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Characterization of R Factor β-Lactamases by the Acidimetric Method

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The properties and regulation of β-lactamases produced by certain R factors derived from ampicillin-resistant strains of *Escherichia coli* and *Klebsiella* were characterized. β-Lactamase activity was determined by the acidimetric method with phenol red as indicator. The sensitivity of this assay was increased by employing phosphate buffer at a final concentration of 0.4 mM; as little as 0.05 unit (1 unit equals the amount of β-lactamase that hydrolyzes 1 μmole of benzyl penicillin per hr at pH 7.6 and 25 C) could be detected. This assay is rapid and convenient and appears to be superior to other methods currently employed to assay R factor β-lactamases. Two classes of β-lactamases were distinguished on the basis of substrate profile, heat inactivation, and K_m values. The regulation of most of these R factor β-lactamases, like certain other R factor enzymes, is subject to cyclic adenosine monophosphate-mediated catabolite repression.

R factors may confer resistance to penicillins and cephalosporins through the production of a β-lactamase (6, 7). This phenomenon is widespread: R factors mediating β-lactamase synthesis have been identified in England, Japan, Greece, France, Mexico, and the United States in *Pseudomonas aeruginosa* and the *Enterobacteriaceae*, i.e., *Escherichia*, *Klebsiella*, *Aerobacter*, *Salmonella*, *Shigella*, and *Proteus* (6, 9, 11, 14, 15, 19, 23, 32). Several distinct R factor β-lactamases have been recognized, and classification systems based on enzymological, physicochemical, and immunological data have been described (14, 23, 27, 34).

Most of the many methods for measuring β-lactamase activity (4, 13, 22, 25, 28) either require high concentrations of substrate or enzyme or are laborious to perform, or both. A sensitive assay is required for R factor β-lactamases, since most of those studied to date generally have relatively low specific activities. The microiodometric method (21) is sufficiently sensitive and has been used widely; however, problems with this procedure (see Discussion) resulted in an investigation of another assay. This paper describes the modification of an acidimetric method for studying the properties and regulation of certain R factor β-lactamases.

MATERIALS AND METHODS

Bacterial strains and R factors. The R factors used in this study were derived from clinical isolates selected for resistance to 50 μg of ampicillin per ml (Table 1). All R factors were transferred by conjugation into *E. coli* K-12 1100 (F^-). No β-lactamase activity was detected in extracts prepared from this strain.

Preparation of bacterial extracts. All bacterial strains were grown in minimal medium (8) at 37 C with shaking; the medium was supplemented with 0.2% glycerol or glucose and 10 mM adenosine 3′,5′-cyclic phosphate (cyclic AMP) where indicated. Cultures used as a source of enzyme were prepared by transferring 2 ml of a 16-hr culture into 35 ml of minimal liquid medium and incubating to an optical density of 0.2 (Lumetron spectrophotometer, 490 nm). The bacterial cells were collected by centrifugation at 12,000 × g for 15 min, washed with 1 mM sodium phosphate buffer (pH 7.6), resuspended in 4 ml of the same buffer, and disrupted by sonic treatment for 2 min at 70 w (Branson Instruments, Inc.) while cooled in an ammonium sulfate-ice bath; the suspension was then clarified by centrifugation at 30,000 × g for 30 min at 0 C. Protein concentration in the extract was measured by the method of Lowry et al. (17).

Antibiotics and chemicals. Benzyl penicillin was obtained from General Biochemicals (Chagrin Falls, Ohio). Ampicillin, methicillin, oxacillin, cloxacillin, and phenethicillin were donated by Bristol Laboratories, Syracuse, N.Y. Cephalothin, cephaloridine, and phenoxymethylpenicillin were donated by Eli Lilly & Co., Indianapolis, Ind. The following chemicals were also employed in this study: p-chloromercuribenzoate (pCMB), Calbiochem, Los Angeles, Calif.; cyclic AMP, Schwarz-Mann, Orangeburg, N.Y.; and phenol red, Sigma Chemical Co., St. Louis, Mo.

TABLE 1. *Description of R factors*

R factor	Source	Drug resistance pattern[a]
B22	*E. coli*	Tet Str Cml Amp
B30	*E. coli*	Tet Str Cml Amp Sul
B68	*Klebsiella*	Cml Amp
RK5	*Klebsiella*	Tet Str Cml Amp
U143	*E. coli*	Tet Str Cml Amp Sul

[a] Tet, Str, Cml, Amp, and Sul denote resistance to tetracycline, streptomycin, chloramphenicol, ampicillin, and sulfonamides, respectively.

Assay for β-lactamase activity. A 1.2-ml reaction mixture contained phenol red (0.0013%, w/v), benzyl penicillin (0.2 mM), sodium phosphate buffer, pH 7.6 (0.4 mM), and bacterial extract. The final concentration of buffer included the contribution from the bacterial extract. All reagents were initially adjusted to pH 7.6. They were mixed thoroughly in a cuvette, and absorption was measured at 558 nm and 25 C for 5 min with a Gilford 2000 recording spectrophotometer. The acidimetric assay was standardized by measuring the change in optical density at 558 nm after the addition of HCl to the reaction mixture; 0.84 μmole of HCl resulted in a decrease in absorbance of 1 optical density unit. One unit of β-lactamase activity was defined as that amount of enzyme which hydrolyzed 1 μmole of benzyl penicillin in 1 hr at 25 C and pH 7.6.

RESULTS

Acidimetric method. When the enzyme cleaves the β-lactam ring of the antibiotic (penicillin), the acid (penicillinoic) formed can be detected by means of a pH indicator. The concentration of acid correlates directly with the color change of the indicator and can be followed colorimetrically. This method was first described by Saz et al. (28) to measure the activity of a penicillinase extracted from *Staphylococcus aureus;* phenol red was used as the indicator, and the phosphate buffer was used at a final concentration of 4 mM. The concentration of buffer affects the detection of (penicillinoic) acid production; increased sensitivity can be obtained by decreasing the amount of buffer (Fig. 1). We have therefore employed a reaction mixture with a total phosphate concentration of 0.4 mM.

The initial velocity was directly proportional to the volume of bacterial extract used as the source of β-lactamase (Fig. 2). Although most of the experiments in this report were performed with 0.1 to 0.5 unit of enzyme activity, other studies indicated that the linear relationship between enzyme concentration and enzyme activity was maintained up to 0.75 unit of enzyme. Under these experimental conditions, the phenol red acidimetric method detected as little as 0.05 unit of β-lactamase. This

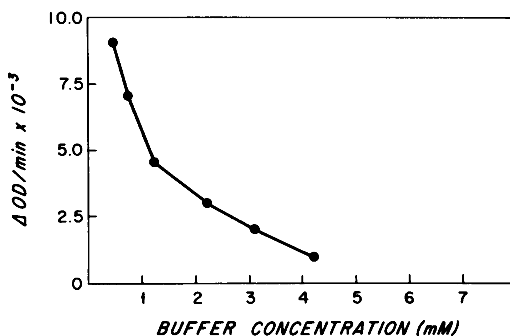

FIG. 1. *Effect of buffer concentration on initial reaction velocity of the β-lactamase mediated by R factor U143. A 10-μliter amount of bacterial extract (885 μg of protein/ml) was added to the reaction mixture, and β-lactamase activity was measured in the presence of different concentrations of buffer as described in Materials and Methods. The substrate was benzyl penicillin at 0.2 mM.*

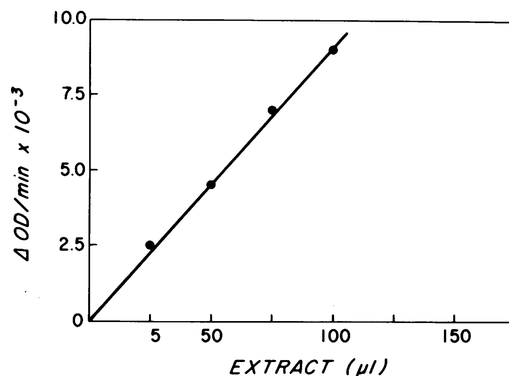

FIG. 2. *Effect of enzyme concentration on enzyme activity. The bacterial extract employed in this experiment had been prepared from E. coli K-12 1100/RK5 and contained 930 μg of protein per ml of extract. The substrate was benzyl penicillin at 0.2 mM.*

assay was employed in the study of the β-lactamases of certain R factors.

Specific activities of certain R factor β-lactamases. The enzymes are synthesized constitutively: the specific activities of the β-lactamases were identical when the cells were grown in the presence or absence of 5 μg of benzyl penicillin per ml of growth medium. The specific activities of the tested enzymes for benzyl penicillin varied from 7 to 952 units/mg of protein (Table 2).

Substrate profiles. A comparison of the activity for a range of substrates demonstrated that the enzymes produced by R factors B22 and RK5 hydrolyzed semisynthetic penicillins at a high rate, whereas those produced by R factors U143, B30, and B68 had little or no

TABLE 2. *Specific activities and substrate profiles of the β-lactamases produced by E. coli K-12 1100 carrying different R factors*[a]

Substrate[b]	R factor				
	RK5	B22	U143	B68	B30
Benzyl penicillin	100 (7)[c]	100 (19)	100 (62)	100 (554)	100 (952)
Ampicillin	464	489	67	78	71
Methicillin	339	259	<0.03	<0.06	<0.01
Oxacillin	200	163	5	3	<0.01
Cloxacillin	157	144	<0.03	<0.06	<0.01
Phenethicillin	157	134	26	15	17
Carbenicillin	86	78	8	11	<0.01
Cephaloridine	50	34	53	40	44
Cephalothin	14	5	12	5	6

[a] Expressed as units per milligram of bacterial protein.
[b] Final concentration of substrate in assay mixture was 0.2 mM.
[c] Rates of hydrolysis relative to benzyl penicillin (specific activity expressed as units per milligram of bacterial protein).

activity against these antibiotics. Cephalosporin antibiotics were hydrolyzed by all of the β-lactamases to the same small extent (Table 2).

K_m values. The K_m values for benzyl penicillin with β-lactamases produced by R factors B22 and RK5 were 8 and 17 μM, respectively, whereas the K_m values for the β-lactamases produced by R factors U143, B30, and B68 were 300, 115, and 83 μM, respectively.

Heat inactivation. No activity was detected after heating extracts of the β-lactamases mediated by B22 and RK5 at 55 C for 2 min. In contrast, the β-lactamases produced by U143, B30, and B68 were heat-stable: 85% of the original activity was retained after heating these extracts at 55 C for 20 min (Fig. 3).

Effect of pCMB. Some β-lactamases of gram-negative bacteria are inhibited more than 80% by 0.5 mM pCMB (29). The activity of all of the R factor β-lactamases was inhibited by 0.5 mM pCMB to approximately 55%.

Regulation of β-lactamase synthesis. The synthesis of the R factor enzymes chloramphenicol acetyl transferase, streptomycin adenylyl transferase, and kanamycin phosphotransferase is subject to catabolite repression (12, 33). When cultures carrying the different R factors were grown in various carbon sources, the synthesis of four of the β-lactamases was subject to cyclic AMP-mediated catabolite repression (see Table 3). The specific activity of β-lactamase in strains grown on glucose was lower than that of cells grown on glycerol ($P < 0.02$ by t test). With the exception of the β-lactamase produced by RK5, the addition of cyclic AMP increased enzyme activity significantly in glucose-grown cells ($P < 0.02$ by t test).

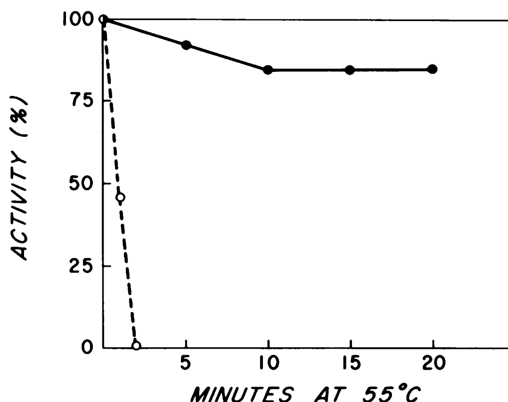

FIG. 3. *Heat inactivation curves for the β-lactamases mediated by R factor B22 (O) and R factor U143 (●). Bacterial extracts, at a protein concentration of 878 and 885 μg/ml, respectively, were heated at 55 C for various times, and then β-lactamase activity was measured and compared with that of unheated controls. Amounts of 10 and 50 μliters of extracts prepared from E. coli K-12 1100/U143 and 1100/B22, respectively, were used in the assay mixture.*

DISCUSSION

The results of these experiments indicate that the acidimetric method can be applied to the assay of the β-lactamases produced by R factors. Enzyme kinetics are linear with time and enzyme concentration, and are dependent on the presence of a β-lactam antibiotic. Neither the bacterial extract nor the β-lactam antibiotic reacted with any other component in the assay mixture. In addition, bacterial extracts did not contain any inhibitors: (i) enzyme

TABLE 3. *Effect of cyclic 3',5'-AMP (cAMP) and carbon sources on β-lactamase activity*

Bacterial strain	Carbon Source (0.2%)	Specific activity[a] of β-lactamase ±SE
1100/U143	Glucose	41 ± 3.5
	Glucose + cAMP	63 ± 5.1
	Glycerol	70 ± 3.0
1100/B68	Glucose	83 ND
	Glucose + cAMP	122 ND
	Glycerol	145 ND
1100/B30	Glucose	396 ± 32
	Glucose + cAMP	632 ± 23
	Glycerol	867 ± 54
1100/B22	Glucose	9 ± 0.3
	Glucose + cAMP	18 ± 0.5
	Glycerol	14 ± 1.6
1100/RK5	Glucose	5 ± 0.6
	Glucose + cAMP	5 ± 1.0
	Glycerol	8 ± 0.7

[a] Specific activity as units of β-lactamase activity per milligram of bacterial protein; ND = not determined.

activity of different bacterial extracts was additive and (ii) reaction velocity was unaffected by the addition of bacterial extract prepared from the parental (R^-) strain.

Although there are other procedures for assaying β-lactamase activity, many are not as sensitive, versatile, or rapid (see introduction) as the acidimetric method. The microiodometric method (21) has been used to study R factor β-lactamases because this assay is sensitive, and it can be used to measure enzyme kinetics. However, the following problems may be encountered when the microiodometric method is used to measure β-lactamase activity of preparations with low specific activities: (i) marked inhibition of the enzyme by iodine (11); (ii) a variation in the structure of different starch preparations which, in turn, affects the sensitivity and reproducibility of the assay (21; Burman, *personal communication*); (iii) limited substrate concentration (it is not practical to use high concentrations of antibiotic because the spontaneous reduction of the iodine becomes too great and kinetics can not be performed [3; see below]); and (iv) difficulty in accurate measurement of K_m values due to nonspecific decolorization of starch-iodine by the proteins in the bacterial extract (3). Novick (21) reported that the microiodometric method underestimates β-lactamase activity by 40% when compared to the macroiodometric method (25). However, Sykes and Nordstrom (31) demonstrated that the microiodometric method correctly estimates enzyme activity provided that measurements are initiated 15 to 20 min after mixture of the assay constituents, the time required for the development of a steady state between the formation of (penicillinoic) acid and its oxidation by iodine.

When the activities of these R factor β-lactamases were assayed in parallel by the acidimetric and microiodometric (21) methods, it was necessary to decrease the concentration of benzyl penicillin in the reaction mixture from 200 to 20 μM because of nonspecific reduction of iodine. By serial dilution of a single extract, it was found that the microiodometric method was sensitive to 0.025 unit of β-lactamase, whereas the acidimetric method was sensitive to 0.050 unit of β-lactamase. The specific activity of a β-lactamase (U143) with a high K_m value was threefold lower when measured by the microiodometric method, with penicillin G as substrate. However, the specific activity of an R factor β-lactamase (B22) with a low K_m value was 10-fold lower when measured by the microiodometric method as compared to the acidimetric method. Therefore, the two assay procedures are only comparable when measuring β-lactamase activity of enzymes with high K_m values, and the two methods give different results with β-lactamases which have low activities and low K_m values.

The R factor β-lactamases investigated can be divided into two well-defined groups, type I and type II. Type I β-lactamases, such as those mediated by R factors B22 and RK5, are heat-sensitive, have low K_m values, and efficiently hydrolyze semisynthetic penicillins such as methicillin and cloxacillin. Type II enzymes, produced by R factors U143, B30, and B68, are heat-stable, have high K_m values, and do not hydrolyze methicillin or cloxacillin. When this classification is applied to other described R factor β-lactamases, the available data (substrate profiles) suggest that the following R factor β-lactamases are similar to type I: R TEM (6), R 7268 (6), R GN14 (9, 34), R1 (16), R381 (15), R B1H9 (19), r^2 (11), two R factors of *P. aeruginosa* (32), and 10 R factors described by Jack and Richmond (14). The enzymes determined by R 1818 (6), R GN 238 (9, 34), and r^7 and r^{16} (11) appear to be similar to type II. Study of purified preparations of the β-lactamases will be necessary to define further the properties of the β-lactamases described in this

report and to determine the accuracy and completeness of this classification system.

Although several R factor β-lactamases have been purified and characterized (5, 7, 9, 19, 26, 27, 34), relatively little is known about the regulation of the synthesis of such enzymes. One approach to the problem of the regulation of R factor enzyme has involved the comparison of specific activities and levels of resistance produced by a given R factor in different bacterial strains. The expression of certain R factors has been found to vary in different hosts, resulting in different levels of resistance (4-6, 26, 30); e.g., when R_{TEM} was transferred to a range of enteric bacteria, the expression of the gene for β-lactamase varied 20- to 30-fold (26). These results suggest that chromosomal loci may affect the synthesis of an R factor β-lactamase. Such regulation of episomal genes by chromosomal loci has been directly documented: mutation of the amp locus in *E. coli* K-12 resulted in enhanced synthesis of both episomal and chromosomal-mediated β-lactamases (20).

R factor enzymes may also be regulated by catabolite repression, the suppressed formation of certain enzymes during growth in glucose, and certain other cultural conditions (18); recent studies indicate that cyclic AMP and certain proteins play a role in the expression of this phenomenon (1, 10, 24, 35). The R factor enzymes chloramphenicol acetyl transferase, streptomycin adenylyl transferase, and kanamycin phosphotransferase are subject to cyclic AMP-mediated catabolite repression (12, 33). In addition, we found that four of five R factor β-lactamases studied were responsive to catabolite repression (Table 3). This regulatory mechanism is not universal for R factor β-lactamases, since the levels of β-lactamase produced by R factor R1 were the same in cultures grown on glucose and glycerol (2). Further studies on the regulation of other R factor β-lactamases appear to be indicated. Experiments in progress are aimed at determining whether the catabolite regulation of these and other R factor-mediated enzymes utilize the same effector protein(s) as those found to affect the synthesis of certain enzymes involved in carbohydrate metabolism in *E. coli* (10, 35).

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