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Identification of β-Lactamases by Analytical Isoelectric Focusing: Correlation with Bacterial Taxonomy

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SUMMARY

β-Lactamases (EC. 3.5.2.6) can be directly compared by analytical isoelectric focusing. Using this technique, 242 strains from five Gram-positive and 16 Gram-negative genera were examined. A preparation of each strain focused as a single group of bands which did not match the pattern of any R factor-associated β-lactamase. None of the strains was known to carry an R factor and resistance transfer experiments were unsuccessful. The enzymes studied were therefore thought to be chromosomally mediated. The isoelectric points ranged from 3·9 to 8·7 and were not related to the substrate profiles or other biochemical properties. The chromosomal β-lactamases appeared to be specific for genus, species and sub-species, and strains that produced identical β-lactamases had identical bacterial characteristics. Correlation of bacteriological differences with differences in β-lactamase patterns is discussed with particular reference to strains of *Escherichia coli* and *Klebsiella* spp. Since β-lactamases may be universally produced by bacteria, separation of the enzymes by analytical isoelectric focusing could be used in bacterial taxonomy.

INTRODUCTION

β-Lactamases that cannot be distinguished biochemically and immunologically can be differentiated by analytical isoelectric focusing (Matthew *et al.*, 1975). Using this very sensitive technique, the different enzymes produced by various strains each appear as a pattern of bands (usually a group comprising a main band and satellites) which can be compared visually. Chromosomal and R factor-mediated β-lactamases produced by a single strain appear as separate entities and two groups of bands have always been seen in the isoelectric focusing patterns of 175 strains known to carry transmissible R factors mediating resistance to β-lactam antibiotics (Matthew *et al.*, 1975; and unpublished observations). Each strain described in this paper produced β-lactamase that focused as a single group of bands. We believe that all these β-lactamases are specified by the bacterial chromosome, and we present evidence that the enzymes are specific for genus, species and sub-species. Since the grouping of organisms by their β-lactamase patterns correlates with conventional taxonomic grouping, comparison of chromosomally-mediated β-lactamases may be used as a single criterion for assigning strains to sub-species delineated by combinations of other available tests.

METHODS

Strains. All strains, except *Neisseria gonorrhoeae*, were from either the National Collection of Type Cultures (NCTC), the National Collection of Industrial Bacteria (NCIB), the

Table 1. *Reference strains*

The isoelectric points of the β-lactamases produced by the reference strains are shown in Table 2.

Organism	Strain no.	Origin	Reference
Enterobacter cloacae	53		Smith* (1963*b*)
	P99	Blood culture	Fleming,* Goldner & Glass (1963)
	214	Clinical isolate	Hennessey* (1967)
Escherichia coli	D31	K12, RC711 derivative	Burman, Nordström & Boman* (1968)
	214T		Smith* (1963*a*)
	741	Isolated in this laboratory, 22 Feb. 1944	
Klebsiella pneumoniae	466	Isolated in this laboratory, 12 Oct. 1939	
Pseudomonas aeruginosa	NCTC8203	Urinary infection	Sabath & Abraham (1964)
Neisseria gonorrhoeae	LH278	Clinical isolate, London Hospital	
	LH271	Clinical isolate, London Hospital	
Staphylococcus aureus	PC1	Osteomyelitis	Novick (1962)

* These authors kindly supplied strains.

American Type Culture Collection (ATCC) or from the culture collection maintained in this laboratory (strain numbers bearing the suffix E). A small group of strains, producing β-lactamases of special interest, are referred to by previously published numbers: these are listed in Table 1, together with the numbers of clinical isolates not maintained in a culture collection. The bacterial characteristics of all the strains were verified using the methods of Cowan & Steel (1965). All serotyping was carried out by the Central Public Health Laboratory, Colindale, London NW9 5HT. There was no epidemiological linkage between the strains.

Resistance transfer. Attempts were made to demonstrate the presence of R factors specifying resistance to β-lactam antibiotics using the method of Datta (1962). *Escherichia coli* K12 cultures, either NCIB1270 or NCIB9482, resistant to nalidixic acid or streptomycin, were used as recipients.

Destruction of β-lactam antibiotics by Klebsiella enzymes. The β-lactamases produced by Klebsiella strains were examined by a modification of the method of Knox & Smith (1961). The strains were sub-cultured on to 0·22 μm pore, 25 mm diameter membrane filters (Millipore) laid on plates containing 10 ml blood agar base no. 2 (Oxoid). These plates were incubated at 37 °C for 18 h and then the membrane filters carrying grown cultures were removed. β-Lactamase activity in the agar was detected by overlaying the plates with a further 10 ml agar medium containing a β-lactam antibiotic and *Staphylococcus aureus* NCIB8244, which was used as the indicator organism (Newsom, Marshall & Harris, 1974). Growth of *Staph. aureus* in the region of filter discs indicated destruction of a β-lactam antibiotic; the range of antibiotics destroyed gave a qualitative indication of the β-lactamase type. The test organisms were never in direct contact with antibiotic and the method therefore assessed only constitutive enzyme production.

Induction of β-lactamase. Cultures of *Enterobacter cloacae* were grown in brain–heart

infusion broth (Difco) for 2 h on a rotary shaker. β-Lactam antibiotic was then added to induce enzyme production. Cephalosporin C, cephaloridine, benzyl penicillin or methicillin was added at a final concentration of 500 μg ml^{-1}. Cultures were shaken at 140 rev. min^{-1} for a further 16 h at 37 °C before the cells were harvested.

Preparation and analytical isoelectric focusing of intracellular β-lactamases. The methods used were described by Matthew *et al.* (1975). Crude intracellular preparations were focused in thin layers of polyacrylamide gel containing mixed ampholytes. The β-lactamases aligned as sharp bands at their isoelectric points (pI) and were located by staining with a chromogenic cephalosporin substrate. The error in measuring the position of a β-lactamase band in the gel was approximately ±0·2 pH, whereas bands of β-lactamase activity were resolved to less than 0·1 pH. Thus the same nominal pI value could be obtained for non-identical β-lactamases (these had non-confluent bands when the enzymes were run in adjacent tracks). Enzymes with similar pI values were always checked for identity or non-identity in adjacent tracks. In different runs, various β-lactamases always appeared in the same positions relative to one aother.

Preparation of antisera. Antisera to β-lactamases from *Ent. cloacae* P99 and *K. aerogenes* 1082E were prepared by the method of Ross & Boulton (1972). *Escherichia coli* D31 β-lactamase was purified by the method described for the *Ent. cloacae* P99 enzyme (Ross & Boulton, 1973) and then used to raise an antiserum. Antiserum to purified *E. coli* TEM β-lactamase was prepared by the method of Jack & Richmond (1970).

Immunoisoelectric focusing. The method of Matthew *et al.* (1975) was used.

RESULTS

β-Lactamases not mediated by R factors

The β-lactamase isoelectric focusing pattern of each strain consisted of a single group of bands. None of the organisms was known to be carrying R factors specifying β-lactamase, and all attempts to transfer β-lactamase-mediated resistance were unsuccessful. None of the strains produced β-lactamase with an isoelectric focusing pattern which matched that of any known R factor-mediated β-lactamase (Datta, Hedges, Matthew & Smith, unpublished observations). It was therefore concluded that all the enzymes studied were chromosomally mediated.

The isoelectric focusing patterns of β-lactamase from an inducible strain were qualitatively identical before and after induction. Figure 1 shows the β-lactamase pattern obtained from preparations of the inducible strain *Ent. cloacae* 214 (Hennessey, 1967) without induction, and after growth in the presence of four β-lactam compounds. None of the enzymes described in this paper was induced because analytical isoelectric focusing was sufficiently sensitive to detect the basal level of enzyme in inducible strains.

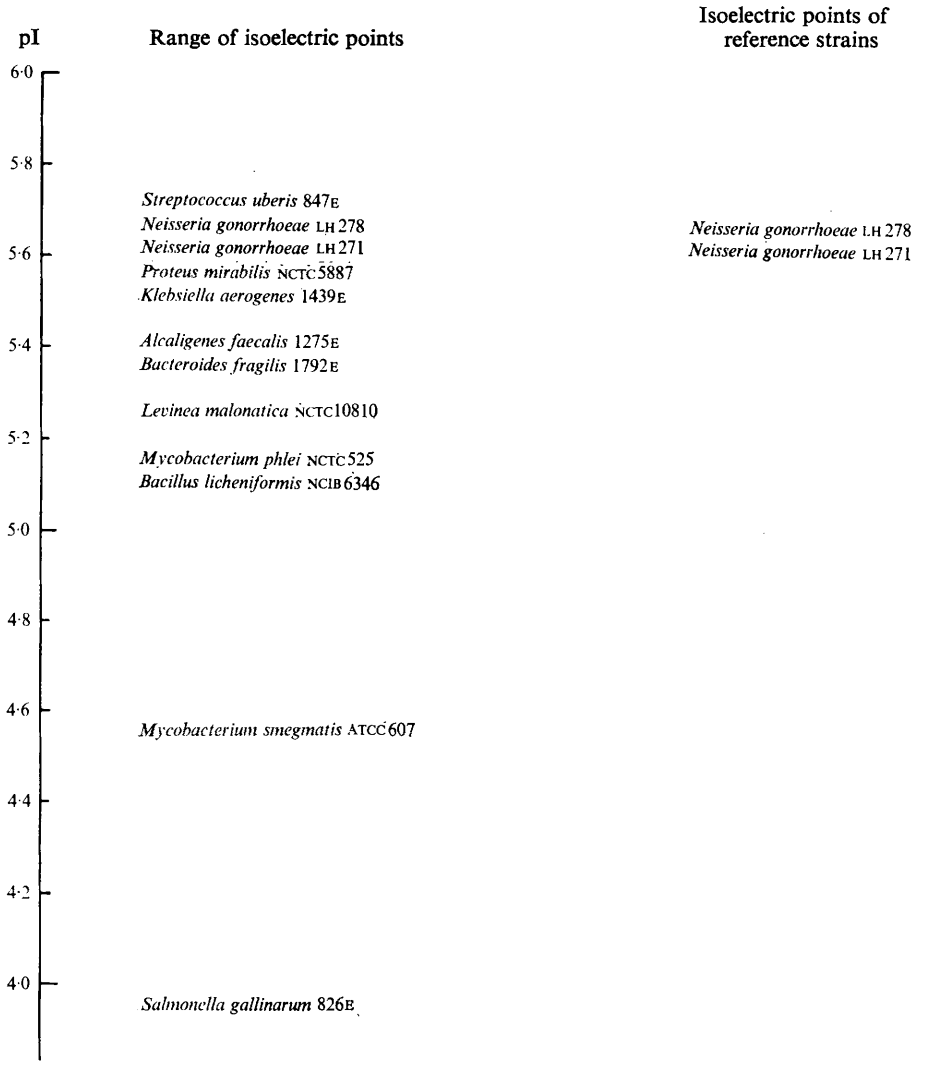
Using this method, β-lactamases were seen in the intracellular preparations of 242 strains from the 21 genera examined. The enzymes had pIs ranging from 3·9 to 8·7. Table 2 shows the highest and lowest observed β-lactamase pI values of strains from each genus and also the pIs of β-lactamases produced by the reference strains (Table 1). The relative pIs of β-lactamases from the various genera were checked by running the enzyme preparations in adjacent tracks, and Table 2 shows that the pI ranges for several genera overlapped, e.g. Levinea and Proteus; Shigella and Enterobacter. Some genera contained strains which produced β-lactamases of widely different pI: e.g. *Bacillus* spp. with enzymes of pI 5·1 to 6·3; and *Salmonella* spp. with enzymes of pI 3·9 to 7·2. Other genera, such as Acinetobacter or Streptococcus, produced β-lactamases with pIs less than 0·5 pH apart. The β-lactamase-

Table 2. *The range of isoelectric points of β-lactamases produced by strains from Gram-positive and Gram-negative genera*

Strains in the left hand column are those that produced β-lactamases with the highest and lowest pI values observed for each genus. The strains in the right hand column are those listed in Table 1.

pI	Range of isoelectric points	Isoelectric points of reference strains
9·0		
8·8		
8·6	*Staphylococcus aureus* PC1 *Acinetobacter inotti* 1786E *Escherichia coli* 1281E *Acinetobacter* sp. 1787E *Serratia marcescens* 1331E *Enterobacter aerogenes* 1687E	*Staphylococcus aureus* PC1
8·4	*Serratia marcescens* 1324E *Citrobacter freundii* 1461E	
8·2	*Shigella sonnei* 1608E *Pseudomonas aeruginosa* NCTC10701 *Escherichia coli* D31 *Bordetella bronchiseptica* 381E	*Escherichia coli* 214T *Escherichia coli* 741 *Escherichia coli* D31
8·0		
7·8	*Klebsiella pneumoniae* 466	*Klebsiella pneumoniae* 466 *Pseudomonas aeruginosa* NCTC8203
7·6	*Proteus rettgeri* 1793E *Citrobacter freundii* 1088E	*Enterobacter cloacae* 214 *Enterobacter cloacae* 53 *Enterobacter cloacae* P99
7·4	*Bacteroides fragilis* 1604E	
7·2	*Salmonella typhimurium* 1343E *Hafnia* sp. 1094E	
7·0	*Shigella flexneri* 1784E	
6·8		
6·6	*Pseudomonas fluorescens* 715E *Enterobacter aerogenes* 1014E	
6·4		
6·2	*Bacillus cereus* NCIB7464 *Streptococcus faecalis* ATCC8043 *Lactobacillus arabinosus* NCIB8030 *Levinea amalonatica* NCTC10805	
6·0		

Table 2. (*cont.*)



producing strains included *E. coli* 741 and *K. pneumoniae* 466, isolated in 1944 and 1939 respectively, before β-lactam antibiotics were widely used in antibacterial therapy; and also species of Neisseria and Streptococcus that were previously thought to lack the enzyme (Richmond & Sykes, 1973).

Genus, species and sub-species specificity of β-lactamases

The β-lactamases produced by strains from one genus always had different pIs from those produced by strains from another genus. This was true even when the genera were fairly closely related taxonomically, and produced enzymes with similar specificities. Figure 2 shows the differences between some β-lactamases from strains of Citrobacter, Enterobacter and Serratia. All of these enzymes are primarily cephalosporinases inhibited by cloxacillin and methicillin.

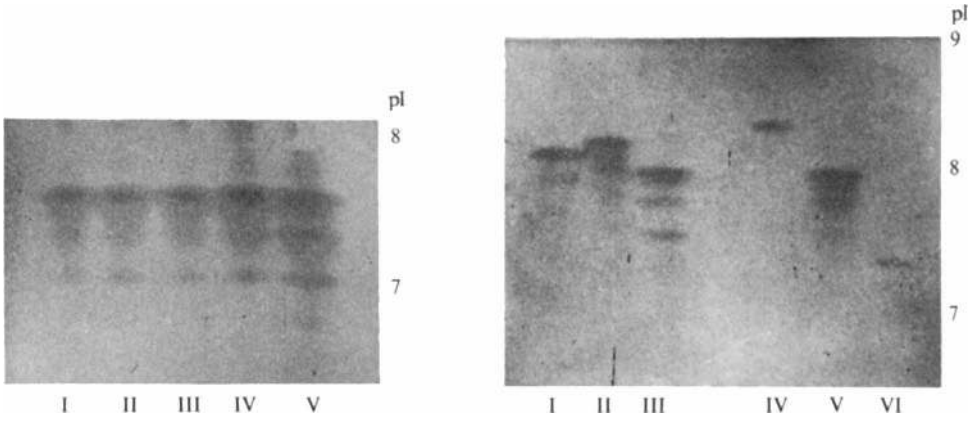


Fig. 1. Uninduced and induced β-lactamase from *Enterobacter cloacae* 214. Cultures of *Ent. cloacae* 214 were grown: I, without inducer; or in the presence of, II, cephalosporin C; III, cephaloridine; IV, benzyl penicillin; V, methicillin; each at a concentration of 500 μg ml^{-1}.

Fig. 2. β-Lactamases with cephalosporinase substrate profiles from strains of different genera. Preparations of the following strains were focused: I, *Serratia marcescens* 1324E; II, *Citrobacter freundii* 1461E; III, *Enterobacter cloacae* 1673E; IV, *Serratia marcescens* 1331E; V, *Citrobacter freundii* 1088E; VI, *Enterobacter cloacae* 1686E.

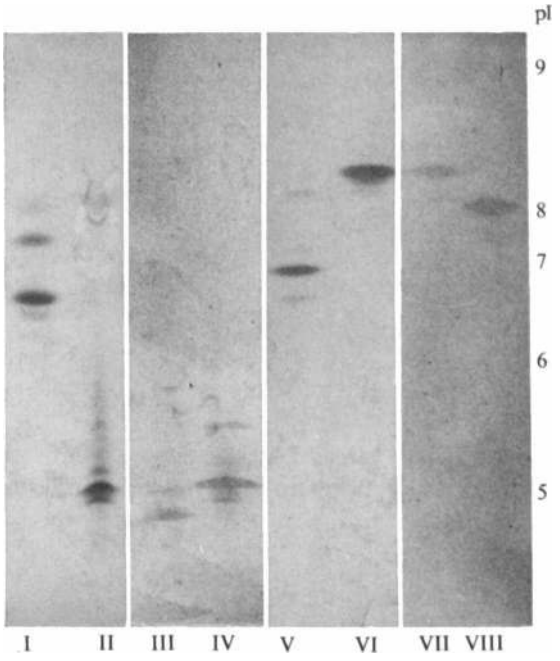
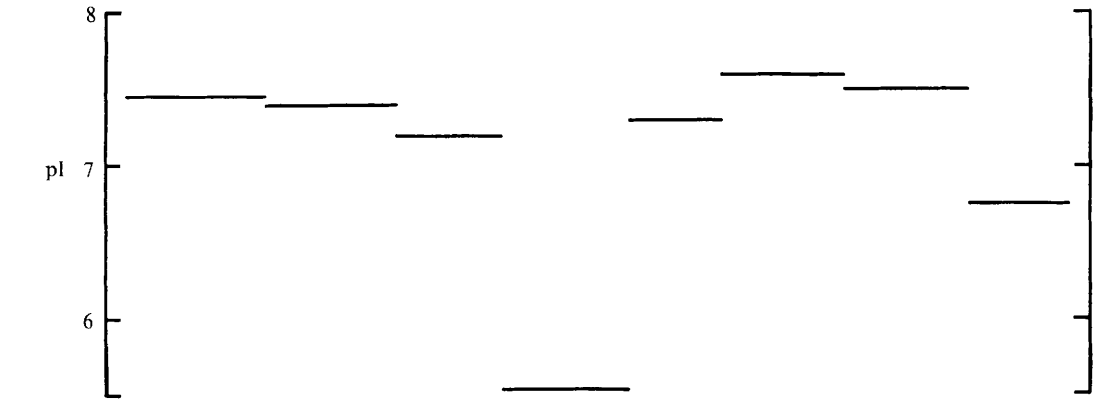


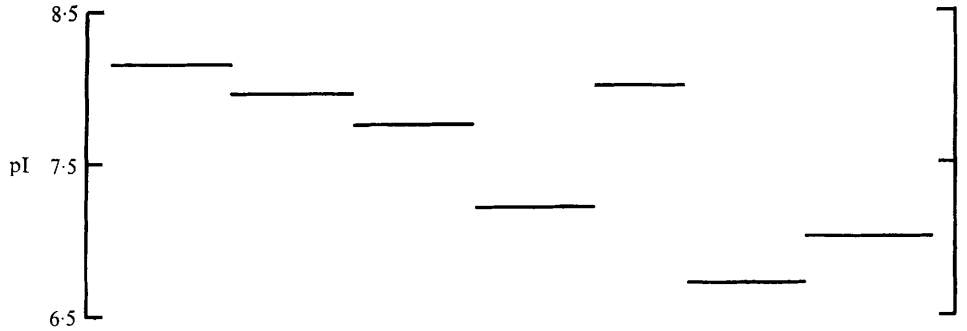
Fig. 3. Species specificity of β-lactamases. Preparations of the following strains were focused: I, *Bacillus cereus* NCIB7464; II, *B. licheniformis* NCIB6346; III, *Mycobacterium fortuitum* ATCC23041; IV, *M. phlei* NCTC525; V, *Shigella flexneri* 1784E; VI, *Sh. sonnei* 1785E; VII, *Enterobacter aerogenes* 1462E; VIII, *Ent. cloacae* P99.



Proteus Ref. strain	*inconstans* (Providencia) 1363E	*inconstans* (Providencia) 1498E	*mirabilis* 431E	*mirabilis* NCTC5887	*morganii* NCTC235	*rettgeri* 1793E	*rettgeri* 1049E	*vulgaris* 1791E

Fig. 4. β-Lactamases produced by strains of *Proteus inconstans* (Providencia), *P. mirabilis, P. morganii, P. rettgeri* and *P. vulgaris.*

Different species from the same genus always produced different β-lactamases. This is illustrated in Fig. 3 by enzymes from strains of *Bacillus cereus* and *B. licheniformis, Mycobacterium fortuitum* and *M. phlei, Shigella flexneri* and *Sh. sonnei,* and *Enterobacter aerogenes* and *Ent. cloacae.* Strains of a single species produced β-lactamases of more than one pattern, as shown in Fig. 4 for strains of *Proteus inconstans* (Providencia), *P. mirabilis* and *P. rettgeri.* However, when preparations of more than a few strains from a single species were examined, it was found that they produced β-lactamases having only a small number of different patterns, which indicated that sub-species differentiation is limited. For instance, Fig. 5 shows that 49 strains of *Pseudomonas aeruginosa* produced only four different β-lactamases: the enzyme pI 8·15 was produced by four strains; enzyme pI 7·95 by 32;



Pseudomonas Ref. strain	*aeruginosa* NCTC10701 (3)	*aeruginosa* 1563E (31)	*aeruginosa* NCTC8203 (11)	*aeruginosa* 150E (0)	*cepacia* 1599E	*fluorescens* 715E	*testosteroni* 1272E

Fig. 5. β-Lactamases produced by strains of *Pseudomonas aeruginosa, Ps. cepacia, Ps. fluorescens* and *Ps. testosteroni.* The number of clinical isolates that produced β-lactamase focusing at the same pI as the enzyme of each reference strain of *Ps. aeruginosa* is shown in brackets below the reference strain number.

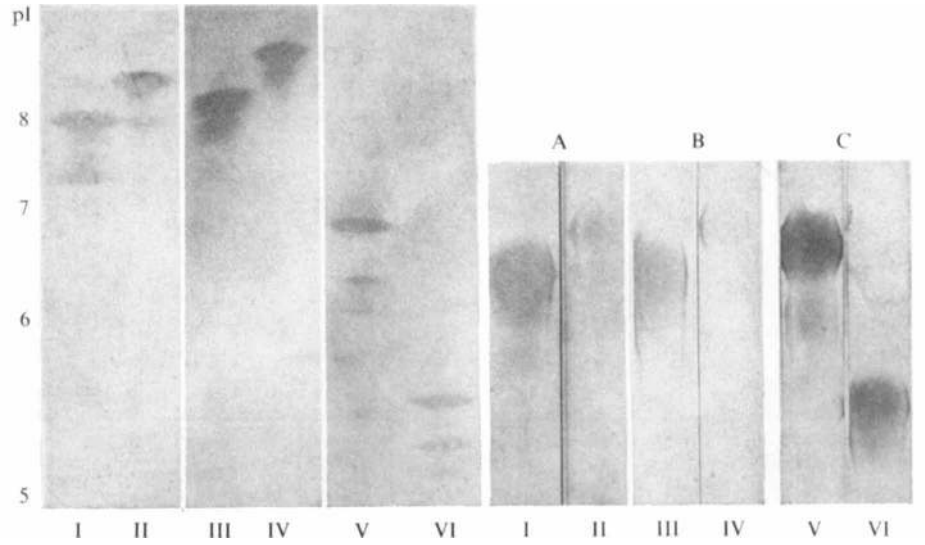
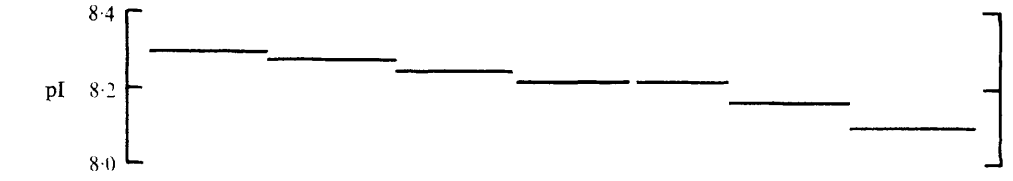


Fig. 6. Separation of pairs of β-lactamases that are serologically identical and have similar biochemical properties. Preparations of the following strains were focused: I, *Enterobacter cloacae* P99; II, *Ent. cloacae* 214; III, *Escherichia coli* D31; IV, *E. coli* 214T; V, *Klebsiella aerogenes* 1082E; VI, *K. aerogenes* 1439E. In immunoisoelectric focusing, troughs were filled with antisera to β-lactamases from the following strains: A, *Ent. cloacae* P99; B, *E. coli* D31; C, *K. aerogenes* 1082E.

enzyme pI 7·5 by 12; and enzyme pI 7·2 by only one. A similar distribution of single-species β-lactamases amongst enzymes of various pI values was obtained for 10 strains of *Citrobacter freundii* that produced only three different β-lactamases, eight strains of *Ent. aerogenes* (four β-lactamases), and 19 strains of *Ent. cloacae* (five β-lactamases).

β-Lactamases from *Ent. cloacae* P99 and 214, that are indistinguishable biochemically (Hennessey & Richmond, 1968) and immunologically, have been differentiated by isoelectric focusing (Fig. 6). This figure also shows the separation of pairs of enzymes with similar properties from *E. coli* D31 and 214T (Smith, Bremner & Datta, 1974) and *Klebsiella aerogenes* 1082E and 1439E (Table 3).

Serotyping has been used conventionally to group strains of *E. coli* into sub-species. Preparations of six different serotypes of *E. coli*, including K12 (1210E), were examined and each produced a different β-lactamase (Fig. 7). A seventh strain, *E. coli* 851ER (rough



E. coli		1740E	1543E	1743E	851ES	851ER	1100E	1210E
Serotype	O	2	8	78	20	rough	111	rough
	K	1	87. 88a. b	80	61	none	58(B4)	none
	H	4	19	minus	minus	minus	2	48

Fig. 7. β-Lactamases produced by various *Escherichia coli* serotypes.

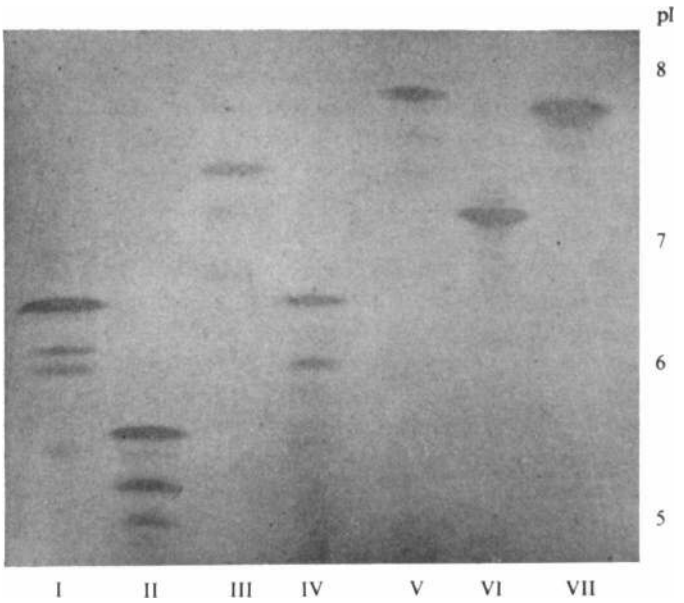


Fig. 8. *β*-Lactamases produced by strains of Klebsiella. Preparations of the following reference strains were focused: I, 1082E (2); II, 1439E (13); III, 1477E (6); IV, 1730E (1); V, 466 (0); VI, 1475E (1); VII, 1319E (0). The numbers in brackets refer to the number of clinical isolates producing *β*-lactamase that focused at the same pI as the enzyme of each reference strain.

variant), produced a *β*-lactamase that could not be separated from the enzyme of the smooth strain, *E. coli* 851ES (Fig. 7).

β-Lactamases from strains of Klebsiella

Preparations from seven strains of Klebsiella had widely different *β*-lactamase patterns, the pIs of the main bands ranging from approximately 5·4 to 7·7 (Fig. 8). The differences in isoelectric focusing patterns reflected the differences between the strains (Table 3). Initially the organisms were characterized by their ability to destroy a range of *β*-lactam antibiotics

Table 3. *The bacteriological properties of Klebsiella reference strains*

Strain properties		Strains						
		1082E	1439E	1477E	1730E	466	1475E	1319E
Antibiotic destruction	Penicillin	+	+	+	+	+	+	+
	Ampicillin	+	+	+	−	−	+	+
	Carbenicillin	+	+	+	+	−	+	+
	Methicillin	+	+	+	−	−	+	+
	Cloxacillin	+	+	+	−	−	+	+
	Cephaloridine	+	+	+	−	+	+	+
	Cephalexin	+	+	−	−	+	−	−
Bacteriological characteristics	Indole	+	+	+	+	−	−	−
	Methyl Red	−	−	+	−	−	−	−
	VP	+	+	−	−	+	+	−
	Dulcitol	+	+	−	+	−	−	−
Number of strains with properties identical to those of reference strains		2	13	6	1	0	1	0

and by conventional bacteriological methods. All strains had 18 positive and five negative diagnostic features in common, but four tests were variable for the strains and served to differentiate the groups of organisms. Two groups were bacteriologically identical by the criteria shown in Table 3; these strains, represented by reference organisms *K. aerogenes* 1082E and 1439E, were clearly separated by isoelectric focusing (Fig. 8).

None of the Klebsiella β-lactamases focused at pH 5·4, the pI of the enzyme mediated by the TEM R factor (Matthew *et al.*, 1975); nor did any of them cross-react with antiserum to the purified TEM enzyme. This was in contrast to the findings of Sawai, Yamagishi & Mitsuhashi (1973), who reported that Klebsiella β-lactamases, pI 7·2 to 7·87, cross-reacted with antiserum to the enzyme produced by *E. coli* R_{GN14}. This is predominantly the TEM β-lactamase (Matthew *et al.*, 1975).

DISCUSSION

Analytical isoelectric focusing has shown that a wide variety of Gram-negative and Gram-positive bacteria produce β-lactamases with a broad range of isoelectric points. These do not appear to be related to the enzymes' biochemical properties, such as the substrate profiles or susceptibility to β-lactam inhibitors that form the basis of Richmond & Sykes (1973) β-lactamase classification. Enzymes may have similar biochemical properties and differ greatly in isoelectric focusing; for example, the penicillinases produced by *Staph. aureus* PC1 (Novick, 1962) and *B. licheniformis* NCIB6346 (Pollock, 1965) have isoelectric points 3·6 pH apart. Alternatively, enzymes may have very different biochemical properties and yet focus close to one another. Examples are the *N. gonorrhoeae* cephalosporinases (R. B. Sykes, unpublished results) and the broad spectrum β-lactamase specified by RP1 (Richmond & Sykes, 1972, 1973), that both have isoelectric points about 5·6; and the cephalosporinase from *Bacteroides fragilis* 1604E (R. B. Sykes, unpublished results) that has an isoelectric point very close to that of the oxacillin-hydrolysing penicillinase, pI 7·4, specified by R_{GN238} (Yamagishi *et al.*, 1969; Dale & Smith, 1974).

β-Lactamase does not appear to be produced primarily in specific response to exposure of organisms to β-lactam compounds, since the enzyme is produced by strains of *B. licheniformis* (Pollock, 1967), *K. pneumoniae* 466 and Staphylococci (Segalove, 1947), that were isolated before β-lactam antibiotics were used therapeutically, and by organisms isolated from populations never treated with antibiotics (Gardner *et al.*, 1969). If the primary role of β-lactamases were to protect organisms from the antibacterial action of β-lactam compounds, then one would not expect the enzyme, whether inducible or not, to be present universally in bacteria. The retention of the enzyme during the evolutionary differentiation of bacteria suggests that it plays some part in normal bacterial physiology. β-Lactamases are probably localized in the periplasmic membrane (Neu & Winshell, 1972; Sargent, Ghosh & Lampen, 1968) and Saz has demonstrated that in *Staph. aureus* the enzyme appears spontaneously during lag and early-exponential phase of growth and is induced by a peptidoglycan (Ozer, Lowery & Saz, 1970; Sachithanandam, Lowery & Saz, 1974). It seems that the normal role of the enzyme might be to break a β-lactam structure that is a transitory intermediate in bacterial cell-wall metabolism. No such compound has been characterized yet, but our present knowledge of cell-wall structure comes chiefly from the chemistry of the finished product (Costerton, Ingram & Cheng, 1974; Salton, 1964).

Evidence of genus and species variation of some β-lactamases from both Gram-negative and Gram-positive bacteria has been presented by other workers (Hamilton-Miller, 1968; Pollock, 1965; Richmond, 1965; Sawai, Mitsuhashi & Yamagishi, 1968; Thompson *et al.*, 1972). Our results from the examination of 242 strains from 21 genera confirm that β-lacta-

mases are genus, species and sub-species specific. When a species forms a coherent taxonomic group, as do *C. freundii* or *Ent. cloacae*, the various β-lactamases produced by the species differ only slightly in isoelectric point. In contrast, in the genus Klebsiella, described by Brown & Seidler (1973) as a 'taxonomic enigma', strains are very clearly separated by their widely different isoelectric focusing β-lactamase patterns. The isoelectric focusing groupings correlated precisely with bacterial groupings based on four characters which are variable within the genus Klebsiella, according to major taxonomic schemes (Bascomb *et al.*, 1971; *Bergey's Manual of Determinative Bacteriology*, 1974; Cowan & Steel, 1965; Edwards & Ewing, 1971). Ørskov (1957) divided the genus *Klebsiella* into 36 biochemical types, some of which correlated with capsular types. We have demonstrated wide variation in β-lactamase patterns among relatively few Klebsiella strains and suggest that the patterns may correlate with serotypes in Klebsiella as they appear to in *E. coli*.

The isoelectric points of chromosomal β-lactamases could be used as definitive taxonomic characteristics, particularly since the enzymes seem to be exclusively bacterial and of universal occurrence. Moreover, the chromosomal β-lactamase isoelectric focusing pattern alone serves to assign an organism to the sub-species defined by a variety of conventional tests. The results obtained with some strains of *E. coli* suggest that these may be classified directly into serotypes by determining their β-lactamase isoelectric points; and analytical isoelectric focusing is probably quicker, easier and cheaper than raising monospecific antisera. Fox & McClain (1975) found that electrophoretograms of esterases and dehydrogenases were useful in the taxonomic separation of strains of *Micrococcus cryophilus*, *Branhamella catarrhalis* and *Neisseria* spp. Similar results were obtained by El-Sharkawy & Huisingh (1971*a*, *b*) for phytopathogenic genera. That is, the isoelectric points of the enzymes were variable and taxonomically related. It seems likely that the isoelectric points of any widely distributed bacterial enzyme could be used for taxonomic separation of strains.

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REFERENCES

Bascomb, S., Lapage, S. P., Willcox, W. R. & Curtis, M. A. (1971). Numerical classification of the tribe Klebsielleae. *Journal of General Microbiology* **66**, 279–295.

Bergey's Manual of Determinative Bacteriology, 8th edn (1974). Edited by R. E. Buchanan and N. E. Gibbons. Baltimore: The Williams and Wilkins Co.

Brown, C. & Seidler, R. J. (1973). Potential pathogens in the environment: *Klebsiella pneumoniae*, a taxonomic and ecological enigma. *Applied Microbiology* **25**, 900–904.

Burman, L. G., Nordstrom, K. & Boman, H. G. (1968). Resistance of *Escherichia coli* to penicillins. V. Physiological comparison of two isogenic strains, one with chromosomally and one with episomally mediated ampicillin resistance. *Journal of Bacteriology* **96**, 438–446.

Costerton, J. W., Ingram, J. M. & Cheng, K.-J. (1974). Structure and function of the cell envelope of Gram-negative bacteria. *Bacteriological Reviews* **38**, 87–110.

Cowan, S. T. & Steel, K. J. (1965). *Identification of Medical Bacteria.* Cambridge: Cambridge University Press.

Dale, J. W. & Smith, J. T. (1974). R factor-mediated β-lactamases that hydrolyse oxacillin: evidence for two distinct groups. *Journal of Bacteriology* **119**, 351–356.

Datta, N. (1962). Transmissible drug resistance in an epidemic strain of *Salmonella typhimurium*. *Journal of Hygiene, Cambridge* **60**, 301–310.

Edwards, P. R. & Ewing, W. H. (1971). *Identification of Enterobacteriaceae*, 3rd edn. Minneapolis: Burgess.

El-Sharkawy, T. A. & Huisingh, D. (1971*a*). Electrophoretic analysis of esterases and other soluble proteins from representatives of phytopathogenic bacterial genera. *Journal of General Microbiology* **68**, 149–154.

EL-SHARKAWY, T. A. & HUISINGH, D. (1971*b*). Differentiation among *Xanthomonas* species by polyacrylamide gel electrophoresis of soluble proteins. *Journal of General Microbiology* **68**, 155–165.

FLEMING, P. C., GOLDNER, M. & GLASS, D. G. (1963). Observations on the nature, distribution and significance of cephalosporinase. *Lancet* i, 1399–1401.

FOX, R. H. & MCCLAIN, D. E. (1975). Enzyme electrophoretograms in the analysis of taxon relatedness of *Micrococcus cryophilus*, *Branhamella catarrhalis* and atypical Neisserias. *Journal of General Microbiology* **86**, 210–216.

GARDNER, P., SMITH, D. H., BEER, H. & MOELLERING, R. C. (1969). Recovery of resistance (R) factors from a drug-free community. *Lancet* ii, 774–776.

HAMILTON-MILLER, J. M. T. (1968). Wild-type variants of penicillinase from *Klebsiella aerogenes*. *Federation of European Biochemical Societies Letters* **1**, 86–88.

HENNESSEY, T. D. (1967). Inducible β-lactamase in Enterobacter. *Journal of General Microbiology* **49**, 277–285.

HENNESSEY, T. D. & RICHMOND, M. H. (1968). The purification and some properties of a β-lactamase (cephalosporinase) synthesized by *Enterobacter cloacae*. *Biochemical Journal* **109**, 469–473.

JACK, G. W. & RICHMOND, M. H. (1970). A comparative study of eight distinct β-lactamases synthesized by Gram-negative bacteria. *Journal of General Microbiology* **61**, 43–61.

KNOX, R. & SMITH, J. T. (1961). Use of cellulose acetate membranes for detecting penicillinase-producing organisms. *Nature, London* **191**, 926–927.

MATTHEW, M., HARRIS, A. M., MARSHALL, M. J. & ROSS, G. W. (1975). The use of analytical isoelectric focusing for detection and identification of β-lactamases. *Journal of General Microbiology* **88**, 169–178.

NEU, H. C. & WINSHELL, E. B. (1972). Relation of β-lactamase activity and cellular location to resistance of Enterobacter to penicillins and cephalosporins. *Antimicrobial Agents and Chemotherapy* **1**, 107–111.

NEWSOM, S. W. B., MARSHALL, M. J. & HARRIS, A. M. (1974). Enterobacteria, β-lactam antibiotics and β-lactamases in clinical practice. *Journal of Medical Microbiology* **7**, 473–482.

NOVICK, R. P. (1962). Staphylococcal penicillinase and the new penicillins. *Biochemical Journal* **83**, 229–235.

ØRSKOV, I. (1957). Biochemical types in the Klebsiella group. *Acta pathologica et microbiologica scandinavica* **40**, 155–162.

OZER, J. H., LOWERY, D. L. & SAZ, A. K. (1970). Derepression of β-lactamase (penicillinase) in *Bacillus cereus* by peptidoglycans. *Journal of Bacteriology* **102**, 52–63.

POLLOCK, M. R. (1965). Purification and properties of penicillinases from two strains of *Bacillus licheniformis*: a chemical, physicochemical and physiological comparison. *Biochemical Journal* **94**, 666–675.

POLLOCK, M. R. (1967). Origin and function of penicillinase: a problem in biochemical evolution. *British Medical Journal* ii, 71–77.

RICHMOND, M. H. (1965). Wild-type variants of exopenicillinase from *Staphylococcus aureus*. *Biochemical Journal* **94**, 584–593.

RICHMOND, M. H. & SYKES, R. B. (1972). The chromosomal integration of a β-lactamase gene derived from the P-type R-factor RP1 in *Escherichia coli*. *Genetical Research* **20**, 231–237.

RICHMOND, M. H. & SYKES, R. B. (1973). The β-lactamases of Gram-negative bacteria and their possible physiological rôle. In *Advances in Microbial Physiology*, vol. 9, pp. 31–88. Edited by A. H. Rose and D. W. Tempest. New York and London: Academic.

ROSS, G. W. & BOULTON, M. G. (1972). Improvement of the specificity of an antiserum to β-lactamase by absorption with a mutant which does not produce the enzyme. *Journal of Bacteriology* **112**, 1435–1437.

ROSS, G. W. & BOULTON, M. G. (1973). Purification of β-lactamases on QAE-Sephadex. *Biochimica et biophysica acta* **309**, 430–439.

SABATH, L. D. & ABRAHAM, E. P. (1964). Synergistic action of penicillins and cephalosporins against *Pseudomonas pyocyanea*. *Nature, London* **204**, 1066–1069.

SACHITHANANDAM, S., LOWERY, D. C. & SAZ, A. K. (1974). Endogenous, spontaneous formation of beta-lactamase in *Staphylococcus aureus*. *Antimicrobial Agents and Chemotherapy* **6**, 763–769.

SALTON, M. R. J. (1964). *The Bacterial Cell Wall*. Amsterdam: Elsevier.

SARGENT, M. G., GHOSH, B. K. & LAMPEN, J. O. (1968). Localization of cell-bound penicillinase in *Bacillus licheniformis*. *Journal of Bacteriology* **96**, 1329–1338.

SAWAI, T., MITSUHASHI, S. & YAMAGISHI, S. (1968). Drug resistance of enteric bacteria. XIV. Comparison of β-lactamases in Gram-negative rod bacteria resistant to α-aminobenzyl penicillin. *Japanese Journal of Microbiology* **12**, 423–434.

SAWAI, T., YAMAGISHI, S. & MITSUHASHI, S. (1973). Penicillinases of *Klebsiella pneumoniae* and their phylogenetic relationship to penicillinases mediated by R factors. *Journal of Bacteriology* **115**, 1045–1054.

SEGALOVE, M. (1947). The effect of penicillin on growth and toxin production by enterotoxic staphylococci. *Journal of Infectious Diseases* **81**, 228–243.

SMITH, J. T. (1963*a*). Penicillinase and ampicillin resistance in a strain of *Escherichia coli*. *Journal of General Microbiology* **30**, 299–306.

SMITH, J. T. (1963*b*). Sulphydryl groups essential for the penicillinase activity of *Aerobacter cloacae*. *Nature, London* **197**, 900–901.

SMITH, J. T., BREMNER, D. A. & DATTA, N. (1974). Ampicillin resistance of *Shigella sonnei*. *Antimicrobial Agents and Chemotherapy* **6**, 418–421.

THOMPSON, J. S., SEVERSON, C. D., STEARNS, N. A. & KASIK, J. E. (1972). Immunological distinction of mycobacterial beta-lactamases. *Infection and Immunity* **5**, 542–546.

YAMAGISHI, S., O'HARA, K., SAWAI, T. & MITSUHASHI, S. (1969). The purification and properties of penicillin β-lactamases mediated by transmissible R factors in *Escherichia coli*. *Journal of Biochemistry (Tokyo)* **66**, 11–20.