Comparative Electrophoretic Profiles of Esterases, and of Glutamate, Lactate and Malate Dehydrogenases, from *Aeromonas hydrophila*, *A. caviae* and *A. sobria*

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Esterases, and glutamate, lactate and malate dehydrogenases of 64 *Aeromonas hydrophila*, *A. caviae* and *A. sobria* strains, were analysed by polyacrylamide agarose gel electrophoresis and by thin layer isoelectrofocusing. On the basis of the isoelectric points of malate dehydrogenase from the three species and the mobility of lactate dehydrogenase from *A. sobria*, 8 species specific zymotypes were defined: three for *A. hydrophila* strains, three for *A. caviae* strains and two for *A. sobria* strains. These zymotypes correlated with previously established DNA hybridization groups. The other electrophoretic data were found to be less useful for distinction between *A. hydrophila* and *A. sobria* strains, but supported differentiation into zymotypes for *A. caviae* strains. The two-dimensional electrophoretic profile established by plotting isoelectric point against electrophoretic mobility of the major esterase illustrated the degree of enzyme polymorphism among the strains of the three species. Variation in electrophoretic patterns within *A. hydrophila* and *A. caviae* might provide useful epidemiological markers.

INTRODUCTION

Motile *Aeromonas* strains are widely distributed in nature, especially in water (Hazen *et al.*, 1978), including drinking water (Le Chevallier *et al.*, 1982). These bacteria have been implicated in various diseases in a wide range of poikilothermic animals. In man, they cause infections of wounds exposed to contaminated water, and severe septicaemia resulting from intestinal contamination in immunocompromised patients (Wolff *et al.*, 1980; Picard *et al.*, 1984).

Aeromonas hydrophila, *A. caviae* and *A. sobria* were clearly separated on the basis of both numerical analysis of characters (Popoff & Veron, 1976) and DNA hybridization (Popoff *et al.*, 1981). Each of these species contained several hybridization groups: three for *A. hydrophila*, three for *A. caviae* and two for *A. sobria*. These groups were biochemically indistinguishable from one another within each species.

The aim of this work was to evaluate isoelectrofocusing and polyacrylamide agarose gel electrophoresis of esterases, and of glutamate, lactate and malate dehydrogenases, for the interspecies and intraspecies differentiation of motile aeromonads.

METHODS

Strains and cultivation. The 64 test strains, including reference strains hybridized by Popoff *et al.* (1981) and fresh strains isolated at Beaujon hospital, are listed in Table 1. Bacteria were inoculated in Fernbach flasks containing 500 ml L broth (Lennox, 1955) without glucose. Flasks were vigorously shaken for 18 h at 30 °C in a reciprocating water bath shaker at about 70 oscillations min⁻¹. Each strain was cultivated at least twice.

Abbreviations: CE, conventional electrophoresis; DFP, di-isopropyl fluorophosphate; GDH, glutamate dehydrogenase; IEF isoelectrofocusing; LDH, lactate dehydrogenase; MDH, malate dehydrogenase; M_F , electrophoretic mobility; pI, isoelectric point.

Table 1. *Strains examined*

Organism	Code	Strain*	Code	Strain*
<i>A. hydrophila</i>	1	HB 92	20	HB 176
	2	PV 218 ^b	21	HB 186
	3	HB 27	22	HB 18
	4	PV 214 ^b	23	HB 56
	5	PV 216 ^b	24	HB 164
	6	PV 219 ^b	25	PV 213 ^d
	7	HB 2340	26	PV 212 ^d
	8	HB 29	27	HB 2374
	9	HB 93	28	HB 22
	10	HB 91	29	HB 2376
	11	HB 42	30	PV 543 ^d
	12	PV 314 ^c	31	HB 165
	13	HB 99	32	HB 2393
	14	PV 235 ^c	33	HB 2344
	15	PV 316 ^c	34	HB 16
	16	PV 201 ^c	35	HB 2
	17	HB 59	36	HB 2392
	18	HB 63	37	HB 55
	19	HB 182	38	HB 2380
<i>A. caviae</i>	39	PV 239 ^e	48	PV 238 ^e
	40	HB 72	49	HB 101
	41	HB 175	50	HB 102
	42	HB 88	51	PV 544 ^f
	43	HB 67	52	PV 268 ^f
	44	HB 2395	53	PV 247 ^f
	45	HB 77	54	PV 545 ^f
	46	PV 230 ^e	55	HB 96
	47	PV 222 ^e	56	PV 546 ^g
<i>A. sobria</i>	57	PV 208 ^h	61	PV 243 ⁱ
	58	PV 228 ^h	62	PV 236 ⁱ
	59	PV 215 ^h	63	PV 224 ⁱ
	60	PV 217 ^h	64	PV 209 ⁱ

* PV, reference strains examined by Popoff & Veron (1976), and subsequently hybridized by Popoff *et al.* (1981); HB, fresh strains isolated at Beaujon hospital, Clichy, France; *b-i*, hybridization groups 218, 316, 212, 239, 545, 546, 208 and 224 respectively. Others not determined.

Electrophoretic analysis. Preparation of extracts, inhibition by di-isopropyl fluorophosphate (DFP), heat inactivation, conventional electrophoresis (CE), isoelectrofocusing (IEF), estimation of electrophoretic mobility (M_F value), establishment of correspondence of bands characterized in the two electrophoretic techniques and esterase staining were all as described previously (Goullet, 1980; Goullet & Picard, 1984). The M_F values were used only comparatively. Glutamate dehydrogenase (GDH) was stained according to the method of Baptist *et al.* (1969) and lactate dehydrogenase (LDH) and malate dehydrogenase (MDH) according to the method of Siciliano & Shaw (1976).

RESULTS

Characterization of enzyme bands

Isoelectrofocusing. Good resolution with distinct banding was obtained only for MDH and one esterase (Figs 1 to 3); the isoelectric point (pI) could not be determined for GDH because of poorly defined banding. The other enzymes were not detected. With a narrow pH gradient from pH 5 to pH 8, MDH gave a sharp and well-stained main band, used for pI determination, and 1 to 3 anodic minor bands. With a narrow pH gradient from pH 2.5 to pH 5, and α -naphthyl acetate as substrate, an intensely coloured esterase band (used for pI determination) accompanied by an anodic satellite band was observed.

Polyacrylamide agarose gel electrophoresis. This method was more sensitive than IEF for detecting the enzymes; satisfactory banding patterns were obtained for all enzymes analysed

Fig. 1. Zymograms of *A. hydrophila*. (a) Strain 5 (hybridization group 218) from zymotype H₁; (b) strain 12 (hybridization group 316) from zymotype H₂; (c) strain 30 (hybridization group 212) (ATCC 7946) from zymotype H₃. For IEF of MDH, the main enzyme band focused as a sharp band which broadened rapidly during the staining.

(Figs 1 to 3). The MDH bands varied in thickness among the strains, the better-defined cathodic limit was chosen for determination of relative mobility. LDH gave 1 to 3 bands, the most intensely stained one being used for mobility determination. GDH gave a single band or two distinctly separated bands. Three kinds of esterases, designated as major (M), fast (F) and slow (S), were differentiated by hydrolytic activity and by sensitivity to heat and to DFP. The band M, which was identified as the esterase obtained by IEF, consisted of a sharp and intensely stained band which hydrolysed α - and β -naphthyl acetates and butyrates. This esterase remained active after 10 min treatment at 60 °C and was resistant to 10⁻³ M-DFP. Esterase F was resolved into 1 to 4 bands; the mobility of the anodic band was chosen for relative mobility determination. This esterase hydrolysed α - and β -naphthyl acetates and was inactivated by 10⁻³ M-DFP and by heat treatment. Esterase S, which was similar in hydrolysing activities and sensitivity to DFP and to heat to esterase F, always migrated more slowly than esterase M. LDH, GDH, MDH, esterase M and esterase F (except for strain 56) were detected in all strains.

Ranges of pI and mobilities of enzyme bands among the strains

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 in several ways. For pI values, all the samples coalesced as a single line after IEF when they were loaded at different positions on the gel, and samples remained in the same line with no additional bands being observed when IEF was continued for an additional 30 min period to condense the focused region. All values of both pI and M_F were tested by comparing

Fig. 2. Zymograms of *A. caviae*. (a) Strain 47 (hybridization group 239) from zymotype C_1 ; (b) strain 54 (hybridization group 545) from zymotype C_2 ; (c) strain 56 (hybridization group 546) from zymotype C_3 . For IEF of MDH, the main enzyme band focused as a sharp band which broadened rapidly during the staining.

Fig. 3. Zymograms of *A. sobria*. (a) Strain 58 (hybridization group 208) from zymotype S_1 ; (b) strain 63 (hybridization group 224) from zymotype S_2 . For IEF of MDH, the main enzyme band focused as a sharp band which broadened rapidly during the staining.

Table 2. Ranges of isoelectric points (pI) and mobilities (M_F) of enzyme bands in strains of *A. hydrophila*, *A. caviae* and *A. sobria*

Zymotype . . .	<i>A. hydrophila</i>			<i>A. caviae</i>			<i>A. sobria</i>	
	H ₁	H ₂	H ₃	C ₁	C ₂	C ₃	S ₁	S ₂
Strain code . . .	1-11	12-24	25-38	39-48	49-55	56	57-60	61-64
Malate dehydrogenase								
pI range	5.8	6.1	6.15	5.65	5.75	6.2	5.7	5.7
M_F range	20*	25*	25*	30†	30	25	29, 30	29, 30
No. of variants	2	3	2	1	1	1	2	2
Lactate dehydrogenase								
M_F range	25-30	23-28	24-27	23	23-25	26	17-19	26-29
No. of variants	5	4	5	1	3	1	2	2
Glutamate dehydrogenase								
M_F range	19-27	19-28	18-28	19-27‡	19-24	20	23-27	24-26
No. of variants	8	10	9	7	5	1	3	3
Major esterase (M)								
pI range	3.95-4.2	3.85-4.45	4.1-4.6	4.3§	3.95-4.2	4.5	4.4-4.45	4.2-4.9
No. of variants	6	5	8	2	4	1	2	2
M_F range	33-43	30-45	29-46	35-40	39-45	31	24-31	24-37
No. of variants	9	11	8	6	6	1	3	3
Fast esterase (F)								
M_F range	63-71	53-70	61-69	54-65¶	50-51	-	68-72	67-69
No. of variants	13	11	13	4	3	0	3	3

* For the most frequently recovered variant.

† Accompanied by a second band at $M_F \approx 26$.

‡ Including the two bands (see text).

§ Except strain 39 (pI = 4.25).

|| M_F range of the faster bands.

¶ This esterase migrated as a single band with exception of strain 55.

each new strain with reference strains in all electrophoretic runs; 4 to 12 extracts of the same strain were tested and had the same pI in IEF and the same electrophoretic relationship in CE. Table 2 shows the pI and M_F values of enzymes detected in the strains.

Malate dehydrogenase. On the basis of the pI values of this enzyme, which ranged from 5.65 to 6.2, seven zymotypes were defined. (i) *A. hydrophila* strains (Fig. 1): zymotype H₁ (11 strains including four reference strains of hybridization group 218) with a pI of 5.8; zymotype H₂ (13 strains including four reference strains of hybridization group 316) with a pI of 6.1; zymotype H₃ (14 strains including three reference strains of hybridization group 212) with a pI of 6.15. (ii) *A. caviae* strains (Fig. 2): zymotype C₁ (10 strains including four reference strains of hybridization group 239) with a pI of 5.65; zymotype C₂ (seven strains including four reference strains of hybridization group 545) with a pI of 5.75; zymotype C₃ (strain 546 with a pI of 6.2). (iii) *A. sobria* strains (Fig. 3): zymotype S with a pI of 5.7. MDH had identical mobilities within each zymotype of *A. hydrophila* and *A. caviae* defined by IEF, except for strains 1 and 11 (zymotype H₁), strains 3 and 16 (zymotype H₂) and strain 37 (zymotype H₃); in addition, strains of zymotype C₁ gave two distinct bands. In *A. sobria* strains, MDH had two mobilities.

Lactate dehydrogenase. Strains of *A. hydrophila* and *A. caviae* had LDH which had identical mobility in strains of zymotype C₁ but variations among the strains of zymotypes C₂ and H₁, H₂ and H₃. Strain 546 (zymotype C₃) was distinguished from other *A. caviae* strains by faster mobility of LDH. In *A. sobria* strains, LDH had two clusters of mobility (Fig. 3) which defined two zymotypes: zymotype S₁ ($M_F \approx 17$ and $M_F \approx 19$) for the four reference strains of hybridization group 208, and zymotype S₂ ($M_F \approx 26$ and $M_F \approx 29$) for the four reference strains of hybridization group 224.

Glutamate dehydrogenase. In strains of *A. hydrophila* and *A. sobria*, GDH had a high electrophoretic polymorphism. In *A. caviae*, GDH had a single band in strains of zymotypes C₂ and C₃ and, in strains of zymotype C₁, one anodic band ($M_F \approx 27$) (except for two strains) was accompanied by another electrophoretically variable band.

Fig. 4. Two-dimensional electrophoretic profile of esterase M from *A. hydrophila*, *A. caviae* and *A. sobria* strains. Each allozyme is defined by pI and M_F . ●, *A. hydrophila*; ▲, *A. caviae*; ■, *A. sobria*.

Esterases. The pI value of esterase M varied from 3.85 to 4.9 (Figs 1 to 3). The strains of each zymotype defined by the pI of MDH had esterases which had 4 to 8 distinct pI values, with the exception of strains of zymotype C₁, the esterases of which had only two distinct pI values. The last one of these (pI = 4.3) was identical for nine strains of this zymotype. CE revealed high variability of esterases M and F. Esterase S was detected in only some strains of the three species. A two-dimensional electrophoretic profile (Goullet & Picard, 1985), established by plotting pI against electrophoretic mobility for esterase M (Fig. 4), showed that several mobility variants fall into a single pI, and that conversely, several pI variants occurred for a single mobility. Thus the number of allozymes resolved by this procedure (40) was considerably higher than the number resolved by CE (19) or by IEF (18).

Copies of diagrammatic representations of isoelectric focusing patterns of malate dehydrogenases and of esterases, and electrophoretic patterns of lactate, glutamate and malate dehydrogenases and esterases from all the test strains, have been deposited with the British Library Lending Division, Boston Spa, Yorkshire LS23 7BQ, UK, as Supplementary Publication no. SUP 28021 (11 pp.). (Copies may be obtained from the BLLD on demand; wherever possible, requests should be accompanied by prepaid coupons, held by many university and technical libraries and by the British Council.)

DISCUSSION

Comparison of data obtained by IEF and CE showed that the latter technique was more sensitive for both detection of enzyme activity and evaluation of electrophoretic variability. The failure to detect enzyme activity (LDH, GDH, esterases F and S) by IEF has already been observed for some esterases produced by *Yersinia* strains (Goullet & Picard, 1984), and inactivation of enzymes at acidic or basic pI values, or interactions between carrier ampholytes and proteins, could offer an explanation.

On the basis of pI values of MDH produced by strains of the three *Aeromonas* species, and M_F values of LDH produced by strains of *A. sobria*, eight species-specific zymotypes were defined. These zymotypes correlated respectively with the eight biochemically indistinguishable groups previously defined on the basis of DNA hybridization (Popoff *et al.*, 1981). The three zymotypes of *A. hydrophila*, and the zymotypes C₁ and C₂ of *A. caviae*, were observed in fresh isolates as well as in reference hybridized strains. The other electrophoretic data were less useful for distinction between *A. hydrophila* and *A. sobria* strains but supported the individuality of the three zymotypes of *A. caviae*. Strains of zymotype C₁ were distinguished from strains of the other *Aeromonas* zymotypes by two bands of MDH ($M_F \approx 26$ and $M_F \approx 30$), and from strains of zymotype C₂ by the electrophoretic homogeneity of LDH, the anodic band of GDH and the higher pI values of esterase M (Table 2). Strain 56 (zymotype C₃) was differentiated from the other *A. caviae* strains by a distinct pI value of esterase M (4.5), by distinct mobilities of LDH,

MDH and esterase M, and by the absence of esterase F. Consequently this particular enzyme profile is in agreement with the possibility of this strain representing a third hybridization group of *A. caviae* (Popoff *et al.*, 1981). Strains of *A. hydrophila* had the highest variability whereas strains of *A. caviae* were electrophoretically less variable. Strains of zymotype C₁ were the most homogeneous set encountered in this study. Strains of *A. sobria* appeared to be polymorphic but the number of strains examined was too low to be considered completely representative of the species. The two-dimensional electrophoretic profile of esterase M refined considerably the degree of enzyme polymorphism among the strains of the three species, and showed that for the same pI value, the mobilities of the enzymes from *A. caviae* strains were generally greater than those from *A. hydrophila* strains (Fig. 4).

Methods of typing *Aeromonas* for epidemiological purposes are required because of the prevalence of these bacteria in the aquatic environment (Hazen *et al.*, 1978), and their capacity to cause infections, particularly in immunocompromised hosts (Wolff *et al.*, 1980; Cookson *et al.*, 1981). The high degree of enzyme polymorphism among the strains of *A. hydrophila* and *A. caviae* might provide epidemiological markers. Thus IEF of MDH and esterase could be proposed as a first screening, which could be subsequently refined by CE of esterases, and eventually of LDH and GDH. Electrophoretic typing of esterases, which has the advantage of detecting three kinds of enzymes using one substrate (α -naphthyl acetate) in the same electrophoretic run, has recently demonstrated the epidemiologic complexity of nosocomial infections caused by *A. hydrophila* and *A. caviae* (Picard & Goullet, 1984).

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