Some Relationships between R-Factor and Chromosomal β -Lactamase in Gram-Negative Bacteria

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1. The β -lactamases specified by *Klebsiella aerogenes* 418 and the R-factor R-7268 have been partially purified. 2. The molecular weights of the *K. aerogenes* strains 418 and 373, *Aerobacter cloacae* 53, R-7268 and R-TEM β -lactamases were all about 20000; that of the enzymes from *Escherichia coli* strains 419 and 214T was about 31000. 3. These enzymes were also compared by means of their K_m values for benzylpenicillin and ampicillin, and their behaviour on starch-gel electrophoresis. 4. The β -lactamases specified by the two *Klebsiella* strains, the *Aerobacter* strain, and the R-factors R-TEM and R-7268 were found to comprise a broadly similar group. However, within this group, only two enzymes seemed to be identical, namely those specified by the two R-factors. The two *E. coli* strains produce identical β -lactamases which are very different from the '*Klebsiella/Aerobacter*-type' enzymes.

β -Lactamase [penicillinase, or penicillin (cephalosporin) β -lactam amidohydrolase, EC 3.5.2.6] has been found in a number of bacteria (see reviews by Citri & Pollock 1966; Smith, Hamilton-Miller & Knox, 1969) and some of these enzymes have been purified from Gram-positive bacteria (see Citri & Pollock, 1966), Gram-negative species (Hennessey & Richmond, 1968; Huber, Caltrider & Noble, 1968; Lindstrom & Boman, 1968; Lindstrom, Boman & Steele, 1970; Smith, 1970; Jack & Richmond, 1970a) and R-factor-specified β -lactamases (Datta & Richmond, 1966; Neu, 1968; Yamagishi, O'Hara, Sawai & Mitsuhashi, 1969; Lindqvist & Nordstrom, 1970; Neu & Winshell, 1970). However, apart from these, information about the properties of the Gram-negative β -lactamases is rather superficial, being in many cases confined to a cursory examination of substrate specificity. Jack & Richmond (1970b) investigated some properties of a number of β -lactamases from Gram-negative bacteria, and divided them into eight types, principally on the basis of their relative activity against benzylpenicillin, ampicillin and cephaloridine. Ooka, Hashimoto & Mitsuhashi (1970) attempted to classify R-factor β -lactamases on a similar basis. These schemes are however, somewhat arbitrary. Smith & Hamilton-Miller (1963) suggested that each bacterial species may produce a distinct enzyme but, since then, it has been found that even within a species, significant variation in β -lactamase can occur from strain to strain (Hamilton-Miller, 1968). The object of the present work was to investigate

more deeply the properties of the β -lactamases produced by some Gram-negative bacteria, including some R-factor-specified enzymes.

MATERIALS AND METHODS

The bacterial strains used in this study are listed in Table 1, together with the substrate profiles of their β -lactamases; the R-factors used were R-7268 and R-TEM (Datta & Kontomichalou, 1965; Smith, 1969). R-7268 has also been referred to as R-1 (Meynell & Datta, 1966).

Other materials and methods used were as described in the preceding papers (Dale & Smith, 1971; Dale, 1971). Buffer A was 0.025M-sodium phosphate, pH 7.4.

RESULTS

Purification of the β -lactamase from Klebsiella aerogenes 418. Column chromatography was done at room temperature and all other procedures at 4°C.

Steps (i) and (ii). These were performed largely as described by Dale & Smith (1971). Briefly, the bacteria were grown in unsupplemented glucose-DM medium (Davis & Mingioli, 1950), washed and resuspended in buffer A. After ultrasonication, cell debris was removed by centrifugation and the supernatant was treated with protamine sulphate. The precipitate was discarded and the supernatant was fractionally precipitated with ammonium sulphate at first 25% saturation, then 50% saturation.

Step (iii). The redissolved precipitate from step (ii) (50%-saturated ammonium sulphate) was

Table 1. *Substrate specificity of β -lactamases*

Absolute activity is expressed as nmol of benzylpenicillin hydrolysed/min per 10^9 cells (as cell extract). Values for other substrates are expressed as a percentage, of the activity against benzylpenicillin. The values were taken from: Datta & Kontomichalou (1965); Hamilton-Miller (1963, 1967); Hamilton-Miller, Smith & Knox (1965); Smith (1963, 1969, 1970); Smith & Hamilton-Miller (1963). —, Not determined.

	R-factors*		<i>Klebsiella aerogenes</i>		<i>A. cloacae</i>	<i>Escherichia coli</i>	
	7268	TEM	418	373	53†	214T	419
Absolute activity	280	1700	13	1360	826	54	10
Ampicillin	100	144	185	170	124	3	7
Oxacillin	—	—	10	10	34	—	5
6-Aminopenicillanic acid	87	140	55	65	21	7	5
Cephaloridine	110	130	9	69	64	172	230
Cephalosporin C	5	5	—	—	—	407	1250

* With *E. coli* K12 as host.

† Assayed at pH 6 (optimum).

dialysed overnight against 5 litres of 10 mM-sodium phosphate buffer, pH 8.0, and applied to a column of DEAE-cellulose (60 cm $\times$ 4 cm²) previously equilibrated with the same buffer. The enzyme was washed on with 200 ml of buffer, and then a linear gradient of 0–1.0 M-sodium chloride in buffer (total volume 400 ml) was applied. The β -lactamase was eluted at a sodium chloride concentration of about 0.1 M.

Step (iv). The fractions containing enzyme from step (iii) were pooled, and solid ammonium sulphate was added to 60% saturation. The precipitate was collected by centrifugation and redissolved in 2 ml of buffer A. This was then applied to a column (60 cm $\times$ 4 cm²) of Sephadex G-100, which was developed by upward flow with buffer A.

The results obtained by this method are shown in Table 2; it can be seen that a purification of over 1000-fold was achieved in the best fraction from step (iv) and the total yield in pooled eluate from Sephadex was 4% of the starting material. The amount of 'purified' protein was too small to enable any independent criteria of purity to be applied. There was, however, considerable variation in the specific activity of the β -lactamase-containing fractions obtained from Sephadex G-100 in step (iv), which indicates some degree of heterogeneity.

Purification of the β -lactamase specified by R-7268.

A partial purification of the β -lactamase produced by *Escherichia coli* J6-2 (R-7268) was achieved by a method involving fractional precipitation with ammonium sulphate, DEAE-cellulose column chromatography and Sephadex G-100 gel filtration (Dale, 1970). The best fraction obtained by this method had a specific activity of 150 units/mg of protein, which represents a purification of over 200-fold with respect to the crude preparation. However, the enzyme at this stage was still not pure.

After this section of the work had been completed, Lindqvist & Nordstrom (1970) published a more complete purification of this enzyme. By including a preparative polyacrylamide-gel electrophoresis step they were able to obtain a preparation with a specific activity of over 2000 units/mg of protein.

Molecular weights. These were determined in general by the method of Andrews (1964, 1965) by using Sephadex G-100 as described in detail by Dale & Smith (1971). The amount of crude β -lactamase used in each run was between 3 and 30 international units, the variation being due to differences in enzyme activities in the different organisms and the undesirability of applying large volumes (greater than 2 ml) to the column. The results obtained are listed in Table 3. The molecular weight of the R-TEM β -lactamase reported here (20550) does not agree with the value (16700) obtained by Datta & Richmond (1966) which was measured by equilibrium ultracentrifugation. Because of this discrepancy, it seemed necessary to check the molecular weight of R-TEM β -lactamase by centrifugation to see if the method used could account for the difference. Enzyme and marker were layered on a 5–20% gradient of sucrose in buffer A in Strohmaier cells. These were centrifuged for 20 h at 20°C at 26000 rev./min in a S9740 swing-out rotor in a Griffin-Christ Omega II-70 ultracentrifuge. After being left to stop with brake off, the contents of the cells were fractionated from the bottom with the sampling tube in the wall of the Strohmaier cell. Three estimates of the molecular weight were made by using the method of Martin & Ames (1961). By comparison with the molecular weights of chymotrypsinogen (25000), cytochrome *c* (12400), and myoglobin (17800) the results obtained for the molecular weight of R-TEM β -lactamase were 20980, 20800 and 23240 respectively; the

Table 2. *Purification of β -lactamase from K. aerogenes 418*

The volume of the culture was 8 litres. One unit of β -lactamase hydrolyses 1 μ mol of benzylpenicillin/min.

Step	Volume (ml)	Total protein (mg)	Total enzyme activity (units)	Specific activity (units/mg of protein)	Purification
(i) Crude enzyme	115	2498	204.5	0.082	1
(ii) Ammonium sulphate precipitate (50% saturation)	30	948	72.5	0.077	0.9
(iii) DEAE-cellulose	70	9.8	44.6	4.55	55.5
(iv) Sephadex G-100					
Pooled fractions 44-50	21	0.1	8.2	82.0	1000
Fraction 47	3	0.014	1.7	121.5	1482

mean value being 21670. This result supports the value obtained here with exclusion chromatography and suggests that Datta & Richmond's (1966) estimate for the molecular weight of R-TEM β -lactamase is inaccurate.

K_m values. These were measured as described by Dale & Smith (1971), and the results obtained for benzylpenicillin and ampicillin are listed in Table 4, together with the previously published values for these or similar β -lactamases. It will be noted that there is a discrepancy in the values for the enzyme extracted from *Klebsiella aerogenes* 418. Hamilton-Miller (1963) used the spectrophotometric method of Saz, Lowery & Jackson (1961); it is not known whether this difference in assay method could account for the wide differences in the apparent *K_m* value for ampicillin.

Table 4 also shows that Lindstrom *et al.* (1970) obtained a *K_m* value for *E. coli* β -lactamase against benzylpenicillin which is about ten times as high as reported here for *E. coli* strains 419 and 214T. In addition, workers from the same laboratory (Burman, Nordstrom & Boman, 1968; Lindqvist & Nordstrom, 1970) obtained *K_m* values of the R-7268 β -lactamase against both benzylpenicillin and ampicillin, which were also ten times as high as those presented here for the R-7268 enzyme. Our results are to some extent confirmed by the fact that they are in reasonable agreement with both sets of values for R-TEM β -lactamase quoted in Table 4. As discussed below, R-TEM and R-7268 β -lactamases are very similar in many respects, if not absolutely identical. It remains a possibility, therefore, that the calculations of the Swedish workers are incorrect by a factor of 10 in both instances.

Starch-gel electrophoresis. The gels were prepared and run as described by Smithies (1955), by using trays (180 mm $\times$ 60 mm $\times$ 6 mm). The following buffer systems were used: 1, sodium acetate, pH 5 (bridge, 75 mm; gel, 38 mm); 2, sodium phosphate, pH 6 (bridge, 100 mm; gel, 25 mm); 3, sodium borate, pH 8.2 (bridge, 200 mm; gel, 25 mm); 4,

bridge, sodium borate, pH 8.65 (0.3 M); gel, tris-citrate, pH 8.65 (Poulik, 1957).

Crude preparations of β -lactamase were used, applied on filter paper inserts. After electrophoresis, the gels were sliced horizontally, and the cut surfaces were treated with penicillin-iodine as described by Lampen (1967).

Figs. 1-4 show that in all four buffer systems the enzymes specified by the R-factors 7268 and TEM were inseparable. They are quite clearly different from all the other β -lactamases tested, being more acidic. The *K. aerogenes* 418 enzyme is readily separable in all four systems. It should be noted that if the run in borate buffer is continued for a longer time (5½ h) the separation becomes more conclusive. Jack & Richmond (1970b) using borate buffer at pH 8.5 also found a difference in electrophoretic mobility between *K. aerogenes* 418 and *Aerobacter cloacae* 53 β -lactamases. The enzyme produced by *A. cloacae* 53 is similar to *K. aerogenes* 373 β -lactamase, but can be separated from it in acetate buffer, pH 5, or in Poulik's (1957) discontinuous buffer system.

The β -lactamases from *E. coli* strains 214T and 419 were inseparable either in Poulik's (1957) buffer or in acetate buffer; the fact that they move towards the cathode at pH 8.65 but do not move from the origin at pH 5 might be due to insolubility at the latter pH value.

DISCUSSION

The β -lactamases produced by *E. coli* strains 214T and 419 cannot be differentiated on the basis of their molecular weight, mobility on starch-gel electrophoresis, or *K_m* value for benzylpenicillin. The substrate profiles of these two enzymes are also very similar (Hamilton-Miller *et al.* 1965; Hamilton-Miller, 1967). It is therefore very likely that the β -lactamases produced by these two strains are in fact identical, although the level of enzyme activity per bacterial cell is different

Table 3. *Molecular weights of β -lactamases*

Crude β -lactamase was mixed with bovine serum albumin, ovalbumin, cytochrome *c* and chymotrypsinogen and applied to a column of Sephadex G-100 as described by Dale & Smith (1971). The molecular weight of the β -lactamase was estimated from the relationship between elution volume and log (molecular weight) (Andrews, 1964, 1965).

	Mean value	S.D.	No. of runs
<i>E. coli</i> J6-2 (R-TEM)	20550	2180	4
<i>E. coli</i> J6-2 (R-7268)	20600	2610	3
<i>A. cloacae</i> 53	23070	490	3
<i>K. aerogenes</i> 418	20080	1840	5
<i>K. aerogenes</i> 373	18000	350	2
<i>E. coli</i> 214T	31150	1830	3
<i>E. coli</i> 419	31530	1150	3

Table 4. *K_m values (μ M) of β -lactamases*

Experimental details are given in the text. —, Not determined.

	Benzylpenicillin	Ampicillin	Reference
R-1818	4	8	Dale (1971)
R-GN238	5	18	Yamagishi <i>et al.</i> (1969)
R-TEM	14	7	Present work
R-TEM	22	2.3	Datta & Richmond (1966)
R-7268	11	11	Present work
R-7268	100	160	Lindqvist & Nordstrom (1970)
R-GN14	28	32	Yamagishi <i>et al.</i> (1969)
<i>A. cloacae</i> 53	5	19	Present work
<i>K. aerogenes</i> 373	5	22	Present work
<i>K. aerogenes</i> 418 (crude)	7	4	Present work
<i>K. aerogenes</i> 418 (purified)	9	—	Present work
<i>K. aerogenes</i> 418	<130	600	Hamilton-Miller (1963)
<i>E. coli</i> 214T	0.9	—	Present work
<i>E. coli</i> 419	0.7	—	Present work
<i>E. coli</i> K12	12	5	Lindstrom <i>et al.</i> (1970)

Fig. 1. Starch-gel electrophoresis of β -lactamases. Diagrammatic representation of results. The method is described in the text. The cathode (—) is to the left. Electrophoresis was carried out for 6 h at 20 mA in acetate buffer, pH 5.

Fig. 2. Starch-gel electrophoresis of β -lactamases. Diagrammatic representation of results. The method is described in the text. The cathode (—) is to the left. Electrophoresis was for 20 h at 15 mA in phosphate buffer, pH 6.

(Hamilton-Miller *et al.* 1965). This is presumably due to a difference in regulation, which is known to occur in *E. coli* as a result of stepwise mutation

(Smith, 1963; Eriksson-Grennberg, Boman, Jansson & Thoren 1965). Lindstrom *et al.* (1970) have described the purification and characterization of the β -lactamase produced by *E. coli* K12; the gene for this enzyme has been mapped on the K12 chromosome (Eriksson-Grennberg *et al.* 1965;

Eriksson-Grennberg, 1968a,b). The molecular weight and substrate profile of the K12 β -lactamase are very similar to those of the two *E. coli* β -lactamases described here and it is possible that all three enzymes are specified by a similar chromosomal gene.

Another pair of β -lactamases that are indistinguishable are those specified by R-7268 (R-1) and R-TEM, although these two R-factors are otherwise quite distinct. R-TEM, which confers resistance to ampicillin and streptomycin, was isolated in Athens from *E. coli*, whereas R-7268, which confers resistance to chloramphenicol, sulphonamides and kanamycin as well as to ampicillin and streptomycin was isolated in London from *Salmonella paratyphi* (Datta & Kontomichalou, 1965). In addition, R-TEM specifies a much higher degree of β -lactamase activity per cell than does R-7268. Despite these differences, these enzymes may be identical, and support for this view is lent by findings of other workers. The optimum pH values and the specific activities of highly purified preparations are similar for both enzymes (Datta & Richmond, 1966; Lindqvist & Nordstrom, 1970), and M. H. Richmond (unpublished work, cited by

Lindqvist & Nordstrom, 1970) has shown that R-7268 and R-TEM β -lactamases cross-react immunologically.

Lindqvist & Nordstrom (1970) found that the molecular weight of R-7268 β -lactamase was 22000; this result is supported by the value of 20600 reported here. On the other hand, Datta & Richmond (1966) reported the molecular weight of R-TEM β -lactamase to be 16700. This apparent difference in the molecular weights of the two enzymes can be resolved by the results reported here, which show that R-TEM β -lactamase has a molecular weight of 20550 when measured by exclusion chromatography, and 21670 by centrifugation. If these values are taken instead of the estimate of Datta & Richmond (1966) then the β -lactamases of R-TEM and R-7268 do not differ significantly in any respect. It should be noted that Datta & Richmond (1966) used *E. coli* strain TEM as the host, whereas the present work was done with *E. coli* strain K12 as the host. However, it is unlikely that host strain differences could account for the molecular weight discrepancy for the β -lactamase of R-TEM, since Dale & Smith (1971) and Dale (1971) have shown, with R-1818, that even species differences do not result in alterations in R-factor β -lactamase.

The remainder of the β -lactamases studied here, from *K. aerogenes* strains 418 and 373 and *A. cloacae* 53, although similar in several respects are not identical when examined closely. When this work was initiated, it was thought possible that the β -lactamases of these bacterial species might be the same as those mediated by R-factors TEM and 7268. This has not turned out to be the case, since these R-factor enzymes can be readily distinguished from the bacterial β -lactamases, particularly by starch-gel electrophoresis.

Jack & Richmond (1970b) studied the β -lactamases produced by a range of Gram-negative

Fig. 3. Starch-gel electrophoresis of β -lactamases. Diagrammatic representation of results. The method is described in the text. The cathode (—) is to the left. Electrophoresis was for 3 h at 10 mA in borate buffer, pH 8.2.

Fig. 4. Starch-gel electrophoresis of β -lactamases. Diagrammatic representation of results. The method is described in the text. The cathode (—) is to the left. Electrophoresis was for 5.5 h at 10 mA in Poulik's (1957) discontinuous buffer system, pH 8.65.

bacteria and attempted to classify them primarily by their enzymic activity on ampicillin and cephaloridine relative to that with benzylpenicillin. However, substrate profiles are notoriously susceptible to variation from one laboratory to another, presumably because of differences in technique. In addition, it would seem that a number of β -lactamases do not fit their classification scheme, e.g. R-GN238 (Yamagishi *et al.* 1969), *K. aerogenes* 415 and *E. coli* 214T (Hamilton-Miller *et al.* 1965); on the other hand, the R-1818 β -lactamase would be classified with a number of enzymes with which it has virtually nothing else in common (Dale, 1971). It is possible that if more β -lactamases were studied, a more or less continuous spectrum of substrate specificity would be observed. The same criticisms can be levelled against other schemes of classification based on substrate profiles, e.g. Ooka *et al.* (1970). It can be concluded that molecular weights and starch-gel electrophoretic mobilities are more specific criteria for the comparison of β -lactamases.

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