per	1968	62		
	HB70.39	1970	20		LM2.73	1973	63		
	HB70.42	1970	21		LM4.73	1973	64		
	HB70.43	1970	22		<i>Prov. stuartii</i> †	Biotype 5	LM1.71	1971	65
	HB72.87	1972	23				LM4.72	1972	66
	HB72.89	1972	24				LM14.72§	1972	67
<i>P. morganii</i>	LM3.70	1970	25	LM15.72			1972	68	
	LM17.72	1972	26	LM16.72§			1972	69	
	CR6.72	1972	27	LM17.72			1972	70	
	CR12.72	1972	28	FM38			1971	71	
	CR541B72	1972	29	HB71.31			1971	72	
	IP55140	—	30	HB71.32§	1971	73			
	FM39	1971	31	HB71.53	1971	74			
	HB71.28	1971	32	HB71.54	1971	75			
	HB71.34	1971	33	HB71.55	1971	76			
	HB71.43	1971	34	Biotype 6	LM4.58§	1958	77		
	HB71.46	1971	35		LM7.62§	1962	78		
	HB71.84	1971	36		LM1.65	1965	79		
<i>P. rettgeri</i> *	Biotype 1	CR1.71	1971		LM2.70§	1970	80		
		CR4.71	1971						
		FM46	1971						
		HB71.36	1971						
		HB71.37	1971						
		HB71.47	1971						
		HB71.48	1971						
				43					

* Biotypes according to Namioka & Sakazaki (1958).

† Biotypes designation following characteristics defined by Ewing (1962) and Richard (1966).

‡ LM, Collection of L. Le Minor, Institut Pasteur, Paris, France; CR, collection of C. Richard, Institut Pasteur, Tangier, Morocco; FM, Laboratory of Microbiology, Faculty of Medicine, Paris, France; HB, Laboratory of Bacteriology, Beaujon Hospital, Paris-Clichy, France; IP, collection of l'Institut Pasteur, Paris; per, Laboratory of Bacteriology, St Joseph Hospital, Paris, France. The CR, FM and HB strains were isolated from diverse materials (e.g. urine, faeces, blood, pus). The LM strains were isolated from diverse materials from various geographical locations.

§ Strain 67 isolated from human urine in Clermont-Ferrand, France; strain 69 from meat in Bourg-en-Bresse, France; strain 73 from human urine in Clichy, France; strain 77 from human faeces in Brazzaville, Africa; strain 78 from human pus in Cayenne, South America; strain 80 from human pus in Antibes, France.

METHODS

Bacteria and growth conditions. The 80 strains of *Proteus* and *Providencia* listed in Table 1 were inoculated in Fernbach flasks containing 500 ml L broth (Lennox, 1955) without glucose. Flasks were incubated at 37 °C in a reciprocating water bath shaker (Monod & Lapierre model, Etablissement Lecourt, Guyancourt, 78 France) at about 70 oscillations/min. Each strain was cultivated at least twice.

Preparation of extracts, heat denaturation, inhibition by di-iso-fluoropropyl-phosphate (DFP) and protein estimation were as described previously (Goulet, 1973).

Electrophoresis. Horizontal slab acrylamide-agarose gel electrophoresis was performed using 7 % (w/v) acrylamide and discontinuous tris-glycine buffer pH 8.7 (Uriel, 1966). To compare relative mobilities the bacterial extracts were inserted side by side into the same gel and in some experiments the order of the extracts was changed. Bromophenol blue was the marker dye. The extracts were subjected to a constant voltage gradient of 7 V/cm until the marker dye had travelled 13 cm. The M_F value was the ratio (%) of the distance moved by the esterase band to the distance moved by the dye front.

Esterase staining. The method of Uriel (1961) was employed. The substrates (0.2 mg/ml) used to detect and differentiate between the various zones of esterase activity were α -naphthyl acetate, α -naphthyl butyrate, β -naphthyl acetate and β -naphthyl butyrate (Sigma). Naphthanil diazo blue B (Dajac Laboratories, Philadelphia, Pennsylvania, U.S.A.) was the dye coupler.

RESULTS

Esterase band differentiation by acetyl esters

All the strains examined possessed at least one anodal band of strong esterase activity. Three main types of esterolytic activity were distinguished by the extent of hydrolysis of acetyl esters: (i) α -esterase bands hydrolysing preferentially α -naphthyl acetate; (ii) β -esterase bands hydrolysing preferentially β -naphthyl acetate; (iii) $\alpha\beta$ -esterase bands degrading both α - and β -naphthyl acetates (Fig. 1).

Proteus vulgaris

Two characteristic esterase bands were defined (Fig. 2*a*). The α -esterase band hydrolysed both α -naphthyl acetate and α -naphthyl butyrate. It reacted weakly with β -naphthyl acetate. This esterase was inhibited by DFP (10^{-5} M) and inactivated after 10 min at 60 °C. The β -esterase band hydrolysed both β -naphthyl acetate and β -naphthyl butyrate. It reacted very weakly with α -naphthyl acetate. This esterase was inhibited by 10^{-4} M-DFP and showed residual activity after 10 min at 60 °C. The α - and β -esterase bands were clearly visible in the 12 strains (Fig. 2*b*) except strain 4 where the α -esterase band was only faint. The α - and β -esterase bands showed several types of electrophoretic mobility. Except for strain 1, all α -esterase bands migrated faster than β -esterase bands. In Fig. 2*b* the strains are arranged in order of increasing mobility of the β -esterase bands. α -Esterase bands range from $M_F \approx 58$ to $M_F \approx 73$ and β -esterase bands from $M_F \approx 52$ to $M_F \approx 67$.

Proteus mirabilis

The strains of *P. mirabilis* were characterized by one set of α -esterase bands and a single β -esterase band (Fig. 3*a*). The three α -esterase bands hydrolysed almost only α -naphthyl acetate; the slowest-moving band was the most active. All bands disappeared after 10 min at 60 °C but remained active in the presence of 10^{-3} M-DFP. The α -bands from different

Fig. 1. Esterase band differentiation by acetyl esters. Substrates used: a, α -naphthyl acetate; b, β -naphthyl acetate. α , α -Esterase band; β , β -esterase band; $\alpha\beta$, $\alpha\beta$ -esterase band.

strains showed either fast or slow migration (Fig. 3*b*). Compared with the slow pattern, the three components of the fast pattern are displaced in a parallel fashion towards the anode. The β -esterase band has characteristics similar to those of *P. vulgaris*. All β -esterase bands of *P. mirabilis* strains exhibited electrophoretic uniformity ($M_F \approx 48$).

Proteus morganii

This was characterized by very strong esterase activity, principally located in a set of $\alpha\beta$ -esterase bands which hydrolysed α -naphthyl acetate, α -naphthyl butyrate, β -naphthyl acetate and β -naphthyl butyrate (Fig. 4*a*). The slowest-moving band was the most densely coloured with each substrate. All bands were inactivated after 10 min at 60 °C and inhibited by 10^{-3} M-DFP. One α -esterase band ($M_F \approx 92$) with two narrow anodic components exhibited activity only with α -naphthyl acetate. This band was inactivated by heat and partially inhibited by 10^{-3} M-DFP. Finally, one diffuse and slower-migrating band ($M_F \approx 16$) reacted weakly with β -naphthyl acetate. Electrophoretic variations of $\alpha\beta$ -esterase bands were observed (Fig. 4*b*).

Proteus rettgeri

In typical cases, *P. rettgeri* shows two major bands, the α -esterase band and the β -esterase band, and one set of minor bands (Fig. 5*a*). The major α -esterase band hydrolysed both α -naphthyl acetate and α -naphthyl butyrate and reacted weakly with β -naphthyl acetate. This esterase was inhibited by 10^{-5} M-DFP and inactivated after 10 min at 60 °C. The β -esterase band migrated more slowly than α -esterase and hydrolysed β -naphthyl acetate. It was weakly active towards β -naphthyl butyrate and relatively stable at 60 °C. The

Fig. 2. *Proteus vulgaris*. (a) Schematic representation of mobility and activity of esterase bands (strain 6). Substrates used: 1, α -naphthyl acetate; 2, α -naphthyl butyrate; 3, β -naphthyl acetate; 4, β -naphthyl butyrate. o, Origin. For each band only preferential hydrolysing activities are represented. The shading in Figs. 2, 3, 4, 5, 7 and 8 indicates the relative intensity of staining: $\blacksquare > \square \times \square > \square \diagup \square > \square \diagdown \square$. (b) Diagram of esterase patterns of the 12 strains arranged in order of increasing mobility of β -esterase bands. $\blacksquare \blacksquare$, α -esterase band hydrolysing both α -naphthyl acetate and α -naphthyl butyrate; $\square \square$, β -esterase band.

Fig. 3. *Proteus mirabilis*. (a) Schematic representation of mobility and activity of esterase bands (strain 15). For key, see Fig. 2. (b) Diagram of esterase patterns of the 12 strains arranged in order of increasing mobility of α -esterase bands. $\blacksquare \blacksquare$, α -esterase band hydrolysing α -naphthyl acetate; $\square \square$, β -esterase band.

Fig. 4. *Proteus morganii*. (a) Schematic representation of mobility and activity of esterase bands (strain 32). For key, see Fig. 2. (b) Diagram of esterase patterns of the 12 strains arranged in order of increasing mobility of $\alpha\beta$ -esterase bands. $\square$, α -esterase band hydrolysing α -naphthyl acetate; $\blacksquare$, $\alpha\beta$ -esterase band.

Fig. 5. *Proteus rettgeri*. (a) Schematic representation of mobility and activity of esterase bands (strain 40). For key, see Fig. 2. (b) Diagram of esterase pattern of the 12 strains arranged in order of increasing mobility of β -esterase bands. $\square$, α -esterase band hydrolysing α -naphthyl acetate; $\blacksquare$, α -esterase band hydrolysing both α -naphthyl acetate and α -naphthyl butyrate; $\square$, β -esterase band.

Fig. 6. Difference between β -esterase bands of *P. rettgeri* and of *P. vulgaris*. Substrates used: a, β -naphthyl acetate; b, β -naphthyl butyrate. R, *P. rettgeri*; V, *P. vulgaris*. 1, Untreated preparation; 2, heated at 60 °C for 10 min.

α -esterase minor bands consisted of a set of components hydrolysing α -naphthyl acetate and inactivated by heat. Each variety of esterase bands showed several types of mobility (Fig. 5*b*). The major α -esterase band was not detected in strains 37, 41 and 43. No biotype pattern was noted. Figure 6 illustrates the different behaviour of the β -esterase bands of *P. vulgaris* and *P. rettgeri*.

Providencia alcalifaciens

In typical cases four distinct bands may be recognized (Fig. 7*a*). The $\alpha\beta$ -esterase band was broad and intensely coloured. Its hydrolytic activity was similar to that of *P. morganii*; it was inactivated after 10 min at 60 °C and inhibited by 10^{-8} M-DFP. The fast-moving α -esterase band ($M_r \approx 92$) was weak and presented characteristics similar to the corresponding band of *P. morganii*. The other α -esterase band ($M_r \approx 65$) was more visible and hydrolysed both α -naphthyl acetate and α -naphthyl butyrate. It was inactivated by heat and was inhibited by 10^{-5} M-DFP. The β -esterase band was apparently similar to that of *P. rettgeri*; it was detected in all strains except strain 63 and showed two main types of mobility (Fig. 7*b*). Considerable variations were observed both in the number and in the electrophoretic mobility of other esterase bands: the fast-moving α -band is often undetected and the slower α -band showed several types of mobility; the $\alpha\beta$ -esterase band was detected in only six strains and two bands were observed in strains 56 and 61.

Providencia stuartii

Two major bands of α - and β -esterases and one or two minor bands were found (Fig. 8*a*). The α -esterase band ($M_r \approx 75$) hydrolysed α -naphthyl acetate. It disappeared after 10 min

Fig. 7. *Providencia alcalifaciens*. (a) Schematic representation of mobility and activity of esterase bands (strain 55). For key, see Fig. 2. (b) Diagram of esterase patterns of the 16 strains arranged in order of increasing mobility of the β -esterase bands. $\square$, α -esterase band hydrolysing α -naphthyl acetate; $\blacksquare$, α -esterase band hydrolysing both α -naphthyl acetate and α -naphthyl butyrate; $\square$, β -esterase band; $\blacksquare$, $\alpha\beta$ -esterase band.

Fig. 8. *Providencia stuartii*. (a) Schematic representation of mobility and activity of esterase bands (strain 72). The cathodal band is not represented. For key, see Fig. 2. (b) Diagram of esterase patterns of the 16 strains. $\square$, α -esterase band; $\blacksquare$, $\alpha\beta$ -esterase band; $\square$, β -esterase band.

Table 2. Preliminary classification of esterase bands of *Proteus* and *Providencia*

Group of esterases	Acetyl ester preferentially hydrolysed*	Butyryl ester hydrolysed†	In-activation after 10 min at 60 °C	Inhibitory concn of DFP (M)	Electrophoretic band characteristics	Organisms
α A-Esterase bands ( )	α NA	($\pm$)	—	10^{-3}	Fast-moving‡	<i>P. morganii</i> <i>Prov. alcalifaciens</i>
			—	(Resistant to 10^{-3} M)	Several	<i>P. mirabilis</i>
α B-Esterase bands ( )	α NA	++	—	10^{-5}	Single	<i>Prov. stuartii</i> <i>P. vulgaris</i> <i>P. rettgeri</i> <i>Prov. alcalifaciens</i>
β A-Esterase bands ( )	β NA	+	++	10^{-4}	Single (migrating slower than α -esterase band)	<i>P. rettgeri</i> <i>Prov. alcalifaciens</i> <i>Prov. stuartii</i>
β B-Esterase bands ( )	β NA	++	+	10^{-4}	Single (migrating slower than α -esterase-band)	<i>P. vulgaris</i> <i>P. mirabilis</i>
$\alpha\beta$ -Esterase bands ( )	Both α NA and β NA	++	—	10^{-3}	Several Single or double	<i>P. morganii</i> <i>Prov. alcalifaciens</i>

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

† α -Naphthyl butyrate for α -esterase bands, β -naphthyl butyrate for β -esterase bands, α - and β -naphthyl butyrates for $\alpha\beta$ -esterase bands. ++, Strong esterase activity; +, weak activity; $\pm$, very weak activity; —, no activity.

‡ $M_F > 90$.

at 60 °C but remained active in the presence of 10^{-3} M-DFP. The β -esterase band ($M_F \approx 50$) presented characteristics corresponding to those of *P. rettgeri* and *Prov. alcalifaciens*. One very fine minor band reacting with α - and β -naphthyl butyrates was observed. Finally, one diffuse cathodal band reacted with α - and β -naphthyl acetates.

A striking feature was that all the α - and the β -esterase bands presented electrophoretic uniformity with respect to one another (Fig. 8b). To confirm these results, the extracts of strains 67, 69, 73, 77, 78 and 80 (for details of their origins see Table 1) were analysed by acrylamide–agarose electrophoresis in a citrate phosphate buffer system pH 7 (Cocks & Wilson, 1972). Under these conditions the α - and β -esterase bands also presented electrophoretic uniformity (α -bands, $M_F \approx 46$; β -bands, $M_F \approx 27$). Thus despite distinct biotypes and origins, the *Prov. stuartii* strains appeared practically identical in terms of the α - and β -esterase band mobilities.

DISCUSSION

The results obtained by acrylamide–agarose gel electrophoresis and subsequent enzyme staining of extracts from 80 strains of *Proteus* and *Providencia* demonstrated different types of esterase bands.

On the basis of their differential hydrolytic activity, their sensitivity to heat or to DFP and their electrophoretic pattern, a preliminary classification may be proposed (Table 2). The α -esterase bands which hydrolyse α -naphthyl acetate preferentially and are inactivated by heat may be clearly separated into two principal groups: (i) the α A-esterase bands which have a very weak activity or no detectable activity towards α -naphthyl butyrate and

which are resistant or relatively insensitive to DFP; (ii) the α B-esterase bands which possess strong activity towards α -naphthyl butyrate and are highly sensitive to DFP.

The β -esterase bands preferentially hydrolysing β -naphthyl acetate and inhibited by 10^{-4} M-DFP may also be divided into two groups: (i) the β A-esterase bands which react weakly with β -naphthyl butyrate and remain relatively stable after 10 min at 60 °C; (ii) the β B-esterase bands which are more active towards β -naphthyl butyrate but less heat-stable.

The α B-esterase bands of *P. morganii* and *Prov. alcalifaciens* show a high and broad hydrolysing activity. Under the experimental conditions employed these two esterase bands are indistinguishable by their specificity and by their heat and DFP sensitivity but they differ by electrophoretic pattern.

Major esterase band profiles permitted species identification of *P. vulgaris*, *P. mirabilis*, *P. morganii* and *Prov. stuartii*. It is more difficult to differentiate between *P. rettgeri* and *Prov. alcalifaciens* when the α B-esterase band of this latter organism is not detected. Separation could be made eventually by comparing esterase band mobilities and by examining some fainter bands.

The intra-species similarities of esterase patterns vary considerably. The homogeneity of *P. mirabilis* and especially of *Prov. stuartii* contrasts with the heterogeneity of other species. This disparity suggests that the bacteria of the tribe Proteae have not the same degree of intra-specific differentiation in physico-chemical properties of esterases.

The author thanks Madame Colette Gaillard for technical assistance.

REFERENCES

- BAPTIST, J. N., SHAW, C. R. & MANDEL, M. (1969). Zone electrophoresis of enzymes in bacterial taxonomy. *Journal of Bacteriology* **99**, 180–188.
- BOWMAN, J. E., BRUBAKER, R. R., FRISCHER, H. & CARSON, P. E. (1967). Characterization of Enterobacteria by starch-gel electrophoresis of glucose-6-phosphate dehydrogenase and phosphogluconate dehydrogenase. *Journal of Bacteriology* **94**, 544–551.
- COCKS, G. T. & WILSON, A. C. (1972). Enzyme evolution in the Enterobacteriaceae. *Journal of Bacteriology* **110**, 793–802.
- EWING, W. H. (1962). The tribe Proteae: its nomenclature and taxonomy. *International Bulletin of Bacteriological Nomenclature and Taxonomy* **12**, 93–102.
- GOULLET, PH. (1971). Caractérisation et répartition moléculaire des activités estérases de diverses Enterobacteriaceae. *Comptes rendus hebdomadaire des séances de l'Académie des sciences* **D273**, 1884–1887.
- GOULLET, PH. (1973). An esterase zymogram of *Escherichia coli*. *Journal of General Microbiology* **77**, 27–35.
- LENNOX, E. S. (1955). Transduction of linked genetic characters of the host by bacteriophage P1. *Virology* **1**, 190–206.
- MURPHY, T. M. & MILLS, S. E. (1969). Immunochemical and enzymatic comparisons of the tryptophan synthase α subunits from five species of Enterobacteriaceae. *Journal of Bacteriology* **97**, 1310–1320.
- NAMIOKA, S. H. & SAKAZAKI, R. (1958). Étude sur les *Rettgerella*. *Annales de l'Institut Pasteur* **94**, 485–499.
- RICHARD, C. (1966). Caractères biochimiques des biotypes de *Providencia*; leurs rapports avec le genre *Rettgerella*. *Annales de l'Institut Pasteur* **110**, 105–114.
- URIEL, J. (1961). Caractérisation des cholinestérases et d'autres estérases carboxyliques après électrophorèse et immunoélectrophorèse en gélose (application à l'étude des estérases du sérum humain normal). *Annales de l'Institut Pasteur* **101**, 104–119.
- URIEL, J. (1966). Méthode d'électrophorèse dans des gels d'acrylamide-agarose. *Bulletin de la Société de chimie biologique* **48**, 969–982.