JOURNAL OF BACTERIOLOGY, Aug. 1974, p. 351-356

Vol. 119, No. 2
Printed in U.S.A.

R-Factor-Mediated β-Lactamases That Hydrolyze Oxacillin: Evidence for Two Distinct Groups

J. W. DALE AND J. T. SMITH

Department of Pharmaceutics, The School of Pharmacy, University of London, London, WC1N 1AX, England

Received for publication 4 March 1974

The enzymatic and molecular properties of 14 oxacillin-hydrolyzing β-lactamases, all of them R-factor-specified, were studied, and two distinct groups were found. Four of the enzymes had a molecular weight of 24,000, were active against methicillin, and had an electrophoretic mobility of −0.1 cm/h. Eight enzymes had a molecular weight of 45,000, low activity against methicillin, and an electrophoretic mobility of +0.5 cm/h. The remaining two enzymes were similar to those of the second group in being relatively inactive against methicillin, but their molecular weight was lower (42,000) and their electrophoretic mobility was different (−0.1 cm/h). All the enzymes of both groups were sensitive to inhibition by sodium chloride. The two groups were not completely homogeneous in their enzymatic properties; seven possible subtypes could be recognized.

Oxacillin is a semisynthetic penicillin that is usually resistant to hydrolysis by β-lactamase (penicillinase) (penicillin/cephalosporin β-lactam amidohydrolase, EC 3.5.2.6). However, the β-lactamase mediated by the R-factor R46 (R1818) hydrolyzes oxacillin more rapidly than benzylpenicillin (18). Enzymes that do this are not common and have been found only as R-factor-mediated. Two such enzymes have been purified: those specified by R_{GN238} (23) and by R46 (4). Dale and Smith (6) were able to distinguish between these two β-lactamases, particularly on the basis of molecular weight. This difference was later confirmed by Richmond and Sykes (17), who referred to the two enzymatic types as Va and Vb, respectively.

Hedges et al. (12), using substrate specificity profiles, were able to identify five different types of R-factor-mediated β-lactamases that hydrolyzed oxacillin more rapidly than benzylpenicillin. These were termed "oxacillin-hydrolysing β-lactamases." This paper reports an examination of the physical properties of the same β-lactamases (12), together with a comparative study of another five R-factor-mediated oxacillin-hydrolyzing β-lactamases, two of which are being reported for the first time. The relationships between these enzymes were studied to provide information on the origin of R-factor-mediated ampicillin resistance.

MATERIALS AND METHODS

The materials and methods have been described in detail previously (4). Crude enzyme preparations were made as described by Hedges et al. (12); β-lactamase activity was measured by the hydroxylamine method (4). One unit of enzyme activity is that amount of enzyme which will catalyze the hydrolysis of 1 μmol of benzylpenicillin per min at 30 C and pH 7.4. Transfer of R-factors was carried out as described by Smith (18). The sensitivity of the β-lactamases to *p*-chloromercuribenzoate (PCMB) and chloride ions was determined as described by Hedges et al. (12).

Molecular weights were determined by gel filtration on Sephadex G-100 (2, 4). Starch-gel electrophoresis (5, 19) was done in 0.03 M sodium borate (pH 8.5; 200 V, i.e., 15 V/cm); β-lactamase bands were detected by spraying with a mixture of iodine and oxacillin or of iodine and benzylpenicillin (15).

Bacterial strains and R-factors. The strains of *Escherichia coli* K-12 used were: 58.161/*sp met*$^-$; J53 *pro*$^-$, *met*$^-$; and J62 *lac*$^-$, *trp*$^-$, *his*$^-$, *pro*$^-$. The R-factors used are listed in Table 1. All the R-factors were transferred into *E. coli* strain J62, as described by Smith (18), to provide a uniform genetic background; this strain produces an insignificant amount of chromosomally determined β-lactamase compared with that specified by the R-factors.

A large number of strains of gram-negative bacteria, isolated from clinical material, were very kindly provided by D. Maclaren, Stepping Hill Hospital, Stockport, Cheshire, U.K. They were screened, by the membrane technique of Knox and Smith (14), for the production of oxacillin-hydrolyzing β-lactamases. Of 550 isolates producing β-lactamase, four were found that produced an enzyme with significantly higher activity against oxacillin than against benzylpenicillin. One of these isolates was a strain of *E. coli*. From this, ampicillin resistance was readily transferred to *E. coli* strain J62 (frequency of transfer about 5×10^{-3} per donor in 6-h matings). This R-factor was assigned

the number R Ox176. It was found to mediate resistance to ampicillin, streptomycin, and spectinomycin.

The other three isolates were strains of *Proteus mirabilis*, and attempts to transfer their ampicillin resistance to *E. coli* strain J62 were unsuccessful. However, when R-factor RA3 (10), which confers resistance to streptomycin, chloramphenicol, and sulfonamide (but not to ampicillin), was introduced into the *Proteus* strains, they were then able to transfer ampicillin resistance to *E. coli* strain 58.161/*sp* (frequency of transfer about 10^{-7} per donor in 6-h matings). The ampicillin-resistant recipients had not received RA3, since they remained sensitive to streptomycin, chloramphenicol, and sulfonamide, and their recipient ability in the transfer of RA3 from *E. coli* strain J53 was similar to that of strain 58.161/*sp*. Thus, the role played by RA3 in the transfer of ampicillin resistance from *P. mirabilis* to *E. coli* strain 58.161/*sp* is not known. The three ampicillin-resistant *E. coli* exconjugant strains were able to transfer ampicillin resistance to other strains of *E. coli* K-12, including strain J62, when the transfer rate was about 10^{-7} per donor in 6-h matings. No other antibiotic resistance was detected in association with ampicillin resistance. These three R-factors, R Ox166, R Ox179, and R Ox407, are thought to be reisolates of the same R-factor. The β-lactamases seem to be identical as judged by substrate specificity profiles, molecular weights, and electrophoretic mobilities, and the original strains were homologous by the Dienes phenomenon (7). For this reason, we report here the results obtained with only one of the three R-factors, R Ox179.

RESULTS

The properties of the enzymes studied here are shown in Table 2. Some of these results have been reported previously (3, 6, 12) and are reproduced here for comparison.

Level of enzyme activity. Hedges et al. (12) stated that most oxacillin-hydrolyzing β-lactamases have a low specific activity in crude extracts compared to the "TEM-like" enzymes, where the activities ranged from 60 to 1,700 mU/10^9 bacteria. The results quoted here support this conclusion, with the exception of R Ox179 β-lactamase, the level of which is indeed higher than that of a number of TEM-like enzymes.

Effect of PCMB and chloride ions. As reported by Hedges et al. (12), the β-lactamases specified by R_{GN238}, R609b, and R656a are slightly sensitive to 0.5 mM PCMB. Yamagishi et al. (23) stated that R_{GN238} β-lactamase was not inhibited by PCMB at 0.5 or 1 mM. The reason for this discrepancy is not known. None of the other β-lactamases studied here was found to be sensitive to 0.5 mM PCMB.

All the oxacillin-hydrolyzing β-lactamases were inhibited by chloride ions, and there were small but reproducible differences in the degree of sensitivity (Table 2). These differences indicate that the enzymes can be divided into subgroups, and this division is supported by other criteria in Table 2.

Substrate specificity profiles. The activities of nine of the β-lactamases against benzylpenicillin, ampicillin, oxacillin, and cephaloridine have been reported previously (12). It can be seen (Table 2) that the additional five enzymes

TABLE 1. *Origins and characters of R-factors whose β-lactamases are described in this paper*

R-factor	Compatibility group (where known)	Resistance pattern[a]	Origin		Reference
			Bacterial species	Geographical	
R455	FI	ApSmTcCmSu	*Proteus morgani*	S. Africa	11
R_{GN238}	FI	ApSmTcCmSu	*Escherichia coli*	Japan	23
R609b	Iα	ApSmSu	*Salmonella typhimurium*	S. Africa	12
R656a	Iα	ApSmKmSu	*S. typhimurium*	S. Africa	12
R55	C	ApCmSuGk	*Klebsiella pneumoniae*	France	22
R57b	C	ApCmSuGk	*K. pneumoniae*	France	22
R^7	O	ApSmTcSu	*E. coli*	Mexico	8
R^{16}	O	ApSmTcSu	*E. coli*	Mexico	8
R Ox176		ApSm	*E. coli*	U.K., Stockport	Not previously published
R46 (=R1818)	N	ApSmTcSu	*S. typhimurium*	U.K., Brighton	1, 13
R45		ApTcSu	*S. typhimurium*	U.K., Enfield	1, 13
R48		ApSmTcSu	*S. typhimurium*	U.K., Bradford	1, 13
R205		ApTcSu	*S. typhimurium*	Holland	13
R Ox166) R Ox179) R Ox407)		Ap	*Proteus mirabilis*	U.K., Stockport	Not previously published

[a] Abbreviations for resistance markers: Ap, ampicillin; Sm, streptomycin; Tc, tetracycline; Cm, chloramphenicol; Km, kanamycin; Su, sulfonamides; Gk, gentamycin, with low level kanamycin.

TABLE 2. *Properties of oxacillin-hydrolyzing β-lactamases*

R-factor	β-Lactamase absolute activity (mU/10⁹R⁺ bacteria)	Concn of NaCl (mM) to cause 50% inhibition	Molar substrate specificity profile[a]				Molecular weight	Binding to dextran blue	Electrophoretic mobility (cm/h towards cathode)
			Methicillin	Oxacillin	Ampicillin	Cephaloridine			
R455	9.1[b]	5[b]	281	184[b]	161[b]	32.6[b]	24 300	–	−0.12
R_{GN238}	5.8[b]	8[b]	332	197[b]	382[b]	30.0[b]	23 300[c]	–	−0.08
R609b	12.0[b]	7[b]	336	196[b]	404[b]	33.2[b]	24 300	–	−0.12
R656a	18.9[b]	6[b]	335	198[b]	408[b]	31.0[b]	24 400	–	−0.14
R55	7.2[b]	49[b]	32.8	376[b]	173[b]	44.7[b]	41 200	+	−0.13
R57b	7.3[b]	43	29.4	336[b]	178[b]	43.8[b]	42 800	+	−0.13
R⁷	1.7[b]	38[b]	36.1	513[b]	133[b]	61.9[b]	45 200	+	+0.49
R¹⁶	3.1[b]	34[b]	37.7	528[b]	143[b]	55.2[b]	46 100	+	+0.45
R Ox176	5.3	30	26.9	595	178	64.6	44 800	+	+0.48
R46 (R1818)	25.2[b]	15[b]	23.3	646[b]	179[b]	36.6[b]	44 600[d]	+	+0.51
R45	24.5	17	28.1	631	166	41.6	46 100	+	+0.50
R48	14.5	20	26.4	661	168	43.0	46 100	+	+0.50
R205	30.0	17	28.6	667	169	38.7	46 400	+	+0.48
R Ox179	261	11	40.2	870	247	67.2	45 200	+	+0.51

[a] Activity of each enzyme (micromoles of substrate hydrolyzed per minute) was measured against the different substrates. The values obtained are expressed as a percentage of the activity against benzylpenicillin (micromoles per minute).

[b] Data from Hedges et al. (12).

[c] Data from Dale and Smith (6).

[d] Data from Dale (3).

studied here can be similarly classified into types by using these same substrates. However, the 14 enzymes can be divided into two groups solely on the basis of their ability to hydrolyze methicillin. Those β-lactamases specified by R455, R_{GN238}, R609b, and R656a hydrolyzed methicillin about three times as rapidly as benzylpenicillin, whereas the remaining enzymes had little relative activity against this substrate.

It should be noted that some of the figures for relative activities, particularly those for ampicillin, do not agree with those of other workers (17). This could result from different assay methods or from different assay conditions. Richmond and Sykes, for example, do their assays at pH 5.9 (17), whereas we use pH 7.4. Ampicillin has a pK value of 7.2 (16), so this difference in pH values could have a particularly significant effect on its relative rate of hydrolysis. To test this directly, we measured the activity of R46 β-lactamase against ampicillin and benzylpenicillin at different pH values. The pH difference alone can account for the difference between the value of 120 quoted by Richmond and Sykes (17) and that of 179 found in this laboratory (Table 2) for the relative rate of hydrolysis of ampicillin compared with that of benzylpenicillin (Table 3). Thus, in this case at least, different assay methods can show good agreement if the assay conditions are standardized.

Molecular weights. The β-lactamase of R_{GN238} has been distinguished from that of R46 on the basis of its molecular weight (6). Examination of the molecular weights of the other oxacillin-hydrolyzing β-lactamases shows that two main groups of these enzymes exist: a low-molecular-weight group (24,000) and a high-molecular-weight group (>40,000). These correspond with types Va and Vb of Richmond and Sykes' classification (17) and also with the ability of the enzymes to hydrolyze methicillin. The low-molecular-weight β-lactamases hydrolyze methicillin much more rapidly than benzylpenicillin; this is a distinctive feature of this group of enzymes because methicillin is normally refractory to β-lactamase attack. This simple criterion may therefore serve as a rapid method of distinguishing the two groups prior to

TABLE 3. *Effect of pH on relative rates of hydrolysis of benzylpenicillin and ampicillin by R46 β-lactamase*

pH	Ampicillin hydrolysis[a]
6.0	125
6.5	125
7.0	179
7.4	183
8.0	192

[a] Expressed in terms of the rate of hydrolysis of ampicillin (micromoles per minute) as a percentage of the rate of hydrolysis of benzylpenicillin (micromoles per minute) at that pH value. The buffer used was 0.025 M sodium phosphate.

molecular weight determination.

The molecular weights of the β-lactamases mediated by R55 and R57b seem to be lower than those of the remainder of the high-molecular-weight group. The difference, although small, is reproducible and is supported by the fact that these two enzymes are consistently eluted later than ovalbumin (chicken egg albumin), which is one of the markers routinely used (molecular weight 45,000).

Dextran blue binding. An unusual feature of R46 β-lactamase is that it binds to dextran blue (stated by the manufacturers to be a high-molecular-weight dextran with a cibachrome blue chromophore) so that in the presence of dextran blue enzymatic activity is found only at the void volume of the Sephadex column (3). The nature of this binding is not known, although it has been reported to occur with other proteins (9, 20, 21). Table 2 shows that this property is common to all the high-molecular-weight, oxacillin-hydrolyzing β-lactamases (including R55 and R57b), whereas those of the low-molecular-weight group do not bind to dextran blue.

Starch-gel electrophoresis. The starch-gel electrophoresis results (Table 2) also confirm the separation of these enzymes into two major groups. Those with a low molecular weight have a mobility of −0.1 cm/h, whereas those with a molecular weight of 45,000 have a mobility of +0.5 cm/h. The β-lactamases mediated by R55 and R57b are anomalous because, although their molecular weight is greater than 40,000, their mobility on starch-gel electrophoresis is −0.1 cm/h, which is in fact similar to that of the low-molecular-weight group. However, this similarity may be merely coincidental.

It has been reported (3) that two bands were obtained when the R46 β-lactamase was subjected to starch-gel electrophoresis. Early experiments in the present work showed the same effect, with the second band running at +0.2 cm/h. However, such experiments were done with old preparations of crude enzyme, stored frozen at −20 C. When fresh, nonfrozen cell extracts were used, the slower-running band was not seen. This is therefore thought to be an artifact either of storage or of freezing and thawing. It is worth noting that there did not seem to be any difference between the old and fresh enzyme preparations regarding any other property investigated.

In all the experiments, an additional very weak band (mobility +0.9 cm/h) was observed when the starch gels were developed with a benzylpenicillin-iodine mixture, but not when oxacillin was used. J. T. Smith (unpublished data) has shown that the chromosomally determined β-lactamase produced in very small amounts by *E. coli* K-12 strains does not hydrolyze oxacillin. We conclude that it is this enzyme that is responsible for this additional band; this is consistent with the electrophoresis results obtained with two other strains of *E. coli* (5).

DISCUSSION

It can be concluded from these results that there are two main groups of oxacillin-hydrolyzing β-lactamases: first, those with a high activity against methicillin, a molecular weight of about 24,000, no binding to dextran blue, and electrophoretic mobility of −0.1 cm/h; and second, those with little activity against methicillin, a molecular weight of 45,000, binding to dextran blue, and an electrophoretic mobility of +0.5 cm/h. These two groups are not homogeneous but can be divided into types on the basis of their substrate specificity profiles and sensitivity to PCMB or sodium chloride. It would be a mistake, however, to treat these types as rigid subdivisions; in most cases there are only one or two independently isolated enzymes in each type. Where R-factors are similar in compatibility properties, source, and resistance spectrum, the possibility cannot be ruled out that they are reisolates of the same plasmid. Hence, the β-lactamases mediated by such R-factors cannot be said to be of independent origin.

There were two types of enzyme in the low-molecular-weight group: that mediated by R455, on the one hand, and those mediated by R_{GN238}, R609b, and R656a, on the other. R609b and R656a could be variants of the same R-factor (Table 1), although R609b lacks the kanamycin-resistance determinant. They are however completely different from R_{GN238} in origin and compatibility group (Table 1), and yet the

β-lactamases specified by these three R-factors are indistinguishable. However, they can be differentiated from that mediated by R455 by their sensitivity to PCMB and their relative rates of hydrolysis of ampicillin (12). In all other respects, all four enzymes of the low-molecular-weight group were similar and could have originated from a common ancestor.

There were possibly five types of oxacillin-hydrolyzing β-lactamase in the high-molecular-weight group specified by the following R-factors: (i) R^7 and R^{16}, (ii) R46, R45, R48, and R205, (iii) R Ox176, (iv) R Ox179, and (v) R55 and R57b. These types can be distinguished by means of the data provided in Table 2. R^7 and R^{16}, which could be reisolates of the same R-factor (8), were found to specify virtually identical β-lactamases. The β-lactamase mediated by R46 is indistinguishable from those mediated by R45, R48, and R205. These four R-factors, which were all isolated in *S. typhimurium* (Table 1), mediate resistance to ampicillin, sulfonamide, and tetracycline. R46 and R48 carry, additionally, resistance to streptomycin, but a consistent finding in this laboratory is that R46, at least, frequently segregates streptomycin-sensitive clones. Hence, all these four R-factors may have had identical resistance spectra at one time. However, they are different in their geographical source (Table 1), and it is not possible at this stage to be certain if these are reisolates of the same R-factor.

The β-lactamase mediated by R Ox176 illustrated the dangers of attempting a rigid classification of these enzymes. In some respects, it resembles the β-lactamases of R^7 and R^{16}, and in other respects it resembles enzymes of the R46 type, so that its properties are intermediate between those of the two types. R Ox176 was isolated independently from any of the other R-factors studied here with the exception of R Ox179. However, although R Ox176 and R Ox179 were isolated at the same time, they were found in different bacterial species, the two R-factors are compatible with each other (J. W. Dale, unpublished data), and there are significant differences between the β-lactamases specified by them (Table 2). It is likely therefore, that R Ox176 and R Ox179 are in fact genuinely different R-factors.

As outlined above, R Ox179, R Ox166, and R Ox407 are thought to be reisolates of the same R-factor. The most notable feature of the β-lactamase specified by these R-factors is the high enzyme level produced per cell (Table 2). Moreover, it is interesting that the enzyme level produced by these R-factors in the original *P. mirabilis* strains is similar to the level found in *E. coli* strain J62 (J. W. Dale, unpublished data). This contrasts with the behavior of R46 (and some other R-factors), which mediates much lower levels of R-factor-specified β-lactamase in *P. mirabilis* than in *E. coli* (18). This suggests that R Ox179 may carry a difference in some as yet unidentified gene which is responsible for the regulation of β-lactamase synthesis.

R55 and R57b may be reisolates of one R-factor (Table 1), and the β-lactamases they mediate deserve special mention. In most properties they bear a close resemblance to the other enzymes of the high-molecular-weight group. However, the molecular weights of the R55 and R57b β-lactamases are slightly lower than those of the other enzymes in this group, and their electrophoretic mobility is different. It is possible that the β-lactamase gene of these two R-factors has undergone a deletion event resulting in changes in the physical properties of the enzyme molecule. If this is the case, it may be that all the oxacillin-hydrolyzing β-lactamases of molecular weight greater than 40,000 could have originated from a common ancestor.

ACKNOWLEDGMENTS

We thank Lesley P. Sharp and Jeanette Surridge for expert technical assistance and D. Maclaren and N. Datta for supplying bacterial strains.

We also thank the Medical Research Council for financial support for J. W. D.

LITERATURE CITED

1. Anderson, E. S., and N. Datta. 1965. Resistance to penicillin and its transfer in Enterobacteriaceae. Lancet 1:407-409.
2. Andrews, P. 1964. Estimation of the molecular weights of proteins by Sephadex gel-filtration. Biochem. J. 91:222-233.
3. Dale, J. W. 1971. Characterization of the β-lactamase specified by the resistance factor R-1818 in *E. coli* K12 and other gram-negative bacteria. Biochem. J. 123:501-505.
4. Dale, J. W., and J. T. Smith. 1971. The purification and properties of the β-lactamase specified by the resistance factor R-1818 in *E. coli* and *Proteus mirabilis*. Biochem. J. 123:493-500.
5. Dale, J. W., and J. T. Smith. 1971. Some relationships between R-factor and chromosomal β-lactamases in gram-negative bacteria. Biochem. J. 123:507-512.
6. Dale, J. W., and J. T. Smith. 1972. A direct comparison of two unusual R-factor mediated β-lactamases. Biochem. J. 128:173-174.
7. Dienes, L. 1964. Reproductive processes in proteus cultures. Proc. Soc. Exp. Biol. Med. 63:265-270.
8. Evans, J., E. Galindo, J. Olarte, and S. Falkow. 1968. β-Lactamase of R-factors. J. Bacteriol. 96:1441-1442.
9. Haeckel, R., B. Hess, W. Lauterborn, and K. H. Wuster. 1968. Purfication and allosteric properties of yeast pyruvate kinase. Hoppe-Seyler's Z. Physiol. Chem. 349:699-714.
10. Hedges, R. W., and N. Datta. 1971. *fi*⁻ R factors giving

chloramphenicol resistance. Nature (London) **234**:220–221.
11. Hedges, R. W., N. Datta, J. N. Coetzee, and S. Dennison. 1973. R-factors from *Proteus morganii*. J. Gen. Microbiol. **77**:249–259.
12. Hedges, R. W., N. Datta, P. Kontomichalou, and J. T. Smith. 1974. Molecular specificities of R-factor-determined β-lactamases: correlation with plasmid compatibility. J. Bacteriol. **117**:56–62.
13. Jenkins, P. G., and W. T. Drabble. 1971. β-Lactamases of R factors derived from *Shigella* and *Salmonella* strains. J. Bacteriol. **108**:159–165.
14. Knox, R., and J. T. Smith. 1961. Use of cellulose-acetate membranes for detecting penicillinase-producing organisms. Nature (London) **191**:926–927.
15. Lampen, J. O. 1967. Cell-bound penicillinase of *Bacillus licheniformis*; properties and purification. J. Gen. Microbiol. **48**:249–259.
16. Retsema, J. A., and V. A. Ray. 1972. Correlation between the binding of β-lactam antibiotics to *Staphylococcus aureus* and their physical-chemical properties. Antimicrob. Ag. Chemother. **2**:173–180.
17. Richmond, M. H., and R. B. Sykes. 1973. The β-lactamases of gram-negative bacteria and their possible physiological role, p. 31–88. *In* A. H. Rose and D. W. Tempest (ed.), Advances in microbial physiology, vol. 9. Academic Press Inc., New York.
18. Smith, J. T. 1969. R-factor gene expression in gram-negative bacteria. J. Gen. Microbiol. **55**:109–120.
19. Smithies, O. 1955. Zone electrophoresis in starch gels: group variations in the serum proteins of normal human adults. Biochem. J. **61**:629–641.
20. Staal, G. E. J., J. Visser, and C. Veeger. 1969. Purification and properties of glutathione reductase of human erythrocytes. Biochim. Biophys. Acta **185**:39–48.
21. Swart, A. C. W., and H. C. Hemker. 1970. Separation of blood coagulation factors II, VII, IX and X by gel filtration in the presence of Dextran blue. Biochim. Biophys. Acta **222**:692–695.
22. Witchitz, J. L., and Y. A. Chabbert. 1971. High level transferable resistance to gentamicin. J. Antibiot. (Tokyo) **24**:137–139.
23. Yamagishi, S., K. O'Hara, T. Sawai, and S. Mitsuhashi. 1969. The purification and properties of penicillin β-lactamases mediated by transmissible R-factors in *Escherichia coli*. J. Biochem. **66**:11–20.