

## The Use of Analytical Isoelectric Focusing for Detection and Identification of $\beta$ -Lactamases

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### SUMMARY

$\beta$ -Lactamases (EC. 3.5.2.6) from strains of Gram-negative bacteria have been studied using analytical isoelectric focusing. This permits a visual comparison of the patterns of  $\beta$ -lactamase bands produced by enzymes from different organisms. Purification of crude intracellular preparations is unnecessary and the technique is sufficiently sensitive to demonstrate  $\beta$ -lactamase in mutants previously reported to lack the enzyme. R factor RTEM and RPI  $\beta$ -lactamases that have not been distinguished from one another biochemically or immunologically can be differentiated by isoelectric focusing. Conversely, the enzymes specified by the R factors RTEM, RI and R<sub>GN14</sub>, with identical isoelectric focusing patterns, have the same biochemical properties. Chromosomal and R factor-mediated  $\beta$ -lactamases from single strains have been separated and their identities confirmed by immunoisoelectric focusing. R factor-mediated enzymes gave identical isoelectric focusing patterns irrespective of the host strain. Isoelectric focusing can therefore be used to observe the transfer of  $\beta$ -lactamases carried by R factors.

### INTRODUCTION

Isoelectric focusing (Haglund, 1967) is a method of separation in which proteins align themselves as sharp bands at their isoelectric points (pI) in an electrophoretically-produced pH gradient. A high degree of resolution is obtained by the method, because focusing is caused by forces that act against diffusion and proteins are therefore concentrated during their separation. We have used the technique in the analytical mode for the separation of  $\beta$ -lactamases (EC. 3.5.2.6) on thin layers of polyacrylamide gel and have located the focused enzymes with a cephalosporin substrate which changes colour after degradation by  $\beta$ -lactamase. This technique of separation and specific staining of  $\beta$ -lactamases allows demonstration of low levels of activity and presents the different enzymes produced by various strains as patterns of bands that can easily be recognized and compared.

The  $\beta$ -lactamases produced by Gram-negative bacteria are known to differ in their substrate specificities, inhibitor profiles, electrophoretic mobilities, biochemical properties and reactions with antisera (Hamilton-Miller, 1967; Jack & Richmond, 1970; O'Callaghan & Morris, 1972; Richmond & Sykes, 1973; Yamagishi *et al.* 1969). These enzymes have frequently been classified by the relative rates at which they hydrolyse penicillins and cephalosporins. Various methods have been used for estimating the activity of crude and pure enzyme preparations, and when a single strain produces two  $\beta$ -lactamases these have not always been separated before determining the substrate profile. Comparison of profiles determined in different laboratories may therefore be misleading. The use of isoelectric focusing of  $\beta$ -lactamases for direct comparison of different enzymes has been illustrated in

Table 1. *Strains examined by isoelectric focusing*

Organism	Strain no.	Derivation of strains	Reference
<i>Escherichia coli</i>	D3I	K12, RC711 derivative	Burman, Nordström & Boman† (1968)
	TEM	K12 (RTEM)	Datta & Kontomichalou (1965)
	R <sub>GN238</sub>	K12, W3630 (R <sub>GN238</sub> )	Sawai, Mitsuhashi & Yamagishi† (1968)
	R <sub>GN14</sub>	K12, W3630 (R <sub>GN14</sub> )	Sawai <i>et al.</i> (1968)
	R1818	K12, J53 (1818)	Datta† & Kontomichalou (1965)
	R1	K12, RC711 (R1)	Meynell & Datta† (1966)
	RPI-I	K12, W3110 (RPI-I)	Sykes† & Richmond (1970)
	RPI-I*	K12, W3110 (RPI-I*)	Richmond & Sykes† (1972)
	K12-44	K12, 44	Nguyen-Distèche, Pollock, Ghuysen†, Puig, Reynolds, Perkins, Coyette & Salton (1973)
	Hlt4	Transductant from Hlt3 using TPI phage	Burman, Park, Lindström & Boman† (1973)
<i>Klebsiella aerogenes</i>	1082E	Clinical isolate	Marshall† <i>et al.</i> (1972)
	1478E	Clinical isolate	Newsom,† Marshall & Harris (1974)
<i>Enterobacter cloacae</i>	P99	Blood culture	Fleming,† Goldner & Glass (1963)
	1321E	Spontaneous mutant of <i>Ent. cloacae</i> P99	Goldner, Glass & Fleming† (1969)
	214	Clinical isolate	Hennessey† (1967)
	1685E	Clinical isolate	Newsom† <i>et al.</i> (1974)
<i>Proteus mirabilis</i>	1496E	Wound swab	Newsom† <i>et al.</i> (1974)
<i>Proteus rettgeri</i>	1728E	Sputum sample	Newsom† <i>et al.</i> (1974)
<i>Pseudomonas aeruginosa</i>	RPI	Clinical isolate	Richmond & Sykes† (1972)

† These authors kindly supplied strains.

this paper principally by reference to enzymes from *Escherichia coli*. This species was chosen because strains carrying R factors specifying well-characterized  $\beta$ -lactamases were available.

#### METHODS

*Strains.* These are listed in Table 1. The clinical isolates were identified by the methods of Cowan & Steel (1965). Other strains of Gram-negative and Gram-positive bacteria were also examined. These were taken from the culture collection maintained in this laboratory.

*Resistance transfer.* The presence of R factors specifying resistance to  $\beta$ -lactam antibiotics was verified using the method of Datta (1962). *Escherichia coli* K12 cultures, either NCIB1270 or NCIB9482, resistant to nalidixic acid or streptomycin, were used as recipients.

*Preparation of intracellular enzymes.* Organisms were grown in 50 ml volumes of brain-heart infusion broth (Difco) and harvested as described by Marshall *et al.* (1972). Cells were resuspended in water to a final concentration of  $10^9$ /ml. Intracellular enzymes were released by ultrasonic treatment (Ross & Boulton, 1973).

Sand-grinding was used as an alternative method of releasing intracellular enzymes. Cell suspensions, in 5 ml amounts, were ground with sand. Sand and cell debris were removed by centrifugation at 5000 g for 30 min. Some enzymes with low activity were concentrated by freeze-drying. The  $\beta$ -lactamases from *E. coli* TEM and *Enterobacter cloacae* P99 were partly purified using Sephadex G-50 and purified further on QAE-Sephadex (Ross & Boulton, 1973).

*Chemicals.* Acrylamide, *N,N'*-methylenebisacrylamide and riboflavin were obtained from BDH, *N,N,N',N'*-tetramethylethylenediamine from Eastman Organic Chemicals, Rochester,

New York, U.S.A., and pH 3.5 to 10 ampholine from LKB Instruments Ltd, South Croydon, Surrey.

*Analytical isoelectric focusing.* This was carried out on sheets of polyacrylamide gel ( $21.5 \times 16.5 \times 0.15$  cm) supported on glass plates (Awdeh, Williamson & Askonas, 1968; Salaman & Williamson, 1971). The composition of the gel mixture was as follows: acrylamide, 70 mg/ml; *N,N'*-methylenebisacrylamide, 2 mg/ml; *N,N,N',N'*-tetramethylethylenediamine,  $0.25 \mu\text{g/ml}$ ; riboflavin,  $0.002 \text{ mg/ml}$ ; pH 3.5 to 10 ampholine, 20 mg/ml. Samples (up to  $50 \mu\text{l}$ ) were applied near the anode as drops of liquid on the surface of the gel. Isoelectric focusing was carried out at 100 to 400 V for about 40 h. As soon as the current had been switched off, the pH gradient of the gel was read at 0.5 cm intervals from the cathode to the anode using a miniature flat-ended combined glass electrode (Pye-Unicam 403.30 M8 EO7). The diameter of the electrode is 5 mm, compared with a band width of 1 mm or less, so that the pI recorded for a given band could vary by a pH value of  $\pm 0.2$  from run to run. Each pI value given in this paper is the mean of sixteen or more values determined after different electrofocusing runs. After the pH measurements had been recorded, the gel was stained for  $\beta$ -lactamase activity and photographed.

*Staining gels for  $\beta$ -lactamase activity.* The enzyme was usually detected by using a chromogenic cephalosporin as substrate (O'Callaghan *et al.* 1972). This compound, 3-(2,4-dinitrostyryl)-(6R, 7R)-7 $\beta$  (2-thienylacetamido)-ceph-3-em-4-carboxylic acid, E-isomer, will be referred to as cephalosporin 87/312. A sheet of Whatman No. 54 paper which had been dipped in a solution of cephalosporin 87/312 ( $0.5 \text{ mg/ml}$  in  $0.1 \text{ M}$ -phosphate buffer, pH 7.0) was laid over the surface of the gel and then removed, in one continuous rotary motion. The intact substrate molecule is yellow, but becomes pink when the  $\beta$ -lactam bond is broken, so that focused bands with  $\beta$ -lactamase activity appear pink on a yellow background. In some instances, enzyme activity was also detected by the starch-iodine method of Perret (1954), as modified by Ross & Boulton (1972), using benzyl penicillin or cephalosporin C ( $10 \text{ mg/ml}$ ) as substrate.

*Staining gels for protein.* Gels were immersed for 1 h in Coomassie brilliant blue G ( $0.1 \%$ , w/v) dissolved in ethanol-acetic acid-water (35:10:55, by vol.). They were destained in ethanol-acetic acid-water (30:5:65, by vol.).

*Photography.* A record of the results could be obtained only if the plates were photographed. The pink bands associated with  $\beta$ -lactamase activity could not be fixed and the faint pink lines on a yellow background could not be distinguished readily. When a focused gel was treated with cephalosporin 87/312 the  $\beta$ -lactamase bands appeared gradually and by the time the weakest bands were visible, the strongest bands had diffused. Serial photographs were usually taken to obtain a complete record. Kodalith Ortho film (35 mm) was used with transmitted light and a Wratten 58 green filter. The film was processed in PQ developer for 5 min at  $21^\circ\text{C}$  and prints were made, enlarged six times. Relatively early photographs have been used in the Figures as these showed the maximum number of clearly defined main bands.

*Preparation of antisera.* Antiserum against purified TEM  $\beta$ -lactamase (Jack & Richmond, 1970) was provided by R. B. Sykes. Antisera to  $\beta$ -lactamases from *Ent. cloacae* P99 (Ross & Boulton, 1972) and *E. coli* D31, purified by the method described for P99 enzyme (Ross & Boulton, 1973) were provided by M. G. Boulton.

*Immunoisoelectric focusing.* Using a Perspex former, six troughs ( $165 \times 1.0 \text{ mm}$ ) were set into a sheet of polyacrylamide gel on which samples were focused. Antiserum was then added to the troughs. The gel was incubated at  $4^\circ\text{C}$  in a humid atmosphere for 7 days to allow precipitin arcs to form. These were stained for  $\beta$ -lactamase activity.

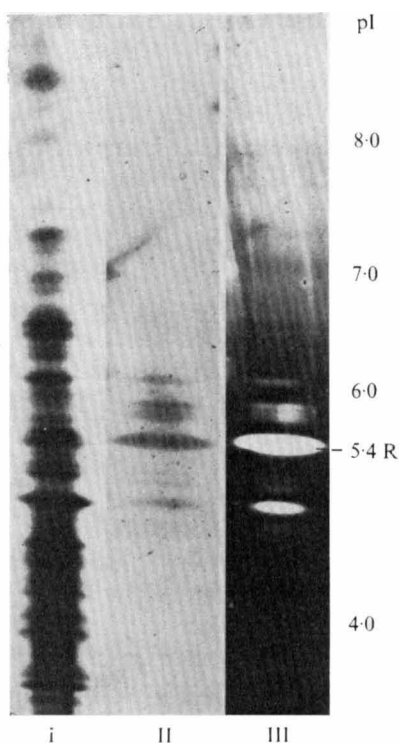


Fig. 1

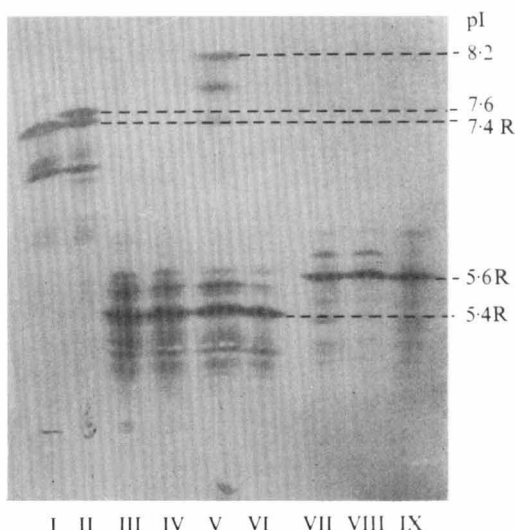


Fig. 2

Fig. 1. An intracellular preparation of *E. coli* TEM stained with: I, Coomassie blue G; II, cephalosporin 87/312; III, benzyl penicillin and starch-iodine. R denotes a band of  $\beta$ -lactamase mediated by an R factor.

Fig. 2. The  $\beta$ -lactamases specified by strains carrying R factors in hosts of different genera. Preparations of the following strains were focused: I, *E. coli* R<sub>GN238</sub>; II, *Klebsiella aerogenes* 1478E; III, *Proteus rettgeri* 1728E; IV, *P. mirabilis* 1496E; V, *Ent. cloacae* 1685E; VI, *E. coli* TEM; VII, *E. coli* RPI-I; VIII, *E. coli* RPI-I\*; IX, *Pseudomonas aeruginosa* RPI. The presence of a transmissible R factor mediating resistance to  $\beta$ -lactam antibiotics was confirmed in every strain except *E. coli* RPI-I\*. In this organism the R factor had become integrated in the chromosome.

## RESULTS

### *The method of detecting and identifying $\beta$ -lactamases by analytical isoelectric focusing*

In focused intracellular preparations, cephalosporin 87/312 stained  $\beta$ -lactamase, revealing patterns of bands qualitatively identical to the patterns revealed by starch-iodine staining, and radically different from the protein bands (Fig. 1).  $\beta$ -Lactamase bands were usually detected with cephalosporin 87/312 because starch-iodine-stained gels faded quickly above pH 8 and gave poor definition of bands with high pI values. Using cephalosporin 87/312, about 0.0002 i.u.  $\beta$ -lactamase could be detected on the focused gel. By taking sequential photographs, samples with a 16 000-fold range of activity could be compared on a single plate.

A  $\beta$ -lactamase pattern could contain several groups of bands (Fig. 2, V); each group of bands usually consisted of one main band and a number of satellite bands (Fig. 2, V, main bands with pI 8.2 and 5.4R, where R denotes a band of  $\beta$ -lactamase mediated by an R factor). The patterns could always be reproduced when replicate experiments were carried

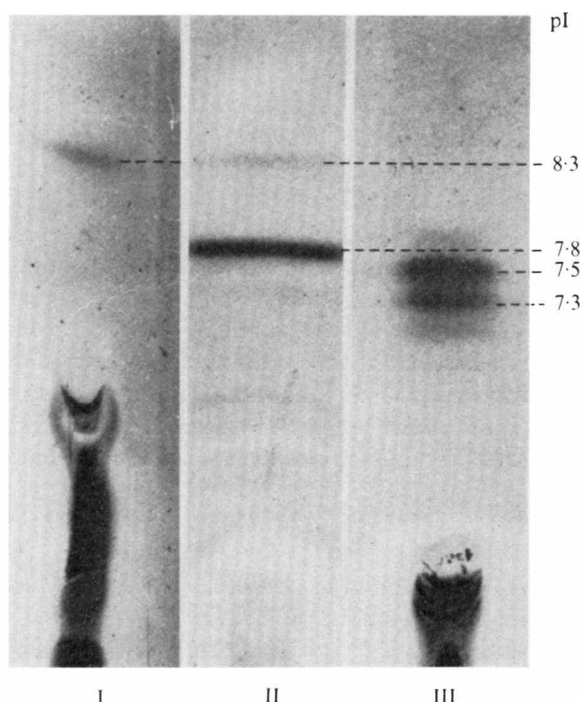


Fig. 3. Bands with  $\beta$ -lactamase activity from intracellular preparations of  $\beta$ -lactamase-negative mutants: I, *E. coli* H114; II, *E. coli* K12-44; III, *Ent. cloacae* 1321E.

out. The intensities of the bands shown in the Figures were not related quantitatively to the amounts of enzyme produced by the organisms, because loadings of enzyme were adjusted so that a qualitative comparison could be made between the principal  $\beta$ -lactamase bands of different patterns.

The same isoelectric focusing patterns were obtained from intracellular preparations of an organism broken by ultrasonic waves or sand-grinding, and from enzyme that had been partly purified by passing the crude preparation through Sephadex G-50 (Ross & Boulton, 1973). When the  $\beta$ -lactamases from *Klebsiella aerogenes* 1082E and *Ent. cloacae* P99 and 214 were further purified on QAE-Sephadex (Ross & Boulton, 1973), their isoelectric focusing patterns differed from those of crude intracellular preparations from the same strains only in the relative intensities of a few of the satellite bands. Experiments were carried out to establish whether the satellite bands were artefacts produced by the conditions of isoelectric focusing. Identical isoelectric focusing patterns were usually obtained when samples were loaded at different ends of the gel, samples were loaded on to different concentrations of ampholine, and when crude sand-ground preparations or partly purified preparations were run. Individual bands, when focused a second time, always appeared in the same positions relative to one another. Enzymes were considered to be identical when the main and satellite bands were confluent, e.g. the confluent main bands (pI 5.4R) and satellites in Fig. 2, III to VI.

*The use of isoelectric focusing to detect  $\beta$ -lactamase in  $\beta$ -lactamase-negative strains*

A number of authors have isolated mutant strains in which they were unable to detect  $\beta$ -lactamase. We examined three such strains (Table 1) and found that bands of  $\beta$ -lactamase activity could be demonstrated after focusing the intracellular preparations (Fig. 3). Two mutants of *E. coli* K12, H14 and K12-44, each had a band (pI 8.3) that appeared to be identical with the main band of the  $\beta$ -lactamase from another strain of *E. coli* K12, D31. Strain K12-44 had an additional and stronger band with pI 7.8. It has been suggested (J.-M. Ghuysen, personal communication) that the pI 7.8 band might be membrane-bound DD-carboxypeptidase-transpeptidase that had been solubilized by ultrasonic treatment in the same way as the corresponding *Streptomyces* enzyme (Frère *et al.* 1974).  $\beta$ -Lactamase from a spontaneous mutant (1321E) of *Ent. cloacae* had a doublet of bands (pI 7.3, 7.5) compared with pI 8.0 for  $\beta$ -lactamase P99 from the parent strain. Bands of  $\beta$ -lactamase activity have also been seen after focusing intracellular preparations from *Haemophilus*, *Neisseria* and *Streptococcus* spp. that were previously thought not to produce the enzyme.

*The use of isoelectric focusing to distinguish between  $\beta$ -lactamases from Escherichia coli*

Strains of *E. coli* have been reported to produce a variety of  $\beta$ -lactamases that differ in their biochemical properties (Richmond & Sykes, 1973). Several of these enzymes are known to be R factor-mediated. The isoelectric focusing patterns from seven strains of *E. coli* K12 were examined (Fig. 4). All preparations had a band at pI 8.3. This band, with its satellites, was the only band seen in the pattern for *E. coli* D31 (Fig. 4, I), a strain that produces a chromosomally-mediated  $\beta$ -lactamase (Boman, Eriksson-Grennberg, Normark & Matsson, 1968). Bands at pI 8.3 from the other six strains were confluent with the D31 band when they were compared in adjacent tracks, and were considered to be identical. This finding suggests that the  $\beta$ -lactamase bands which focus at pI 8.3 are chromosomally specified in strains of *E. coli* K12. The same band has also been seen in 104 other strains of *E. coli* K12 carrying various R factors. The  $\beta$ -lactamase band at pI 8.3 is not clearly visible for all the strains shown in Fig. 4, because it is difficult to obtain a good simultaneous picture of two groups of isoelectric focusing bands present in a single preparation.

In addition to groups of bands at pI 8.3, the focusing patterns of all six strains carrying R factors had a second group of bands that were shown by transfer experiments to be R factor-associated (Fig. 4, II to VII). In the patterns of all strains of *E. coli* carrying R factors, the bands associated with R factor were more strongly expressed than the bands at pI 8.3. Strains carrying the R factors RTEM, R1 and R<sub>GN14</sub> produced the group of bands (main band pI 5.4R) seen in Fig. 4, II to IV. The enzymes mediated by these R factors have previously been reported to have similar biochemical properties (Dale & Smith, 1971; Richmond & Sykes, 1973; Yamagishi *et al.* 1969), and the confluence of main and satellite bands in focusing patterns from these strains confirms that the enzymes are identical.

The association of RTEM-mediated activity with the pI 5.4R main band was confirmed by immunoelectrofocusing against antiserum to *E. coli* TEM  $\beta$ -lactamase (Fig. 5, II). Bands at pI 5.4R cross-reacted with TEM antiserum but not with antiserum to *E. coli* D31  $\beta$ -lactamase. Similarly, the band at pI 8.3 did not cross-react with TEM antiserum (Fig. 5, I). TEM  $\beta$ -lactamase could be detected by isoelectric focusing when it was produced in different genera, e.g. two species of *Proteus*, *Ent. cloacae* 1685E and *E. coli* TEM. Figure 2 (III to VI) shows the confluent bands at pI 5.4R associated with the TEM enzyme.

The isoelectric focusing pattern of a preparation of *Ent. cloacae* 1685E contained two

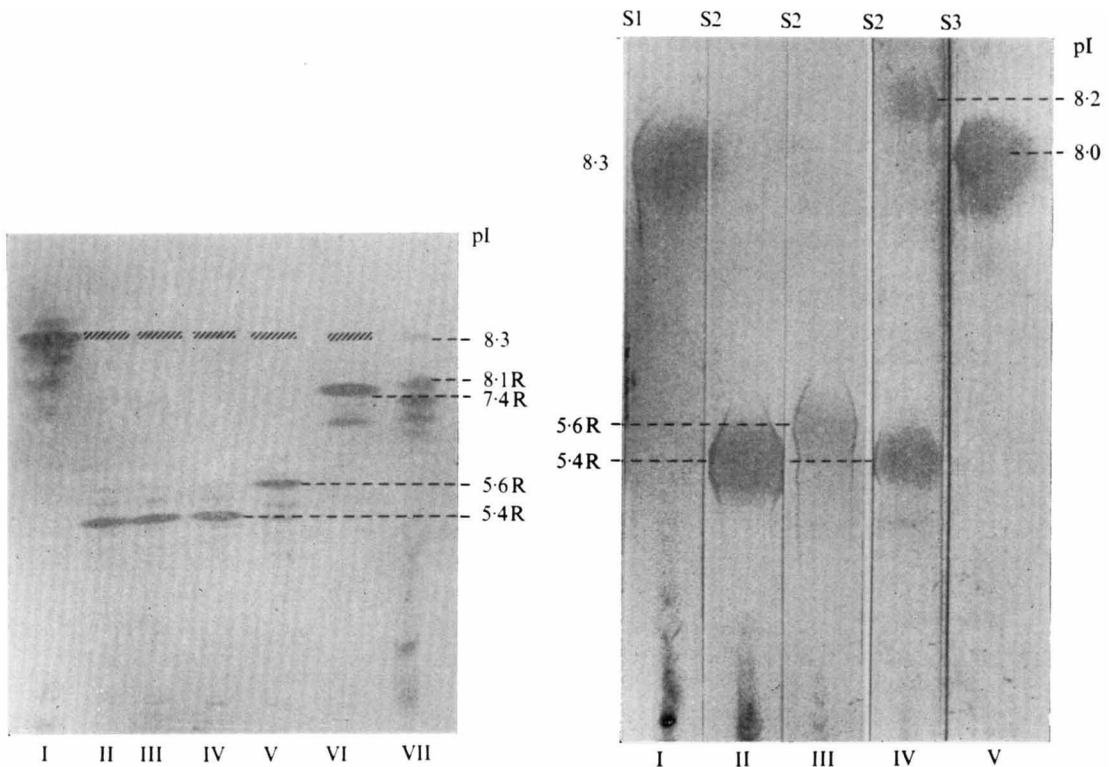


Fig. 4

Fig. 5

Fig. 4. The separation of  $\beta$ -lactamases from *E. coli*. Preparations from the following strains were focused: I, D31; II, R1; III, R<sub>GN14</sub>; IV, TEM; V, RPI-1; VI, R<sub>GN238</sub>; VII, R1818.  $\square$ , Denotes the position of weaker bands that developed slowly and which are seen in later serial photographs.

Fig. 5. Immunoisoelectric focusing of  $\beta$ -lactamases. Preparations from the following strains were focused: I, *E. coli* D31; II, *E. coli* TEM; III, *E. coli* RPI-1; IV, *Ent. cloacae* 1685E; V, *Ent. cloacae* P99. Troughs were filled with: S1, antiserum to purified D31 enzyme; S2, antiserum to purified TEM enzyme; S3, absorbed antiserum to *Ent. cloacae* P99 enzyme. Tracks I to III and IV to V were run on separate plates.

groups of  $\beta$ -lactamase bands (Fig. 2, V; main bands, pI 8.2 and 5.4R). These cross-reacted, respectively, with antiserum to *Ent. cloacae* P99 enzyme and to the TEM enzyme (Fig. 5, IV). These findings indicate that the  $\beta$ -lactamase at pI 8.2 was specified by an *Ent. cloacae* gene and the  $\beta$ -lactamase at pI 5.4R by a gene associated with the TEM R factor.

The RPI R factor which originated in a strain of *Pseudomonas* gives rise to an enzyme identical (Sykes & Richmond, 1970) to the  $\beta$ -lactamase from *E. coli* TEM in its substrate specificity, molecular weight and cross-reaction with antiserum to purified TEM enzyme (Fig. 5, II and III). In spite of these similarities, the isoelectric focusing patterns of the enzymes were clearly different (Fig. 4, IV and V). The RPI-1 R factor (RPI lacking the resistance markers neomycin and tetracycline) is also present in strain *E. coli* RPI-1\*, but in this strain it has been integrated into the chromosome as a stable element (Richmond & Sykes, 1972). The isoelectric focusing patterns for the enzyme RPI were identical whether it was specified by a transmissible R factor in *Pseudomonas* or in *E. coli* K12 or by the R factor integrated into the *E. coli* chromosome (Fig. 2, VII, VIII and IX).

*Escherichia coli* R<sub>GN238</sub> gave an isoelectric focusing pattern with a main band at pI 7.4R

(Fig. 4, VI) which was thought to be the R factor-mediated enzyme. No antiserum to this enzyme was available, but it did not cross-react with antisera to *E. coli* D31 or TEM enzymes. An identical band at pI 7.4R was seen in the electrofocusing pattern from a clinical isolate, *Klebsiella aerogenes* 1478E (Fig. 2, I and II). An R factor was transferred from this strain to *E. coli* K12, and the resulting organism gave an electrofocusing pattern identical with that of *E. coli* R<sub>GN238</sub>. It was originally suggested (Dale, 1971) that the R<sub>GN238</sub> and R1818  $\beta$ -lactamases were the same, but they were later differentiated by molecular weight and enzyme kinetic experiments (Dale & Smith, 1972). For the purpose of this study R1818 was transferred to *E. coli* K12. Isoelectric focusing confirms that the R<sub>GN238</sub> and R1818 enzymes are different (Fig. 4, VI and VII).

#### DISCUSSION

Analytical isoelectric focusing has shown that three  $\beta$ -lactamase-negative mutants do produce  $\beta$ -lactamase, although the isoelectric point is sometimes different from that of the corresponding wild-type enzyme.

A  $\beta$ -lactamase generally focuses as a main band that is accompanied by satellite bands. The close relationship between these is demonstrated by the multiple-linking arcs they give in immunoelectrofocusing against antiserum to a highly purified enzyme preparation. This sort of microheterogeneity does not seem to be the type of artefact that commonly arises in isoelectric focusing (Carlström & Vesterberg, 1967; Frater, 1970; Illingworth, 1972) because it is unaffected by altering the experimental conditions. It is also unlikely that the microheterogeneity is caused by the action of the local intracellular environment on the enzyme, because a  $\beta$ -lactamase mediated by an R factor has an identical isoelectric focusing pattern when it is isolated from hosts of different genera. Post-synthetic deamidation gives rise to microheterogeneity (Awdeh, Williamson & Askonas, 1970), but of a distinctive pattern, quite different from the patterns observed for  $\beta$ -lactamases. At present, the reason why an apparently single  $\beta$ -lactamase focuses as a main band accompanied by satellites is unknown.

Since the different  $\beta$ -lactamases produced by R factors can easily be recognized by analytical isoelectric focusing, this method can be used in epidemiological studies for identifying the enzyme responsible for the acquired resistance of a strain. It clearly distinguishes between the TEM and RP1 enzymes that had not previously been distinguished either biochemically or immunologically. Another potential use of the technique is to demonstrate the presence of R factors specifying  $\beta$ -lactamase in genera such as *Proteus*, in which expression of the gene is impaired (Smith, 1969). The patterns of the  $\beta$ -lactamase specified by the RP1-1 R factor existing as a transmissible plasmid or integrated into the chromosome are identical. It is therefore impossible to say whether an enzyme is chromosomally or R factor-mediated unless the necessary genetic experiments have been done to prove R factor or chromosomal transfer. Isoelectric focusing could also be used for observing the transfer, between species, of plasmids that specify  $\beta$ -lactamases and presumably applied to other R factor-specified enzymes, such as those that inactivate aminoglycosides.

Identical isoelectric focusing patterns are given by the  $\beta$ -lactamases specified by R factors TEM, R1 and R<sub>GN14</sub>, which appear to have identical biochemical properties. It therefore seems probable that one can predict the properties of a  $\beta$ -lactamase if its isoelectric focusing pattern is identical with that of an enzyme that has already been characterized. The question as to whether  $\beta$ -lactamases are elicited in response to  $\beta$ -lactam antibiotics or are enzymes that play a fundamental role in normal bacterial physiology is still unanswered. The latter alternative is supported by the detection of  $\beta$ -lactamases in a wide range of bacteria. To



date, the enzyme has been found, using analytical isoelectric focusing, in 354 strains from 19 genera.

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