Esterases and Phosphatases of *Escherichia coli* Serotypes Isolated from the Pig

By D. B. STEWART AND D. J. STEWART*

*Ministry of Agriculture for Northern Ireland and The Queen's University,
Elmwood Avenue, Belfast, Northern Ireland*

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SUMMARY

Escherichia coli isolates grew poorly in a phosphate-deficient medium. The presence of inorganic phosphate repressed the formation of alkaline phosphatase enzyme but did not affect the formation of acid phosphatase enzyme.

Twenty-two non-enteropathogenic *Escherichia coli* isolates exhibited 16 esterase isoenzyme patterns while 15 enteropathogenic isolates exhibited three patterns. No relationship was found between enteropathogenicity and acid or alkaline phosphatase isoenzyme patterns.

INTRODUCTION

Jensen (1899) was the first to suggest that *Escherichia coli* was associated with diarrhoea in piglets. Since then, many investigators have considered *E. coli* to be involved in enteritis and bowel oedema of pigs.

Sojka (1965) reviewed the literature and concluded that relatively few serotypes were associated with bowel oedema. These serotypes occurred in all countries where the disease was a problem, were mostly haemolytic, appeared to be host-specific and were widely distributed in the environment of the pig. Sojka listed five serotypes, rarely found with bowel oedema, which were most frequently encountered in outbreaks of enteritis, particularly in the young piglet. Two serotypes were frequently found in outbreaks of both postweaning enteritis and bowel oedema.

Moon, Sorensen & Sautter (1966) found the dilatation of intestinal segments of newborn pigs to be a reliable test for enteropathogenicity of *Escherichia coli*. They confirmed the findings of Taylor, Maltby & Payne (1958) that not all strains of a particular serotype commonly associated with enteritis are enteropathogenic, and indicated that the inability of a strain to dilate an intestinal loop was not the result of inability to survive and grow in the loop. Smith & Halls (1967) also found that the reaction of dilatation produced by a strain of *E. coli* in a ligated segment of the small intestine provided useful information on the ability of the strain to produce diarrhoea. They indicated that two essential properties of an enteropathogenic strain were that it must produce enterotoxin and that it must be able to proliferate in the anterior small intestine.

Taylor & Bettelheim (1966) noted that chloroform-killed suspensions of enteropathogenic *Escherichia coli* lost their ability to dilate ligated segments of rabbit intestine and that there was a simultaneous loss of esterase activity. These workers did,

* Present address: Heriot-Watt University, Chambers Street, Edinburgh, Scotland.

however, point out that esterase activity was not responsible for the dilatation effect. In the present paper esterase and phosphatase isoenzyme patterns of *E. coli* serotypes are studied. This is part of a programme of work designed to differentiate further biotypes of *E. coli*.

METHODS

Organisms. The strains used are listed in Table 1, together with details of their antigenic structure and reaction in ligated segments of pig intestine.

Media. The media were Oxoid nutrient broth and a basal medium containing

Table 1. *Strains used, antigenic structure and dilatation reactions*

Strain no.	Original numbering*	Antigenic structure	Source	Dilatation reaction in ligated pig intestine
S1	—	O 34: K?	Pig	—
S2	—	Untypable	Pig	—
S3	—	Untypable	Pig	—
S4	—	O 13: K 11	Pig	—
S5	—	Untypable	Sow	—
S6	E4	O 139: K 82	Pig	—
S7	E145	O 141: K 85a, c	Pig	+
S8	—	O 31: K?	Pig	—
S9	E68, type 1	O 141: K 85a, b, K 88a, b	Pig	+
S10	—	O?: K?	Milk	—
S11	E38	O?: K?	Pig	—
S12	E57	O 138: K 81	Pig	+
S13	E65	O 45: K?	Pig	—
S14	M434	O?: K?	Pig	—
S15	G7	O 8: K 87, K 88	Pig	+
S16	G11	O 18: K?	Pig	—
S17	G515	O 54: K?	Pig	—
S18	P1	O 141: K 85a, b → K 85a, c	Pig	+
S19	P11	O?: K?	Pig	+
S20	P16	O 9: K?	Pig	+
S21	P105	O 138: K 81	Pig	+
S22	P114	O 147: K 89, K 88a, c	Pig	+
S23	P7	Untypable	Pig	—
S24	P13	Untypable	Pig	—
S25	P20	Untypable	Pig	—
S26	P32	Untypable	Pig	—
S27	P45	Untypable	Pig	—
S28	—	O?: K?: K 88a, c	Pig	+
S29	NCTC8007	O 111: B4: H2	Human	—
S30	NCTC8603	O 55: B5: H 6	Human	—
S31	P27	O 141: K 85a, b → K 85a, c	Pig	+
S32	P87	O 141: K 85a, b → K 85a, c	Pig	+
S33	P100	O 141: K 85a, b → K 85a, c	Pig	+
S34	P101	O 141: K 85a, b → K 85a, c	Pig	+
S35	P23	O 141: K 85a, b → K 85a, c	Pig	—
S36	P205	O 45: K?, K 88a, c	Pig	+
S37	P206	O 45: K?	Pig	—

? Antigen not determined.

→ Symbolizes variation from K 85b to K 85c.

* Strains with prefixes E, M or G were obtained from Dr W. J. Sojka. Strains with prefix P were obtained from Dr H. Williams Smith. Strains with prefix NCTC were obtained from the National Collection of Type Cultures, Central Public Health Laboratory, Colindale, London. All other strains were isolated by the authors.

0.12 M-tris, 0.08 M-NaCl, 0.02 M-KCl, 0.08 M-NH₄Cl, 0.003 M-Na₂SO₄, 0.001 M-MgCl₂, 2×10^{-4} M-CaCl₂, 2×10^{-6} M-ZnCl₂ and 0.03 M-glucose. This basal medium was adjusted to pH 7.5 and supplemented with Na₂HPO₄ to give final concentrations of 2, 4, 6, 8, 10 and 12 μ g. inorganic phosphorus (Pi)/ml. These supplemented basal media were designated 2 Pi, 4 Pi, etc. The final concentration of Pi in each medium was determined colorimetrically by the method of Sumner (1944). Oxoid nutrient broth, basal medium and Pi-supplemented media were dispensed in 200 ml. amounts in 1 l. Erlenmeyer flasks and autoclaved at 15 lb/in.² for 15 min.

Culture of organisms. The media were inoculated with washed cell suspensions and incubated in an orbital shaking incubator at 37° for 24 h. The cells were harvested by centrifugation and washed twice with distilled water. Cell yields were measured turbidimetrically.

Preparation of cell extracts. Washed cells were suspended in distilled water to give approximately 50 mg. dry wt bacteria/ml. and sonicated using a Dawe 'Soniprobe' (Dawe Instruments Ltd, Western Avenue, London W. 3). After 5 min. sonic treatment in an ice bath, the preparations were clarified by centrifugation.

Tryptophan levels in the extracts were determined by the method of Opieńska-Blauth, Chareziński & Berbeć (1963). On this basis the extracts were diluted with distilled water to give protein concentrations of about 300 μ g. tryptophan/ml.

Determination of optimum pH for esterase activity. The following reaction mixtures ranging in pH value from 5.3 to 7.3 were incubated at 37° for 1 h.: cell extract of strain S17, 0.1 ml.; tris-maleate buffer solutions (0.1 M), 0.9 ml.; α -naphthyl acetate (0.25 g. in 100 ml. 40% (v/v) acetone), 1 ml. After incubation the reaction was stopped and the colour developed by adding the following solutions: NaOH, 0.1 N, 5 ml.; Fast Red B salt (0.02 g. in 100 ml. 0.1 N-HCl), 1 ml. The reaction mixtures were allowed to stand at room temperature for 30 min. and the extinctions of the blue colour which developed were read at 590 nm. in a Unicam SP600 spectrophotometer (Unicam Instruments, Cambridge) against control cuvettes which contained all reagents except cell extract. The optimum substrate concentration was 1.25 mg. α -naphthyl acetate/ml. and the reaction curve was linear over a time interval of 90 min. with cell extracts containing 200 to 500 μ g. tryptophan/ml. The amounts of α -naphthol released from the buffered substrate solutions were derived from a standard curve for α -naphthol.

Determination of optimum pH for phosphatase activity. Phosphatase activity in cell extracts of strain S17 grown in Oxoid nutrient broth and in medium 8 Pi was determined by measuring the amount of *p*-nitrophenol (NP) released from *p*-nitrophenylphosphate (NPP). The following reaction mixtures ranging in pH value from 2.5 to 10.5 were incubated at 37° for 30 min.: cell extract, 0.1 ml.; 0.4% (w/v) NPP in 'B.T.L. Universal' buffer solutions (Hopkin & Williams Ltd, Chadwell Heath, Essex), 1.9 ml. After incubation the yellow colour of released NP was developed by the addition of 1 ml. 10% (w/v) NaOH and the volume of each reaction mixture was made to 10 ml. with distilled water. The extinctions at 405 nm. were measured against control cells which contained all reagents except cell extract. The optimum substrate concentration was 4 mg. NPP/ml. and the reaction curve was linear over a time interval of 50 min. with cell extracts containing 200 to 500 μ g. tryptophan/ml. Using standard curves the phosphatase activity was expressed as μ moles NP released/mg. cell extract tryptophan/h.

Determination of phosphatase activity in cells grown in basal medium and Pi supple-

mented media. Phosphatase activity at pH 4.0 and pH 9.5 was determined in extracts of strain s17 grown in basal medium and in media 2 to 12 Pi as described above.

Gel electrophoresis. Starch gels were prepared from hydrolysed starch (Connaught Medical Research Laboratories, Toronto, Canada). The tris-citrate buffer for the gel and the sodium borate buffer for the electrode vessels were prepared according to Poulik (1957). Electrophoresis was carried out in a horizontal tank with a constant current of 30 mA (1.6 mA/cm. gel width).

Esterase stain. Gel slices were flooded with freshly prepared substrate solution containing: 1% (w/v) α -naphthyl acetate in 50% (v/v) acetone, 2 ml.; Fast Blue B salt, 50 mg.; tris-maleate buffer, 0.1 M, pH 6.4, 50 ml. (Lawrence, Melnick & Weimer, 1960).

Phosphatase stain. Gel slices were flooded with freshly prepared substrate solutions containing: (a) α -naphthyl phosphate, 10 mg.; Fast Blue B salt, 10 mg.; tris-HCl buffer, 0.1 M, pH 9, 10 ml.; (b) α -naphthyl phosphate, 10 mg.; Fast Blue B salt, 10 mg.; tris-HCl buffer, 0.1 M, pH 7, 10 ml.; (c) α -naphthyl phosphate, 10 mg.; Fast Scarlet GG, 10 mg., 'B.T.L. Universal' buffer, pH 4, 10 ml. Prior to treatment with last solution, the gel slices were rinsed with glacial acetic acid followed by 'B.T.L. Universal' buffer, pH 4.

Fig. 1

Fig. 2

Fig. 1. Effect of the concentration of inorganic phosphorous (Pi) in the growth medium on growth and phosphatase production of *Escherichia coli* s17. Acid phosphatase activity measured at pH 4.0 (○) and alkaline phosphatase activity measured at pH 9.5 (●). Total growth (open blocks) determined after 24 h. at 37° using a nephelometer (10 nephelometer units = 9.4 mg. dry wt bacterial/ml.).

Fig. 2. Effect of pH on phosphatase activity of *Escherichia coli* strain s17 grown in medium 8 Pi (●) and in nutrient broth (○), and on esterase activity of the same strain after growth on nutrient broth (Δ).

RESULTS

Effect of Pi on growth. *Escherichia coli* s17 grew poorly in the basal medium. The effect of supplementing the basal medium with various levels of Pi is shown in Fig. 1.

Optimum pH for esterase activity. The pH value for optimum esterase activity of strain s17 using α -naphthyl acetate as substrate was 6.4 (Fig. 2).

Optimum pH for phosphatase activity. In extracts of cells grown in medium 8 Pi the optimum for phosphatase activity was pH 9.5. The curve (Fig. 2) also showed a shoulder

of activity at pH 7.0 and a smaller peak at pH 4.0. Phosphatase activity in extracts of cells grown in 'Oxoid' nutrient broth which contained 84 μ g. Pi/ml. was much smaller than that in extracts of cells grown in medium 8 Pi. With extracts of such cells the optimum for activity was pH 4.0 (Fig. 2).

Effect of Pi on production of phosphatase enzymes. Phosphatase activities at pH 4.0 and 9.5 in extracts of cells grown in media containing various levels of Pi are shown in Fig. 1.

Fig. 3. Numbering of esterase isoenzymes. Isoenzyme E4, which was just visible on gels, was designated with suffix *a*. Isoenzymes which moved slightly faster than E4 or E5 were designated E4*b* or E5*b*, and isoenzymes which moved slightly faster than E4 and were just visible were designated E4*ab*. Figure shows relative positions of all the esterase isoenzymes detailed in this study.

Esterase isoenzyme patterns. Esterase isoenzymes were numbered in decreasing order of negative charge (Fig. 3). The esterase isoenzyme patterns of the 37 strains of *Escherichia coli*, grown in nutrient broth, are recorded in Table 2.

Phosphatase isoenzyme patterns. Gels containing extracts of cells grown in medium 8 Pi stained readily when flooded with substrate solutions buffered at pH 9 and 7. Identical isoenzyme patterns appeared with both solutions. These isoenzymes were designated alkaline phosphatases and were numbered P1 to P10 in decreasing order of negative charge. The alkaline phosphatase isoenzymes of *Escherichia coli* strains s1 to s30 are recorded in Table 3.

Table 2. *Esterase isoenzymes of strains of Escherichia coli*

Iso-enzyme no.*	Strain no. (s)																																					
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	
E1	.	.	.	.	.	.	.	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	+	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.
E2	.	.	.	.	.	.	.	.	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
E3	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.
E4 ^a	.	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
E4 ^{ab}	.	.	.	.	.	.	.	.	+	.	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
E4	.	.	.	.	.	.	+	+	.	.	.	+	+	.	+	+	+	+	+	+	+	+	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
E5 ^b	.	.	.	.	.	.	.	+	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
E5	+	+	+	+	+	+	+	+	+	+	+	+	.	.	+	+	+	+	+	+	+	+	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
E6	.	.	+	+	+	+	+	+	+	+	+	+	.	.	+	+	+	+	+	+	+	+	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
E7	.	.	.	.	.	.	.	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
E8	.	.	.	.	.	.	.	.	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
E9	.	+	+	+	.	.	+	.	.	+	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
E10	.	.	.	.	.	.	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

* Isoenzymes numbered in order of decrease in negative charge.

Table 3. *Alkaline phosphatase isoenzymes of strains of Escherichia coli*

Iso-enzyme no.*	Strain no. (s)																			
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
P1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
P2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
P3	.	.	.	+	+	.	+	+	+	+	+	+	+	+	+	+	+	+	+	+
P4	+	+	+	+	+	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.
P5	.	+	+	+	+	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.
P6	.	+	+	+	+	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.
P7	.	+	+	+	+	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.
P8	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
P9	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
P10	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

* Isoenzymes numbered in order of decrease in negative charge.

Table 4. *Acid phosphatase isoenzymes of strains of Escherichia coli*

Iso-enzyme no.*	Strain no. (s)																			
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
AP1	.	+	+	.	+	.	+	+	+	.	.	+	.	.	.	+	+	.	.	.
AP2	+	+	+	+	+	.	+	+	+	.	.	+	+	+	+	+	+	+	+	+
AP3	+	.	.	+	.	+	.	.	.	+	+	+	+	+	+	+	+	+	+	+
AP4	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
AP5	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
AP6	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
AP7	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
AP8	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
AP9	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
AP10	+	.	.	+	.	+	+	+	+	.	+	+	+	+	.	+	.	+	+	+

* Isoenzymes numbered in order of decrease in negative charge.

When gel slices containing the same extracts were stained for phosphatase activity at pH 4.0, faint traces of the alkaline phosphatase isoenzymes appeared and in addition a further set of isoenzymes, designated acid phosphatases, developed. Alkaline phosphatases in gels containing extracts of bacteria grown in 'Oxoid' nutrient broth stained faintly when the gels were flooded with substrate solutions buffered at pH 7 and 9. When substrate solution at pH 4 was used, no alkaline phosphatase isoenzymes appeared but acid phosphatase isoenzymes developed readily. These isoenzymes were numbered AP1 to AP10 and the patterns for strains S1 to S30 were recorded (Table 4). Fig. 4 illustrates the relative positions of the acid and alkaline phosphatase isoenzymes of strains S1 to S16.

Fig. 4. Relative positions of acid (open blocks) and alkaline (solid blocks) phosphatase isoenzymes of *Escherichia coli* strains S1 to S16.

DISCUSSION

Esterase patterns of *Escherichia coli* isolates were found to be highly reproducible. Since cell extracts were adjusted to similar protein concentrations and precautions were taken to ensure a constant ' E_t value' for the front artefact (Norris, 1964), the variations described for E4 and E5 isoenzymes (Fig. 3) were considered to be distinct properties of the strains. The following results of preliminary experiments supported this contention. In a mixture of cell extracts of strains S10 and S13, E4a and E5 isoenzymes of strain S10 were separated from E4 and E5b isoenzymes of strain S13. An extract of strain S35 with twice the protein concentration of an extract of S34 exhibited a much weaker E4 isoenzyme, i.e. E4a than the E4 isoenzyme of strain S34. The relatively weak staining of isoenzymes labelled with the suffix *a* is thought to be due to the presence of smaller amounts of these particular isoenzyme proteins in the cell extracts.

On the basis of esterase isoenzyme patterns the 37 isolates studied could be divided into 18 groups. The esterase groupings and the distribution of isolates producing a positive reaction in ligated segments of pig intestine are shown in Table 5. While there does not appear to be a direct correlation between enteropathogenicity and esterase isoenzymes, the narrow grouping of enteropathogenic isolates would suggest that there may be some relationship.

Table 5. *Distribution of groups of esterases among strains of Escherichia coli*

Esterase isoenzymes													Total no. of isolates
1	2	3	4ab	4a	4	5b	5	6	7	8	9	10	
+	.	+	.	.	.	+	.	+	.	.	.	.	1
+	.	.	+	.	.	+	.	+	.	.	.	.	1
+	.	.	.	.	+	+	.	+	.	.	.	.	2
+	.	.	.	.	.	+	.	+	.	.	.	.	2
.	+	.	.	.	.	+	.	.	+	.	.	.	1
.	+	.	.	.	.	+	.	.	.	+	.	.	1
.	.	+	.	.	.	.	+	+	.	.	+	.	2
.	.	.	+	.	.	.	+	+	.	.	+	.	1
.	.	.	.	+	.	.	+	+	.	.	.	.	2
.	.	.	.	.	+	+	.	+	.	.	.	.	1
.	.	.	.	.	+	.	+	+	.	.	.	.	13(10)*
.	.	.	.	.	+	.	+	+	.	.	+	.	4(4)
.	.	.	.	.	+	.	.	+	.	.	.	.	1
.	.	.	.	.	.	.	+	.	.	.	.	.	1
.	.	.	.	.	.	.	+	+	.	.	.	.	1(1)
.	.	.	.	.	.	.	+	+	.	.	+	.	1
.	.	.	.	.	.	.	.	+	.	.	.	.	1
.	.	.	.	.	.	.	.	+	.	.	+	+	1

* Numbers in parentheses represent number of isolates producing a +ve reaction in ligated segments of pig intestine.

The *Escherichia coli* isolates possessed two electrophoretically distinct phosphatase enzymes. The pH optima found for the two enzymes are in broad agreement with those described by Torriani (1960). The formation of the alkaline phosphatase enzyme was repressed by Pi in the growth medium, whereas the formation of acid phosphatase was unaffected.

The acid and alkaline phosphatase isoenzyme patterns of 30 isolates defined 12 and 14 groups respectively. Neither of these groupings corresponded to grouping on the basis of esterase isoenzyme patterns. There does not appear to be any relationship between the phosphatase isoenzyme patterns of *Escherichia coli* isolates and enteropathogenicity.

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