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Characterization of the β-Lactamase Specified by the Resistance Factor R-1818 in *E. coli* K12 and Other Gram-Negative Bacteria

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1. The amino acid composition of the β-lactamase from *E. coli* (R-1818) was determined. 2. The R-1818 β-lactamase is inhibited by formaldehyde, hydroxylamine, sodium azide, iodoacetamide, iodine and sodium chloride. 3. The K_m values for benzylpenicillin, ampicillin and oxacillin have been determined by using the R-factor enzyme from different host species. The same values were obtained, irrespective of the host bacterium. 4. The molecular weight of the enzyme was found to be 44600, and was the same for all host species. 5. The relationship of R-1818 and R-GN238 β-lactamases is discussed.

The resistance (R-) factor R-1818 (also known as R-46 or R-Brighton), which confers on its host bacterium resistance to ampicillin, streptomycin, sulphonamides and tetracycline, has been shown to specify the production of a β-lactamase [penicillinase, penicillin (cephalosporin) β-lactam amidohydrolase, EC 3.5.2.6] with an unusual substrate 'profile' (Datta & Kontomichalou, 1965; J. M. T. Hamilton-Miller, unpublished work, quoted by Smith, 1969; Dale & Smith, 1971*a*); in particular, it is 6–7 times as active against oxacillin (normally considered a 'penicillinase-stable' penicillin) as against benzylpenicillin. Smith (1969) transferred this R-factor to several different species of Gram-negative bacteria, and found that a similar amount of R-factor specific β-lactamase was produced in *Escherichia coli*, *Serratia marcescens*, *Alkalescens*, and *Klebsiella aerogenes*. In *Proteus mirabilis*, however, the R-factor β-lactamase activity per organism was only one-twentieth of that in the other species. Dale & Smith (1971*a*) were unable to detect any difference in the properties of the R-1818 β-lactamase when obtained from *E. coli* or *P. mirabilis* harbouring a high-activity β-lactamase mutant of R-1818. Neu & Winshell (1970) found, with R-B1H9, that the β-lactamase specified was similar in several different host species. It has also been shown for episomes carrying genetic determinants for alkaline phosphatase (Signer, Torriani & Levinthal, 1961) or β-galactosidase (Falkow, Wohlheiter, Citarella & Baron, 1964; Colby & Hu, 1968) that the properties of the enzyme specified by the episome are not affected by a change of host species. The object of the present work was to characterize the R-1818 β-lactamase further, and to extend the comparison of its properties to other host species.

MATERIALS AND METHODS

The bacterial strains used were those described by Smith (1969). For details of other materials and methods, see the previous paper (Dale & Smith, 1971*a*). Crude enzyme preparations were used except where otherwise noted.

RESULTS AND DISCUSSION

Effect of inhibitors. A number of substances were tested for possible inhibitory effects on a crude preparation of R-1818 β-lactamase from *E. coli*, without preincubation, by using the hydroxylamine assay. The majority of the compounds tested (Table 1), including EDTA and *p*-chloromercuribenzoate, had no effect at the concentrations used.

During the investigation of the effect of pH on the activity of R-1818 β-lactamase (Dale & Smith, 1971*a*) it was observed that the enzyme was apparently inhibited in tris–HCl buffer and that the inhibition decreased with increasing pH (Dale, 1970). It was estimated that if it was the Cl^- that was responsible for the decreased enzyme activity then a Cl^- concentration of 16mM was required to cause 50% inhibition. When the effect of sodium chloride on enzyme activity was tested directly, it was found that a concentration of 16mM caused 50% inhibition of the R-1818 β-lactamase (see Fig. 1). Yamagishi, O'Hara, Sawai & Mitsuhashi (1969) have described the inhibition of R-GN238 β-lactamase by sodium chloride.

Table 1. *Effect of possible inhibitors on R-1818 β-lactamase*

	Concn. (mM)	Activity remaining (%)
o-Iodosobenzoic acid	10	100
N-Ethylmaleimide	10	100
Formaldehyde	10	40
Hydroxylamine	10	0
EDTA	1	100
p-Chloromercuribenzoate	1	100
Potassium cyanide	1	100
Phenylhydrazine	1	100
2,4-Dinitrophenol	1	100
Sodium fluoride	1	100
Sodium chloride	16	50
Sodium azide	2.5	50
Iodoacetamide	10	50
Iodine	0.07	50

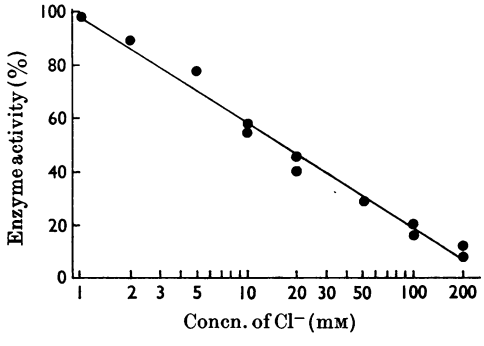

Fig. 1. Effect of Cl^- concentration on the activity of β-lactamase from *E. coli* (R-1818). A crude β-lactamase preparation from *E. coli* (R-1818) in phosphate buffer was used with oxacillin as substrate.

K_m *values.* These were determined as described by Dale & Smith (1971*a*), by using the micro-iodometric assay method; correction was made for the non-specific rate of decolorization of the starch–iodine by the proteins in the sample. The lower the specific activity of the preparation, the more necessary this correction becomes; in some cases, the non-specific rate can be so high as to render it impossible to determine the K_m value with any degree of accuracy. The results obtained with benzylpenicillin, ampicillin and oxacillin as substrate (Table 2) show that the affinity of the R-1818 β-lactamase for these substrates is not affected by the bacterial strain harbouring the R-factor. Dale & Smith (1971*a*) also showed that purified preparations of the R-1818 β-lactamase from *E. coli* J6-2 and *P. mirabilis* F67 had the same K_m values for a range of substrates.

The fact that R-1818 β-lactamase has a comparatively high K_m for oxacillin (Table 2) is noteworthy. Whereas at saturating substrate concentrations (2mg/ml in the hydroxylamine assay) oxacillin is hydrolysed at 6.7 times the rate of benzylpenicillin (Dale & Smith, 1971*a*), at progressively lower concentrations this ratio is decreased and eventually reversed. Pollock (1965) introduced the term 'physiological efficiency' (defined as $V_{max.}/K_m$) as a convenient way of describing the behaviour of enzymes at substrate concentrations below their K_m. However, the approximation on which this idea is based is not really valid unless the substrate concentration is less than one-tenth of the K_m value (see Dale, 1970).

Attempts were made to measure the K_m of R-1818 β-lactamase for cloxacillin, but it was found that at a substrate concentration of 200 μM, the rate was barely measurable, even with a high concentration of enzyme. It is not practicable to use substrate concentrations much higher than this with the micro-iodometric assay, as the spontaneous rate of reduction of iodine becomes too great. By using the hydroxylamine assay, it was estimated that the K_m value for cloxacillin was greater than 1mM; however, since this method requires relatively large changes in penicillin concentration for the rate to be measurable, it was not possible to obtain an accurate value for the K_m.

Molecular weights. These were determined by Sephadex-gel filtration (Andrews, 1964, 1965) as described by Dale & Smith (1971*a*).

During preliminary runs with the R-1818 β-lactamase, in which Blue Dextran was included, the enzyme was eluted from the column at the void volume, simultaneously with the Blue Dextran. This result was unexpected since preparative runs on Sephadex G-100 had indicated that R-1818 β-lactamase was considerably retarded (Dale & Smith, 1971*a*). The most likely explanation is that the enzyme binds in some way to the Blue Dextran; Haeckel, Hess, Lauterborn & Wüster (1968) described a similar effect with yeast pyruvate kinase. In contrast, however, R-1818 β-lactamase was not easily dissociated from the Blue Dextran, and was strongly inhibited by it (Dale, 1970).

The molecular-weight results obtained by exclusion chromatography are given in Table 3; it can be shown by analysis of variance that there is no statistically significant difference in the values obtained for the different host bacteria. The overall mean value is 44560, with the standard deviation 1300.

The molecular weights of a few other R-factor specified β-lactamases have been reported (Lindqvist & Nordstrom, 1970; Neu & Winshell, 1970; Dale & Smith, 1971*b*); R-1818 β-lactamase is considerably larger than any of these. Indeed, as far

Table 2. *K_m values (μM) of R-1818 β-lactamase from various host bacteria*

All values were obtained with crude enzyme preparations except those marked * for which purified enzyme preparations (Dale & Smith, 1971*a*) were used.

	Benzyl-penicillin	Ampicillin	Oxacillin
E. coli J6-2 (R-1818)	4.4	8.4	88.8*
E. coli K12 (R-1818)	2.6	6.8	74.3
S. marcescens (R-1818)	4.1	10.5	121
Alkalescens (R-1818)	4.4	9.0	84.1
P. mirabilis F67 (R-1818)*	3.8	5.9	92.5
K. aerogenes 418 (R-1818)	Not determined		

Table 3. *Molecular weight of R-1818 β-lactamase from various sources*

The molecular weights were determined by using Sephadex G-100. About 2 units of crude β-lactamase were used in each run.

	Mean	S.D.	No. of runs
E. coli J6-2 (R-1818)	44140	1630	3
E. coli K12 (R-1818)	44570	740	3
S. marcescens (R-1818)	45870	230	2
Alkalescens (R-1818)	46130	300	2
P. mirabilis F67 (R-1818)	43900	70	2
P. mirabilis 130 (R-1818)	43560	1000	2
K. aerogenes 418 (R-1818)*	43930	2000	2
Pooled results	44560	1300	16

* R-1818 β-lactamase only.

as is known, this is the highest molecular weight yet reported for any β-lactamase.

Starch-gel electrophoresis. The method for this was as described by Dale & Smith (1971*b*); the enzyme was apparently inactivated in acetate buffer, pH 5, since no spots were observed. Oxacillin was the substrate used for the location of the β-lactamase, to avoid any interference from other β-lactamases, but multiple (usually two) bands were frequently obtained, rendering comparison of the different preparations difficult. This effect is not yet understood and taken at face value would suggest the presence of isoenzymes. However, it is thought to be an artifact for two reasons: first, only one band was obtained on acrylamide-gel electrophoresis (Dale & Smith, 1971*a*); secondly, the pattern of bands on starch gels is not consistent from one run to the next, even with the same sample of enzyme.

However, it was established that there was no consistent difference between the β-lactamases obtained from the different host species; all moved slowly towards the cathode at pH 8.2 and towards the anode at pH 8.65. No correction was made for electro-endosmosis.

Comparison of R-1818 and R-GN238 β-lactamases. Yamagishi *et al.* (1969) described the purification and properties of the β-lactamase specified by the R-factor GN238; this appears to be the only β-lactamase so far described in detail that resembles the R-1818 β-lactamase, so it is worth comparing the results they obtained with those described in this paper and by Dale & Smith (1971*a*). This comparison is set out in Table 4.

(*a*) Specific activity. From these values, it appears that R-GN238 β-lactamase has a much lower specific activity, both in the crude extract and as a purified preparation, than does the R-1818 enzyme. These results must be viewed with caution, however, as there seems to be an error in their values: Yamagishi *et al.* (1969) apparently obtained 107g of protein from 70g (wet wt.) of cells.

(*b*) Substrate specificity. The most unusual feature of R-1818 β-lactamase is the fact that it hydrolyses oxacillin (normally considered a 'penicillinase-stable' penicillin) at about 6.7 times the rate at which it hydrolyses benzylpenicillin (Dale & Smith, 1971*a*). Unfortunately, Yamagishi *et al.* (1969) did not measure the rate of hydrolysis of oxacillin by R-GN238 β-lactamase, but it is significant that the rate of hydrolysis of cloxacillin was about three times that of benzylpenicillin; this is similar to the results obtained with R-1818 β-lactamase (Table 4). The overall picture with the

Table 4. *Comparison of R-1818 and R-GN238 β-lactamase*

The results for R-GN238 are from Yamagishi *et al.* (1969).

	R-1818	R-GN238
Specific activity (units/mg of protein)		
Crude	0.46	0.0223*
Purified	143	3.36*
Relative activity		
Benzylpenicillin	100	100
Phenethicillin	387	155
Ampicillin	175	450
Cloxacillin	201	292
6-Aminopenicillanic acid	68	263
Cephaloridine	43	36
K_m (μM)		
Benzylpenicillin	4.0	5.1
Phenethicillin	15.5	6.4
Ampicillin	8.0	15.9
Cloxacillin	—	13.0
6-Aminopenicillanic acid	61.4	26.3
Cephaloridine	58.7	111.0
pH optimum (95% activity)	7.0–7.6	7.2–7.8
Isoelectric point	8.3	8.3
Inhibitors		
p-Chloromercuribenzoate (1 mM)	No effect	No effect
EDTA (1 mM)	No effect	No effect
Iodine	50% inhibition at 0.07 mM	60% inhibition at 5 mM
Chloride	50% inhibition at 16 mM	71% inhibition at 14 mM

* These values are possibly in error (see the text).

other penicillins is broadly similar in the two cases, though clearly not identical. However, substrate-specificity results are notoriously susceptible to variation according to the methods used. The substrate specificity of R-GN718 β-lactamase (Ooka, Hashimoto & Mitsuhashi, 1970) is also similar to that of R-1818 β-lactamase but no other properties of the enzyme were described.

(*c*) K_m values. Once again there seems to be a broad degree of similarity although there appear to be differences for some substrates, particularly cloxacillin. However, as the determination of the K_m of this substrate presented difficulties, too much weight should not be given to this difference.

(*d*) pH optima. The pH optima and the isoelectric points appear to be virtually identical for the two enzymes.

(*e*) Inhibitors. The R-1818 β-lactamase is considerably more sensitive to iodine than is the R-GN238 enzyme; on the other hand they are both inhibited to a similar extent by Cl^-.

In the absence of a direct comparison of the two enzymes in the same laboratory, it is difficult to reach a definite conclusion about their relationship. However, the high activity against cloxacillin, the high isoelectric point, and the inhibition by Cl^- are in themselves sufficient to suggest the possibility of a close relationship between these two enzymes. On the other hand, the differences apparent from Table 4, if they are not ascribed to differences in methods of measurements, indicate that the two enzymes are not in fact identical.

Amino acid composition. The β-lactamase from *E. coli* (R-1818) was purified as described by Dale & Smith (1971*a*). The redissolved precipitate from step (vi) was centrifuged at 20000*g* for 30min, dialysed exhaustively against deionized water, and then freeze-dried in a pre-weighed tube. The yield was 6.3mg. Portions of the purified enzyme were hydrolysed by heating with 6M-hydrochloric acid under vacuum in a sealed tube, one portion for 24h and another for 72h. The amino acids were then analysed on a Locarte amino acid analyser (I am grateful to Dr M. D. Melamed for doing this analysis). The values for threonine and serine were extrapolated to the value at zero time; for valine and isoleucine the 72h value was taken. Cysteine was not determined. Tryptophan was determined on a third portion by the method of Edelhoch (1967), which also gave a confirmatory result for tyrosine. The number of amino acid residues per molecule was calculated on the basis of a molecular weight of

Table 5. *Amino acid composition of R-1818 β-lactamase*

The purified β-lactamase from *E. coli* J6-2 (R-1818) was used. The values given are molar ratios, taking leucine as 100. The results for threonine and serine were extrapolated to zero time. For valine and isoleucine, the 72h result was used. Tryptophan was determined spectrophotometrically. The amount of protein used for the 72h hydrolysis was too small to allow accurate determination of histidine and proline. The values for *E. coli* K12 are from Lindstrom *et al.* (1970).

	Hydrolysis time			Estimated no. of residues/molecule	
	24h	72h	Average	R-1818	*E. coli* K12
Lys	85.2	82.9	84.1	22	17
His	17.1	—	17.1	5	4
Arg	127.4	120.5	123.9	33	9
Asx	136.8	135.9	136.4	36	24
Thr	42.2	40.2	43.2	11	18
Ser	61.9	50.4	67.8	18	15
Glx	122.3	126.5	124.4	33	32
Pro	8.7	—	8.7	2	19
Gly	82.9	82.1	82.5	22	23
Ala	121.2	115.4	118.3	31	28
Val	80.3	84.6	84.6	22	17
Met	24.3	21.4	22.9	6	4
Ile	71.4	73.5	73.5	19	16
Leu	100	100	100	26	23
Tyr	29.7	26.5	28.1	7	11
Phe	79.8	79.5	79.7	21	7
Cys	—	—	—		0
Trp	—	—	57.0	15	6

44560, ignoring the possible presence of cysteine; the results are given in Table 5, together with those obtained by Lindstrom, Boman & Steele (1970) for the β-lactamase produced by *E. coli* K12. Jack & Richmond (1970) showed that R-TEM, *Aerobacter cloacae* 53 and *Escherichia cloacae* 214 β-lactamases all had similar amino acid compositions (in terms of percentage by weight) to the *E. coli* K12 enzyme. Compared with *E. coli* K12 β-lactamase, and also with other β-lactamases (Citri & Pollock, 1966; Neu & Winshell, 1970), the R-1818 β-lactamase has a very high content of arginine and phenylalanine whereas the proline content is very low. These results are further evidence of the unusual nature of R-1818 β-lactamase.

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