ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Nov. 1990, p. 2184–2192
0066-4804/90/112184-09$02.00/0

Vol. 34, No. 11

Discrimination of Extended-Spectrum β-Lactamases by a Novel Nitrocefin Competition Assay

GENOVEFA A. PAPANICOLAOU† AND ANTONE A. MEDEIROS*

Division of Infectious Diseases and Clinical Microbiology, The Miriam Hospital and Brown University, Providence, Rhode Island 02906

Received 13 March 1990/Accepted 13 August 1990

We describe a nitrocefin competition assay for determining inhibition profiles as a useful adjunct to existing biochemical methods for the discrimination of β-lactamases. The hydrolysis rate of nitrocefin was measured with a plate photometer as the change in A_{480} over 45 min in the presence of 17 inhibitors. Fourteen well-established β-lactamases and 13 extended-spectrum β-lactamases were tested. Correlations with data from isoelectric focusing and amino acid sequencing suggested that the inhibition profile reflects alterations in the active-site configuration of β-lactamases. The method was especially useful in measuring the relative affinities of β-lactamases against poorly hydrolyzed substrates and in screening large numbers of isolates for the detection of new β-lactamase types.

β-Lactamases are the principal determinants of resistance to β-lactam antibiotics for most bacteria (23). The classification of β-lactamases has been based on several criteria, including substrate profiles, inhibition by compounds such as *p*-chloromercuribenzoate, molecular weight, and isoelectric point (6). However, the large number of β-lactamases makes their classification difficult, because many have very close or identical pIs and similar biochemical properties. Newer methodologies such as amino acid sequencing and nucleic acid hybridization have provided a better understanding of the evolution of β-lactamases and have enhanced their classification (1–3, 16). Many new β-lactamases, which are often derived from their progenitors by point mutations and which display different biochemical properties, have recently been identified in clinical isolates (17, 20, 35). Differences in their biochemical properties are often difficult to determine by conventional methods, and amino acid sequencing is time-consuming.

The development of the nitrocefin competition assay (NCA) offers an additional means of discriminating these β-lactamases. It is simple and easy to perform, and thus, a large number of isolates can be screened. In conjunction with data obtained by other methods, NCA yields valuable information regarding the interactions of various β-lactamases with β-lactam compounds.

MATERIALS AND METHODS

Bacterial strains. The β-lactamases tested in this study are listed on Table 1.

Preparation of β-lactamase extracts and isoelectric focusing. Crude sonic extracts of β-lactamases were prepared from the strains grown in brain heart infusion broth (BBL, Cockeysville, Md.) without inducer as described previously (39). Isoelectric focusing was performed on polyacrylamide gels as described previously (22, 39). β-Lactamase activities were revealed with nitrocefin (BBL) as a chromogenic substrate (26).

NCA. Seventeen β-lactams in a single concentration were tested for their ability to inhibit hydrolysis of nitrocefin. Some were competitive substrates and others were inhibitors; for simplicity, all are referred to here as "inhibitors." The concentrations of the inhibitors was determined by measuring approximate 50% inhibitory concentrations for representative enzymes by the microdilution plate assay described below and by choosing the one concentration that was most likely to discriminate between the different classes (data not shown). The final concentrations and suppliers were as follows: potassium clavulanate, 2 μM, Beecham Laboratories, Bristol, United Kingdom; sulbactam, 20 μM, and cefoperazone, 350 μM, Pfizer, Inc., Groton, Conn.; cefpirome sulfate, 750 μM, and cefotaxime, 5,000 μM, Hoechst-Roussel Pharmaceuticals, Inc., Sommersville, N.J.; cefoxitin, 5,500 μM, and imipenem, 980 μM, Merck Sharp and Dohme, West Point, Pa.; ceftazidime, 2,000 μM, and cefuroxime, 1,180 μM, Glaxo Ltd., Greenford, England; ceftriaxone, 750 μM, Hoffmann-LaRoche, Inc., Nutley, N.J.; aztreonam, 1,148 μM, Squibb Institute, Princeton, N.J.; moxalactam, 950 μM, Eli Lilly, Indianapolis, Ind.; cefotetan, 870 μM, ICI Pharmaceutical Laboratories, Wilmington, Del.; cefsulodin, 940 μM, Abbott Laboratories, North Chicago, Ill.; tazobactam, 3 μM, Lederle Laboratories, Pearl River, N.Y.; cloxacillin, 520 μM, Sigma Chemical Co., St. Louis, Mo.; and ceftibuten, 1,180 μM, Schering Corp., Bloomfield, N.J.

Assay mixtures were prepared by dissolving each inhibitor and 100 μM nitrocefin in 0.1 M phosphate buffer (pH 7.0). Fifty microliters of each assay mixture was dispensed into the wells of microdilution plates. Control wells contained nitrocefin alone. The plates were stored at −20°C and were allowed to stand at room temperature for about 30 min prior to use. Crude sonic extracts of β-lactamases were diluted to the point that 50 μl gave an A_{480} in the control wells of approximately 0.2 absorption units when measured at 2 min. The assay was started by the manual addition of 50 μl of diluted enzyme to duplicate wells containing inhibitor and to the control wells by using a multichannel pipette (Titertek). Inoculation started from column 1 and was completed in approximately 20 s. The change in optical density at a wavelength of 480 nm was measured with a plate photometer (Titertek Multiskan). Reading of the A_{480} started from column 1 and finished in about 25 s. Readings were taken at 2,

* Corresponding author.
† Present address: Department of Clinical Microbiology, Karolinska Hospital, S10401 Stockholm, Sweden.

TABLE 1. β-lactamases tested in the NCA

β-Lactamase type	pI	Host species	Plasmid or strain designation	Source	Reference
Well established, plasmid mediated					
TEM-1	5.4	*Escherichia coli*	R6K	Matthew	13
		Neisseria gonorrhoeae	119	Opal	
		Haemophilus influenzae	136	Miriam Hospital	
TEM-2	5.6	*Escherichia coli*	1725E	Matthew	13
SHV-1	7.6	*Escherichia coli*	J53(R1010)	Matthew	3
OHIO-1	7.0	*Escherichia coli*	C600(pDS075)	Shlaes	32
OXA-6	7.68	*Pseudomonas aeruginosa*	PA038(pMG39)	Medeiros	24
CARB-3	5.75	*Pseudomonas aeruginosa*	AP115	Labia	19
Extended spectrum, plasmid mediated					
TEM-3 (CTX-1)	6.3	*Escherichia coli*	J53(pMG227)	Jacoby	33
TEM-4	5.9	*Escherichia coli*	J53-2(pUD16)	Jacoby	27
TEM-5 (CAZ-1)	5.57	*Escherichia coli*	CF604	Sirot	34
TEM-6	5.87	*Escherichia coli*	88083101	Miller	5
TEM-7	5.2	*Escherichia coli*	Cla(pIF100)	Gutmann	10
TEM-101	5.2	*Escherichia coli*	Cla(pBR322-C3)	Gutmann	10
TEM-9 (RHH-1)	5.56	*Escherichia coli*	J53(pMLC28-B5)[a]	Jacoby	36
TEM-10	5.57	*Escherichia coli*	J53-2(pJPQ100)	Jacoby	30
SHV-2	7.6	*Escherichia coli*	JC2926(pBP60-1)	Wiedemann	17
SHV-3	7.0	*Escherichia coli*	J53-2(pUD18)	Jacoby	25
SHV-4	7.75	*Escherichia coli*	J53-2(pUD21)	Jacoby	28
SHV-5	8.2	*Escherichia coli*	J53-2(pAFF2)	Jacoby	12
CAZ-2	5.87	*Escherichia coli*	C600(pCFF34)	Jacoby	9
Chromosomally mediated					
P99	7.66	*Enterobacter cloacae*	81010504-9	Miller	
P99	7.66	*Enterobacter cloacae*	55M	Wiedemann	31
	7.8	*Enterobacter cloacae*	83120215	Miller	
	7.9	*Serratia marcescens*	83120234W	Miller	
	7.9	*Enterobacter aerogenes*	83101021	Miller	
	8.4	*Serratia marcescens*	83110701	Miller	
AmpC	8.5	*Escherichia coli*	87120702	Miller	15
	8.8	*Acinetobacter calcoaceticus*	83111835	Miller	

[a] TEM-9 from *K. pneumoniae* 2633E (36) cloned into vector pMLC28.

5, and 10 min and subsequently at 10-min intervals over 40 min. The percent inhibition was calculated as follows: 100 × [1.00 − (A_{480} of the well containing inhibitor/A_{480} of the control well)] (Fig. 1). Maximum inhibition was usually observed at 2 min, but occasionally it was observed at 5 min. When maximum inhibition exceeded 25%, the rate of decrease of inhibition (slope) was calculated manually from the linear portion of the inhibition curves as follows: (maximum percent inhibition − percent inhibition at 10 min)/10. Data were processed with a microcomputer interfaced to the plate photometer. A reference enzyme, usually TEM-1, was run as a control for each batch of plates used. The number of runs for each enzyme varied from three to seven.

RESULTS

The reproducibility of results for TEM-1 is shown in Table 2, which displays values for repeated determinations with a single preparation of TEM-1 from *Escherichia coli* R6K, for three different preparations of TEM-1 from *E. coli* R6K, and

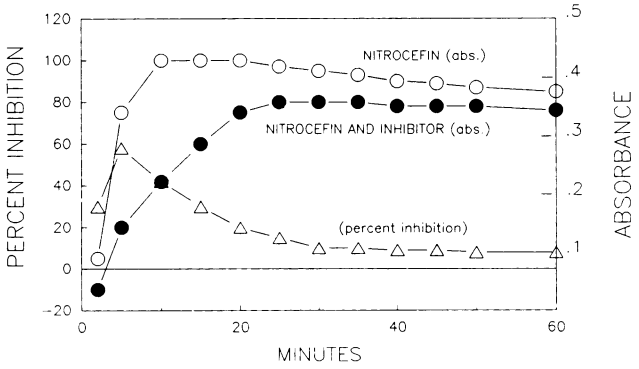

FIG. 1. Calculation of percent inhibition based on A_{480} values of nitrocefin versus those of nitrocefin and inhibitor.

TABLE 2. Reproducibility of inhibition profiles of TEM-1

β-Lactamase Source	Clavulanate		Sulbactam		Tazobactam		Cefoxitin (Avg ± SE)	Cefotetan (Avg ± SE)	Moxalactam (Avg ± SE)	Cefotaxime (Avg ± SE)
	Avg ± SE[a]	SL[b]	Avg ± SE	SL	Avg ± SE	SL				
E. coli R6K[c]	60 ± 3	0.4	86 ± 2	11	97	<0.3	21 ± 1	11 ± 2	3 ± 1	5 ± 2
E. coli R6K[d]	66 ± 2	0.4	86 ± 1	1	91 ± 2	<0.3	23 ± 3	7 ± 4	1	13 ± 3
H. influenzae	69	0.3	85	1.0	92	<0.3	23	10	2	13
N. gonorrhoeae	57	0.7	80	1.4	93	<0.3	17	1	1	6

[a] Average of maximum percent inhibition ± standard error.
[b] SL, Slope, given when maximum inhibition exceeded 25%.
[c] A single preparation from *E. coli* R6K tested multiple times over several months.
[d] Three different preparations from *E. coli* R6K, with two sets of experiments done for each preparation.
[e] ND, Not determined.

for preparations of TEM-1 from *Haemophilus influenzae* 136 and *Neisseria gonorrhoeae* 119. In general, values were slightly lower for the preparation from *N. gonorrhoeae* 119, a relatively weak producer of the TEM-1 enzyme. Standard error bars for both TEM-1 and SHV-1 are given in Fig. 2. Similar values were obtained for the inhibitors not shown in Fig. 2. The greatest variability was observed for enzymes with weak activities against nitrocefin or for inhibitors with a steep slope. There was little variation in values of the maximum percent inhibition, with the standard errors not exceeding ±3% for most enzyme-inhibitor combinations.

Representative β-lactamases of the major classes of plasmid-determined β-lactamases and chromosomal β-lactamases can be distinguished as shown in Fig. 3. Chromosomal β-lactamases, which were exemplified by *Enterobacter cloacae* P99 β-lactamase, were characterized by the lack of inhibition by clavulanate, sulbactam, or tazobactam and strong inhibition by aztreonam, ceftibuten, and cefuroxime. In contrast, the profile of the plasmid-determined β-lactamase TEM-1 was characterized by strong inhibition by clavulanate, sulbactam, and tazobactam and weak or no inhibition by aztreonam, ceftibuten, or cefuroxime. The OXA-6 enzyme differed from TEM-1 in that it was inhibited by aztreonam and cefuroxime, with the percent inhibition decreasing rapidly over 30 min. CARB-3 had a profile similar to that of TEM-1 when the inhibitors shown in Fig. 3 were used. However, CARB-3 could be distinguished from TEM-1 by its reaction with other inhibitors, as shown in Fig. 4. Cloxacillin and imipenem inhibited TEM-1 much more strongly than they inhibited CARB-3. Conversely, cefoxitin and cefotaxime inhibited CARB-3 much more strongly than they inhibited TEM-1.

β-Lactamases that are closely related genetically may have different inhibition profiles by the nitrocefin competition assay. Figure 5 shows the inhibitors that discriminated TEM-7 and SHV-2, which are extended-spectrum β-lactamases, from their progenitors TEM-1 and SHV-1, respectively. Both TEM-1 and SHV-1 were inhibited by tazobactam and imipenem, but the slope of the inhibition curves of tazobactam and imipenem were consistently steeper for SHV-1. Additionally, SHV-1 was inhibited by cefuroxime, while TEM-1 was not. The TEM derivative TEM-7 was

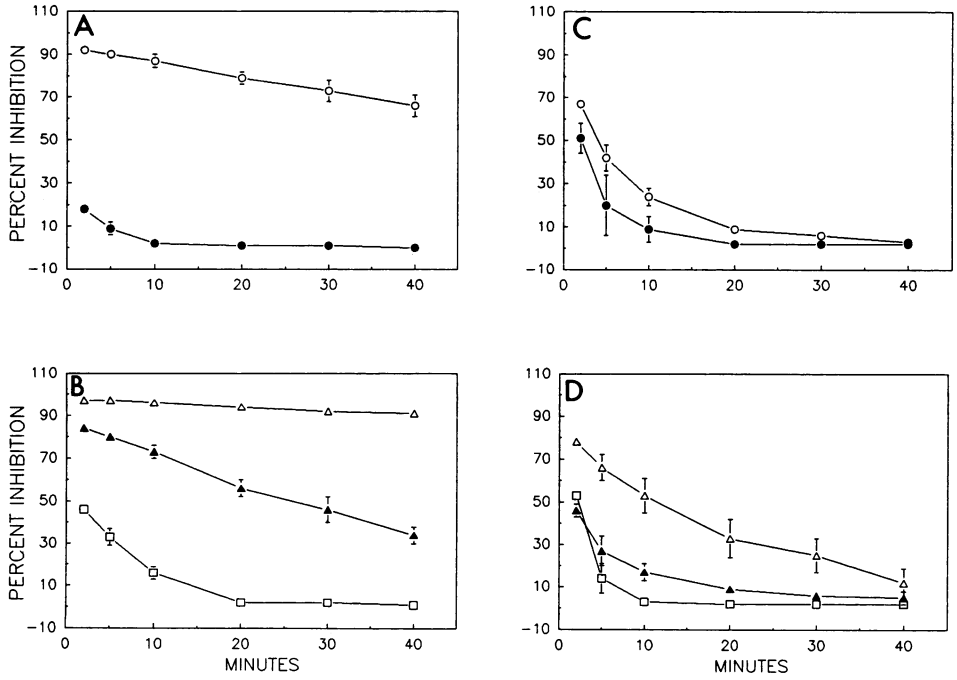

FIG. 2. Inhibition profiles of TEM-1 (A and B) and SHV-1 (C and D) β-lactamases showing ranges of values on multiple determinations. Symbols: ○, imipenem; ●, cefuroxime; △, tazobactam; ▲, cloxacillin; □, cefoperazone.

TABLE 2—*Continued*

Ceftazidime (Avg ± SE)	Aztreonam (Avg ± SE)	Ceftriaxone (Avg ± SE)	Cefuroxime (Avg ± SE)	Cefoperazone		Imipenem		Cloxacillin	
				Avg ± SE	SL	Avg ± SE	SL	Avg ±SE	SL
3 ± 1	6 ± 2	3 ± 1	18 ± 2	46 ± 2	3	92 ± 2	0.7	84 ± 2	1.2
9 ± 3	1 ± 0	1 ± 0	13 ± 5	48 ± 3	2.1	90 ± 1	0.5	81 ± 2	1.4
10	3	1	19	48	2.2	89	0.6	82	1.2
6	1	1	ND[e]	40	3.1	87	0.7	77	1.5

strongly inhibited by cefotetan, in contrast to TEM-1, and was inhibited poorly by aztreonam and ceftriaxone, in contrast to SHV-2. SHV-2 was strongly inhibited by aztreonam and ceftriaxone, which distinguished it from SHV-1.

The inhibition profiles of TEM-1, TEM-2, and eight extended-spectrum β-lactamases derived from TEM are shown in Table 3. All the TEM enzymes were well inhibited by imipenem and cloxacillin. All the extended-spectrum enzymes were well inhibited by cefoxitin, cefotetan, and cefoperazone. TEM-3 was further distinguished by being highly inhibited by aztreonam. The inhibition profile of TEM-5 was distinctive in that it showed weak inhibition by clavulanate, sulbactam, or tazobactam and strong inhibition by cefsulodin. TEM-6 was strongly inhibited by aztreonam and ceftriaxone but was very weakly inhibited by cefsulodin. In contrast, TEM-10 was strongly inhibited by cefsulodin, ceftriaxone, and aztreonam.

The slope of the inhibition curve contributed to the discrimination of the TEM-derived β-lactamases, especially when maximum levels of inhibition were high. For example, the initial percents inhibition by cefoperazone for TEM-3 and TEM-6 were 81 and 93%, respectively, but in 40 min they fell to 11% for TEM-3 (slope, 1.8) and 70% for TEM-6 (slope, 0.6). Similarly, the greater slope of inhibition by cloxacillin helped to discriminate TEM-1 from TEM-10.

Table 4 shows the inhibition profiles of SHV-1, OHIO-1, and extended-spectrum β-lactamases derived from SHV-1. The SHV derivatives could readily be distinguished from the TEM derivatives. All SHV derivatives were inhibited by cefuroxime and were inhibited to a lesser extent by cefotetan than the TEM derivatives were. The much greater slope of tazobactam inhibition of SHV-1 compared with that of TEM-1 helped to discriminate between these two enzymes. Slope differences in cefoxitin inhibition also contributed to the discrimination between SHV-derived and TEM-derived extended-spectrum β-lactamases. SHV-4 could be distinguished from SHV-2 β-lactamase because it was inhibited by ceftazidime and ceftibuten.

Five pairs of plasmid-mediated β-lactamases that could not be discriminated from each other by inhibition profile, although they could readily be discriminated by isoelectric focusing, are shown in Table 5. Additionally, SHV-4 was indistinguishable from SHV-5 by inhibition profile (data not shown). TEM-6 was indistinguishable from CAZ-2 both by inhibition profile and by isoelectric point.

Table 6 shows the inhibition profiles of eight chromosomal β-lactamases. In contrast to most of the plasmid-determined β-lactamases, the chromosomal β-lactamases tested could not be discriminated from each other by their inhibition profiles. All were very strongly inhibited by most cephalo-

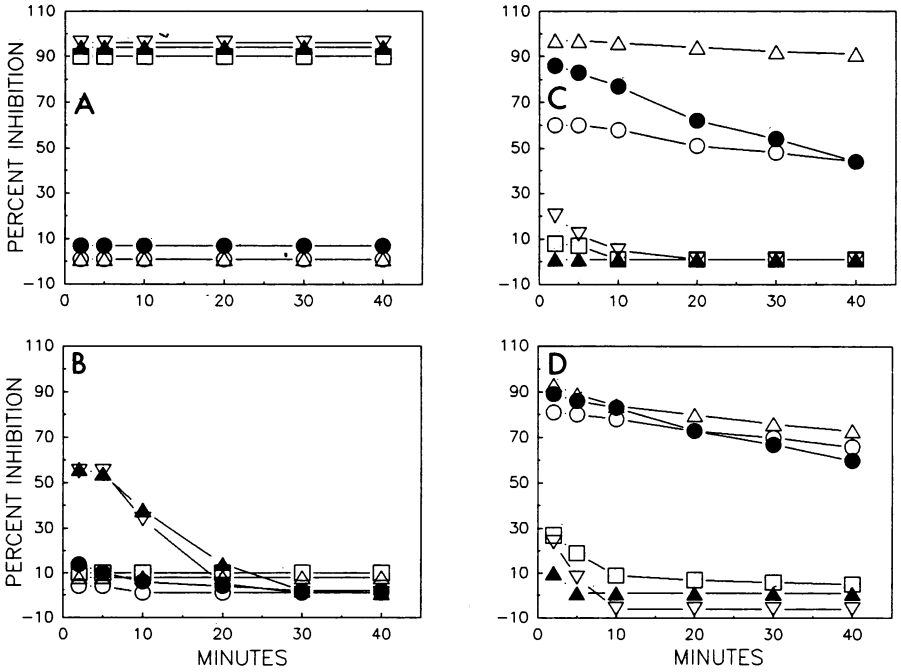

FIG. 3. Inhibitors that discriminate between representative enzymes belonging to different β-lactamase classes (P99 [A], OXA-6 [B], TEM-1 [C], CARB-3 [D]) by NCA. Symbols: ○, clavulanate; ●, sulbactam; △, tazobactam; ▲, aztreonam; □, ceftibuten; ▽, cefuroxime.

TABLE 3. Inhibition profiles of TEM-derived β-lactamases

β-Lactamase	pI	Clavulanate		Sulbactam		Tazobactam		Cefoxitin		Cefotetan		Moxalactam		Cefotaxime		Ceftazidime	
		Avg ± SE[a]	SL[b]	Avg ± SE	SL	Avg ± SE	SL	Avg ± SE	SL	Avg ± SE	SL	Avg ± SE	SL	Avg ± SE	SL	Avg ± SE	SL
TEM-1	5.4	60 ± 3	0.4	86 ± 2	1.1	97 ± 0	<0.3	21 ± 1		11 ± 2		3 ± 1		5 ± 2		3 ± 1	
TEM-2	5.6	57	0.7	85	1.3	97	<0.3	25		7		8		7		10	
TEM-3	6.3	60 ± 5	<0.3	79 ± 4	0.3	71 ± 6	0.3	57 ± 3	0.3	53 ± 5	0.3	24 ± 5		68 ± 2	1.5	1 ± 1	
TEM-5	5.57	33 ± 3	0.6	39 ± 2	0.9	16 ± 4	<0.3	87 ± 3	<0.3	85 ± 2	<0.3	70 ± 3	0.4	75 ± 2	1.5	6 ± 1	
TEM-6	5.87	81 ± 2	0.4	93 ± 2	0.4	93 ± 2	0.3	83 ± 3	<0.3	94 ± 3	<0.3	77 ± 1	<0.3	84 ± 3	1.5	49 ± 6	4.4
CAZ-2	6.0	64 ± 2	0.4	83 ± 1	0.3	78 ± 1	0.5	78 ± 1	<0.3	87 ± 2	<0.3	75 ± 2	<0.3	83 ± 1	1	38 ± 5	2.2
TEM-7	5.41	81 ± 7	0.3	97 ± 1	0.3	86 ± 5	<0.3	83 ± 2	<0.3	68 ± 2	<0.3	33 ± 2	0.3	69 ± 4	1.5	7 ± 1	
TEM-101	5.2	82 ± 5	0.5	93 ± 0	<0.3	93 ± 2	0.4	86 ± 3	<0.3	80 ± 4	<0.3	47 ± 5	<0.3	66 ± 7	1.4	4 ± 2	
TEM-9	5.56	54 ± 6	0.3	76 ± 6	0.5	79 ± 2	<0.3	53 ± 5	<0.3	79 ± 1	<0.3	29 ± 5	<0.3	74 ± 2	1.2	11 ± 2	
TEM-10	5.57	73 ± 3	<0.3	77 ± 2	0.3	69 ± 1	0.4	81 ± 4	<0.3	81 ± 2	<0.3	76 ± 3	<0.3	79 ± 4	0.3	38 ± 0	1.4

[a] Average of maximum percent inhibition ± standard error.
[b] SL, Slope, given when maximum inhibition exceeded 25%.

TABLE 4. Inhibition profiles of SHV-derived β-lactamases

β-Lactamase	pI	Clavulanate		Sulbactam		Tazobactam		Cefoxitin		Cefotetan		Moxalactam		Cefotaxime		Ceftazidime	
		Avg ± SE[a]	SL[b]	Avg ± SE	SL	Avg ± SE	SL	Avg ± SE	SL	Avg ± SE	SL	Avg ± SE	SL	Avg ± SE	SL	Avg ± SE	SL
SHV-1	7.6	51 ± 1	0.8	44 ± 2	4	78 ± 2	1.6	4 ± 2		4 ± 2		1 ± 0		15 ± 7		6 ± 5	
OHIO-1	7.0	50	0.5	54	4	81	1.2	10		2		1		2		14	
SHV-2	7.6	69 ± 3	0.4	86 ± 2	1.2	84 ± 3	0.6	78 ± 3	1.3	7 ± 5		8 ± 4		93 ± 3	0.9	13 ± 4	
SHV-4	8.2	71 ± 1	0.7	88 ± 1	0.7	85 ± 2	0.5	76 ± 0	1.3	35 ± 5	0.5	52 ± 4	0.3	91 ± 2	0.6	63 ± 5	2.6

[a] Average of maximum percent inhibition ± standard error.
[b] SL, Slope, given when maximum inhibition exceeded 25%.

sporins, with there being little decrease in inhibition over time.

DISCUSSION

Nitrocefin has been used previously as a competing substrate to monitor enzyme-inhibitor interactions (11, 14). James (14) used multiple concentrations of inhibitors to calculate a relative substrate affinity index based on a 5-min reaction between enzyme, inhibitor, and nitrocefin. In the NCA described here, we used a panel of 17 inhibitors in a fixed concentration in two microdilution plates and recorded the interaction between the enzyme, substrate, and inhibitor over 45 min. The concentration of inhibitors was chosen to permit optimal discrimination after testing a variety of β-lactamases with a wide range of concentrations of each of the various inhibitors. The discrimination of β-lactamase types by NCA is based on the relative affinities of their active sites for the different inhibitors and the stability of the enzyme-inhibitor complex (8).

The method requires only crude sonic extracts of enzyme; thus, it is rapid and simple to perform. Care must be taken, however, to ensure that the extract does not contain multiple highly active β-lactamases; this is usually accomplished by demonstrating a single dominant enzyme on an isoelectric focusing gel. Automation by the use of the plate photometer and the computer interface facilitated the screening of large numbers of isolates for the detection of different types of β-lactamases and permitted quantitation of the maximum percent inhibition of nitrocefin hydrolysis and the decrease in inhibition over time (slopes). Since inhibitors were tested simultaneously on one or two plates, values of maximum inhibition relative to one another were internally controlled. When an inhibitor displays a similar maximum level of

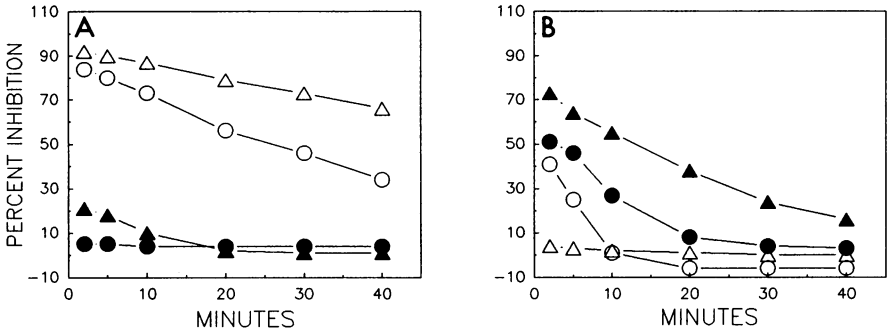

FIG. 4. Inhibitors that discriminate between TEM-1 (A) and CARB-3 (B) β-lactamases by NCA. Symbols: ○, cloxacillin; ●, cefotaxime; △, imipenem; ▲, cefoxitin.

TABLE 3—*Continued*

Aztreonam		Ceftriaxone		Cefsulodin		Cefpirome		Ceftibuten		Cefuroxime (Avg ± SE)	Cefoperazone		Imipenem		Cloxacillin	
Avg ± SE	SL	Avg ± SE	SL	Avg ± SE	SL	Avg ± SE	SL	Avg ± SE	SL		Avg ± SE	SL	Avg ± SE	SL	Avg ± SE	SL
6 ± 2		3 ± 1		1 ± 0		4 ± 1		7 ± 2		18 ± 2	46 ± 2	3	92 ± 2	0.7	84 ± 2	1.2
1		5		2		7		0		0	48	4	89	0.7	82	1.4
89 ± 2	0.8	39 ± 2	3.6	5 ± 1		7 ± 3		8 ± 1		25 ± 4	81 ± 2	1.8	64 ± 5	<0.3	87 ± 3	0.4
30 ± 4	2.9	56 ± 2	3.9	78 ± 3	1.3	8 ± 2		14 ± 5		10 ± 2	72 ± 2	1.6	92 ± 2	<0.3	92 ± 2	0.5
63 ± 5	1.4	73 ± 2	1.6	15 ± 1		44 ± 4	3.5	29 ± 1	2	16 ± 3	93 ± 1	0.6	92 ± 3	<0.3	96 ± 2	<0.3
69 ± 2	1.6	71 ± 1	1.6	23 ± 2		56 ± 2	2.5	22 ± 5		3 ± 1	84 ± 1	0.7	87 ± 1	<0.3	89 ± 2	<0.3
18 ± 2		35 ± 5	3	22 ± 7		26 ± 5	2.3	10 ± 6		1 ± 0	79 ± 6	1.6	90 ± 3	<0.3	96 ± 0	<0.3
18 ± 4		45 ± 2	2.6	31 ± 7	2.0	31 ± 5	2	12 ± 5		9 ± 5	81 ± 3	1.5	95 ± 2	<0.3	94 ± 1	<0.3
24 ± 7		24 ± 3		4 ± 3		2 ± 1		2 ± 1		1 ± 0	77 ± 4	1.0	85 ± 0	<0.3	88 ± 1	0.3
78 ± 2	0.5	73 ± 2	0.7	63 ± 3	0.9	26 ± 4	1.4	24 ± 3		16 ± 3	72 ± 5	0.6	84 ± 0	<0.3	84 ± 4	<0.3

TABLE 4—*Continued*

Aztreonam		Ceftriaxone		Cefsulodin		Cefpirome		Ceftibuten		Cefuroxime		Cefoperazone		Imipenem		Cloxacillin	
Avg ± SE	SL	Avg ± SE	SL	Avg ± SE	SL	Avg ± SE	SL	Avg ± SE	SL	Avg ± SE	SL	Avg ± SE	SL	Avg ± SE	SL	Avg ± SE	SL
1 ± 0		4 ± 2		1 ± 0		1 ± 0		10 ± 5		51 ± 7	4.3	53 ± 2	5.1	67 ± 1	4.3	46 ± 3	2.9
5		11		1		3		10		59	4.3	59	5.5	61	3.8	56	1.9
85 ± 3	1.3	89 ± 2	1.2	7 ± 5		27 ± 5	1.6	19 ± 5		54 ± 5	3.5	64 ± 3	1.5	76 ± 1	0.6	91 ± 2	0.7
96 ± 0		88 ± 0	1.6	27 ± 3	2.6	17 ± 4		56 ± 4	3.9	63 ± 1	4.6	55 ± 2	1.3	60 ± 3	0.9	87 ± 0	0.5

inhibition for two β-lactamases, comparison of slopes may be of further help in discriminating them.

The inhibition data can be grouped into semiquantitative categories, as shown in Fig. 6 and 7, which display algorithms that permit presumptive discrimination of TEM- and SHV-type β-lactamases based on a limited number (n = 10) of inhibitors. The differences in inhibition are often sufficiently large that a visual reading of a single plate containing these 10 inhibitors at 2 and 10 min could presumptively discriminate a reference and a test β-lactamase run on the same plates. The high cost of nitrocefin remains a limitation of this and other methods that are based on its use.

Other methods for characterizing β-lactamases are often difficult to perform. Determination of the kinetic constants V_{max} and K_m of β-lactamases requires partially pure enzymes and is laborious to perform against a large variety of

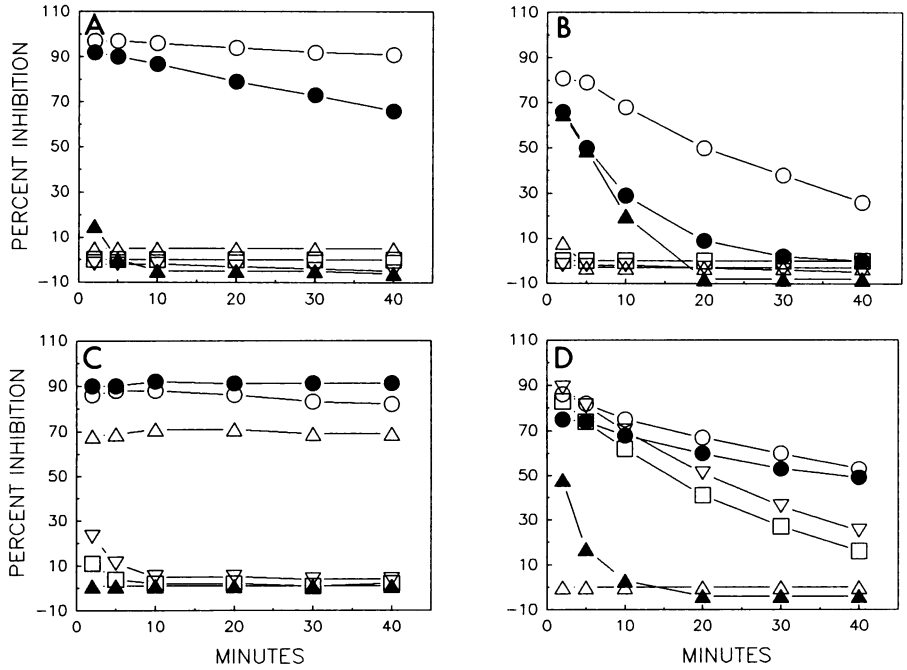

FIG. 5. Inhibitors that discriminate between TEM-1 (A), SHV-1 (B), TEM-7 (C), and SHV-2 (D). Symbols: ○, tazobactam; ●, imipenem; △, cefotetan; ▲, cefuroxime; ▽, aztreonam; □, ceftriaxone.

TABLE 5. Pairs of β-lactamases with indistinguishable inhibition profiles

β-Lactamase type	pI	Amino acid[a]												
		Near active site at position[b]:					Distal to active site at position[c]:							
		102	162	234	235	236	39	49	50	51	59	201	203	261
TEM-1	5.4	Glu	Arg			Gly	I Gln I						Gln	Thr
TEM-2	5.6	Glu	Arg			Gly	I Lys I						Gln	Thr
TEM-4	5.9	Lys	Arg			Ser	I Gln I						Gln	I Met I
TEM-3	6.3	Lys	Arg			Ser	I Lys I						Gln	I Thr I
TEM-101	5.2	Glu	Ser			Gly	I Gln I						Gln	Thr
TEM-7	5.41	Gly	Ser			Gly	I Lys I						Gln	Thr
SHV-1	7.6			Gly	Glu		I Arg	Arg	Pro	Gly	Asn I	Arg		
OHIO-1[d]	7.0			Gly	Glu		I Ser	Ser	Gly	Arg	Asp I	Arg		
SHV-2	7.6			Ser	Glu							I Arg I		
SHV-3	7.0			Ser	Glu							I Leu I		

[a] Amino acid residues are numbered as dscribed by Sutcliffe (37) for TEM derivatives. The corresponding residues for SHV derivatives are numbered two less than those for TEM-1 (3).
[b] Boxes III, V, and VII, of Joris et al. (16).
[c] Not adjacent to boxes I to VII of Joris et al. (16).
[d] OHIO-1 as compared with SHV-1 has nine additional substitutions in positions not adjacent to any of the conserved boxes and a terminal deletion at position 279 (D. Shlaes, personal communication).

β-lactams. Slow hydrolysis rates, poor affinity of the substrate for the β-lactamases, or low molar coefficients (7, 8) add to the difficulty of the biochemical characterization of β-lactamases. Moreover, compounds like carbapenems, clavams, and sulfones may result in irreversible inactivation of the β-lactamases (38).

Isoelectric focusing alone has become insensitive for differentiation as the number of extended-spectrum β-lactamases has grown (29). DNA hybridization has been proposed as an alternative for identifying β-lactamases; however, many variables may affect the final results (21). Amino acid sequencing has contributed to a better understanding of the structure-activity relationships of these enzymes, in that substitutions that occur near the regions that determine the active-site configuration may result in enzymes with identical pIs but altered kinetic properties (16–18). NCA may facilitate discrimination in such cases (see Table 5). For example, the SHV-1 and SHV-2 β-lactamases both have pIs of 7.6, but they have very distinct inhibition profiles. The single serine substitution at position 236 by which SHV-2 is derived from SHV-1 is essential for the extended spectrum of activity of SHV-2 (4). Similarly, TEM-5 (pI 5.57), TEM-10 (pI 5.57), and TEM-9 (pI 5.56) β-lactamases, which are virtually indistinguishable by isoelectric focusing, have distinct inhibition profiles by NCA and have different kinetic parameters (30, 34, 36).

On the other hand, amino acid substitutions in positions that are not located near the critical domains may result in enzymes that have different pIs but similar biochemical properties (18). Such β-lactamases may be indistinguishable by kinetic constant determinations or inhibition profiles. For example, TEM-7 β-lactamase (pI 5.41), which is derived

TABLE 6. Inhibition profiles of chromosomal β-lactamases

Host species	pI	Clavulanate (avg[a])	Sulbactam (avg)	Tazobactam (avg)	Cefoxitin		Aztreonam		Cefuroxime		Ceftibuten		Cefotaxime		Ceftazidime		Cefoperazone	
					Avg	SL[b]	Avg	SL	Avg	SL	Avg	SL	Avg	SL	Avg	SL	Avg	SL
E. cloacae 81010504	7.66	5	2	5	94	<0.3	92	<0.3	92	<0.3	88	0.6	91	0.3	96	0.2	88	1.1
E. cloacae 55M	7.66	14	0	15	92	<0.3	94	<0.3	94	<0.3	88	<0.3	93	<0.3	90	0.5	90	1.3
E. cloacae 83120215	7.8	4	5	0	98	<0.3	97	<0.3	98	<0.3	94	<0.3	94	<0.3	94	0.5	ND[c]	
E. aerogenes 83101021	7.9	13	5	0	98	<0.3	98	<0.3	98	<0.3	94	<0.3	95	<0.3	92	<0.3	80	1.2
S. marcescens 83120234	7.9	6	2	0	90	<0.3	91	<0.3	88	<0.3	84	<0.3	90	<0.3	83	0.5	79	0.9
S. marcescens 83110701	8.4	1	10	0	80	<0.3	81	<0.3	85	<0.3	76	0.6	80	<0.3	72	0.7	78	0.5
E. coli 87120702	8.5	1	1	0	95	<0.3	93	<0.3	93	<0.3	88	<0.3	95	<0.3	84	0.3	84	1
A. calcoaceticus 83111835	8.8	6	9	0	87	<0.3	86	<0.3	86	<0.3	78	<0.3	85	<0.3	88	<0.3	64	<0.3

[a] Average of maximum percent inhibition.
[b] SL, Slope.
[c] ND, Not determined.

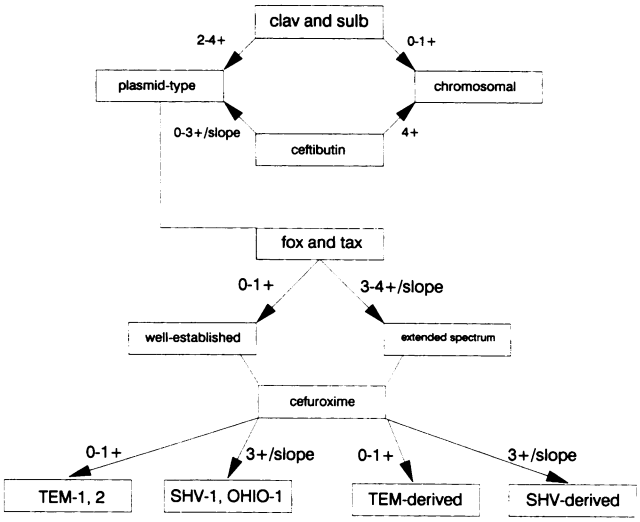

FIG. 6. Algorithm for discriminating between plasmid-determined and class I chromosomal β-lactamases: 1+, less than 25% inhibition; 2+, up to 50% inhibition; 3+, up to 75% inhibition; 4+, more than 75% inhibition. Slope indicates a relatively rapid decrease in inhibition, i.e., >0.7. clav, Clavulanate; sulb, sulbactam; fox, cefoxitin; tax, cefotaxime.

from TEM-2 (pI 5.6) by a serine substitution at position 236, has the same inhibition profile as TEM-101 (pI 5.2), which is a laboratory-constructed mutant of TEM-1 (pI 5.4) with a serine substitution at position 236 (10). Likewise, TEM-4 and TEM-3 have identical inhibition profiles and share common amino acid substitutions at positions 162 and 236; the amino acids at these positions are responsible for catalytic activity (29). The more basic amino acids at positions 37 and 201 apparently result in higher isoelectric points. Similarly, the OHIO-1 β-lactamase (pI 7.0), which has the same nucleotide sequences as SHV-1 at positions that determine active-site configuration (D. Shlaes, personal communication), has an identical inhibition profile.

An amino acid substitution at a site distal to those that determine active-site configurations appears, also, to explain the differences in isoelectric points between SHV-2 and SHV-3. When the sequence of SHV-5 becomes known, a similar substitution may account for the difference between it and SHV-4. TEM-6 and CAZ-2, on the other hand, are indistinguishable both by inhibition profile and isoelectric point, suggesting that they may be the same β-lactamase.

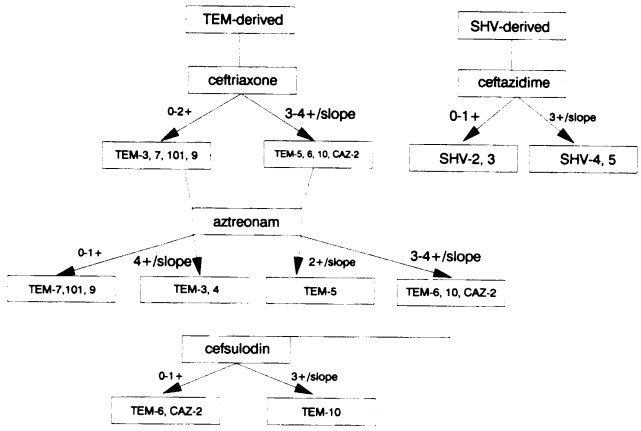

FIG. 7. Algorithm for discriminating between TEM- and SHV-derived extended-spectrum β-lactamases. Inhibition numbers are as described in the legend to Fig. 6.

The continuing evolution and appearance of new β-lactamase types has created the need for additional methods of discriminating them. As new amino acid sequences or active-site structures have become available, assignment to molecular classes may become routine. However, at this time many β-lactamases remain unclassified on a molecular level (6). NCA used in conjunction with isoelectric focusing and more conventional biochemical characterization enables better discrimination of β-lactamase types. The method also contributes to a better understanding of the relationships between active-site structure and function of β-lactamases.

ACKNOWLEDGMENTS

We thank C. O'Gara, E. Papa, and R. Dickenson for expert technical assistance and C. Breggia for secretarial assistance.

LITERATURE CITED

1. **Ambler, R. P.** 1980. The structure of beta-lactamases. Philos. Trans. R. Soc. London Ser. B **289**:321–331.
2. **Ambler, R. P., and G. R. Scott.** 1978. Partial amino acid sequence of penicillinase coded by *E. coli* plasmid R6K. Proc. Natl. Acad. Sci. USA **75**:3732–3736.
3. **Barthelemy, M., J. Peduzzi, and R. Labia.** 1988. Complete amino acid sequence of p453-plasmid mediated PIT-2 beta-lactamase (SHV-1). Biochem. J. **251**:73–79.
4. **Barthelemy, M., J. Peduzzi, H. B. Yaghlane, and R. Labia.** 1988. Single aminoacid substitution between SHV-1 beta-lactamase and cefotaxime hydrolyzing SHV-2 enzyme. FEBS Lett. **231**: 217–220.
5. **Bauernfeind, A., and G. Horl.** 1987. Novel R-factor borne beta-lactamase of *Escherichia coli* conferring resistance to cephalosporins. Infection **15**:257–259.
6. **Bush, K.** 1989. Characterization of β-lactamases. Antimicrob. Agents Chemother. **33**:259–263.
7. **Bush, K., J. Freudenberger, and R. Sykes.** 1982. Interaction of aztreonam and related monobactams with beta-lactamases from gram negative bacteria. Antimicrob. Agents Chemother. **22**: 414–420.
8. **Bush, K., and R. B. Sykes.** 1984. Interaction of beta-lactam antibiotics with beta-lactamases as a source for resistance, p. 1–31. *In* L. E. Bryan (ed.), Antimicrobial drug resistance. Academic Press, Inc., Orlando, Fla.
9. **Chanal, C. M., D. L. Sirot, R. Labia, A. Petit, A. Morand, J. L. Sirot, and R. A. Cluzel.** 1988. Comparative study of a novel plasmid-mediated β-lactamase, CAZ-2, and the CTX-1 and CAZ-1 enzymes conferring resistance to broad-spectrum cephalosporins. Antimicrob. Agents Chemother. **32**:1660–1665.
10. **Collatz, E., N. Tran Van, D. Billot-Klein, R. Williamson, and L. Gutmann.** 1989. Substitution of serine for arginine in position 162 of TEM-type beta-lactamases extends the substrate profile of mutant enzymes TEM-7 and TEM-101 to ceftazidime and aztreonam. Gene **78**:349–354.
11. **Eliasson, I., and C. Kamme.** 1985. Characterization of the plasmid mediated beta-lactamase in *Branhamella catarrhalis* with special reference to substrate affinity. J. Antimicrob. Chemother. **15**:139–149.
12. **Gutmann, L., B. Ferre, F. W. Goldstein, N. Rizk, E. Pinto-Schuster, J. F. Acar, and E. Collatz.** 1989. SHV-5, a novel SHV-type β-lactamase that hydrolyzes broad-spectrum cephalosporins and monobactams. Antimicrob. Agents Chemother. **33**:951–956.
13. **Hedges, R. W., N. Datta, P. Kontomichalou, and J. T. Smith.** 1974. Molecular specificities of R factor determined beta-lactamases: correlation with plasmid compatibility. J. Bacteriol. **117**:56–62.
14. **James, R.** 1983. Relative substrate affinity index values: a

method for identification of beta-lactamase enzymes and prediction of successful beta-lactam therapy. J. Clin. Microbiol. **17**:791–798.

15. **Jaurin, B., and T. Grundstrom.** 1981. AmpC cephalosporinase of *Escherichia coli* K12 has a different evolutionary origin from that of beta-lactamase of the penicillinase type. Proc. Natl. Acad. Sci. USA **78**:4897–4901.
16. **Joris, B., J. M. Ghuysen, G. Dive, A. Renard, O. Dideberg, P. Charlier, J. M. Frere, J. A. Kelly, J. C. Boyington, P. C. Moews, and J. R. Knox.** 1988. The active site-serine penicillin-recognizing enzymes as members of the streptomyces R61 DD-peptidase family. Biochem. J. **250**:313–324.
17. **Kliebe, C., B. A. Nies, J. F. Meyer, R. M. Tolxdorff-Neutzling, and B. Wiedemann.** 1985. Evolution of plasmid-coded resistance to broad-spectrum cephalosporins. Antimicrob. Agents Chemother. **28**:302–307.
18. **Labia, R., M. Barthelemy, J. Peduzzi, A. Morand, K. Tiwari, H. B. Yaghlane, J. Sirot, and D. Sirot.** 1989. Properties of novel plasmid mediated beta-lactamases hydrolyzing third generation cephalosporins. Their position in class A beta-lactamases. Med. Mal. Infect. Hors Ser. **22**:27–36.
19. **Labia, R., M. Guionie, and M. Barthelemy.** 1981. Properties of three carbenicillin-hydrolyzing beta-lactamases (CARB) from *Pseudomonas aeruginosa*: identification of a new enzyme. J. Antimicrob. Chemother. **7**:49–56.
20. **Labia, R., A. Morand, K. Tiwari, J. Sirot, D. Sirot, and A. Petit.** 1988. Interactions of new plasmid mediated beta-lactamases with third generation cephalosporins. Rev. Infect. Dis. **4**:885–891.
21. **Levesque, R. C., A. A. Medeiros, and G. A. Jacoby.** 1987. Molecular cloning and DNA homology of plasmid mediated beta-lactamase genes. Mol. Gen. Genet. **206**:252–258.
22. **Matthew, M., A. M. Harris, M. J. Marshall, and G. W. Ross.** 1975. The use of isoelectric focusing for detection and identification of beta-lactamases. J. Gen. Microbiol. **88**:169–178.
23. **Medeiros, A. A.** 1984. Beta-lactamases. Br. Med. Bull. **40**:18–27.
24. **Medeiros, A. A., M. Cohenford, and G. A. Jacoby.** 1985. Five novel plasmid-determined β-lactamases. Antimicrob. Agents Chemother. **27**:715–719.
25. **Nicolas, M.-H., V. Jarlier, N. Honore, A. Philippon, and S. T. Cole.** 1989. Molecular characterization of the gene encoding SHV-3 β-lactamase responsible for transferable cefotaxime resistance in clinical isolates of *Klebsiella pneumoniae*. Antimicrob. Agents Chemother. **33**:2096–2100.
26. **O'Callaghan, C. H., A. Morris, S. M. Kirby, and A. H. Shingler.** 1972. Novel method for detection of β-lactamases by using a chromogenic cephalosporin substrate. Antimicrob. Agents Chemother. **1**:283–288.
27. **Paul, G. C., G. Gerbaud, A. Bure, A. M. Philippon, B. Pangon, and P. Courvalin.** 1989. TEM-4, a new plasmid-mediated β-lactamase that hydrolyzes broad-spectrum cephalosporins in a clinical isolate of *Escherichia coli*. Antimicrob. Agents Chemother. **33**:1958–1963.
28. **Péduzzi, J., M. Barthélémy, K. Tiwari, D. Mattioni, and R. Labia.** 1989. Structural features related to hydrolytic activity against ceftazidime of plasmid-mediated SHV-type CAZ-5 β-lactamase. Antimicrob. Agents Chemother. **33**:2160–2163.
29. **Philippon, A., R. Labia, and G. A. Jacoby.** 1989. Extended-spectrum β-lactamases. Antimicrob. Agents Chemother. **33**: 1131–1136.
30. **Quinn, J. P., D. Miyashiro, D. Sahm, R. Flamm, and K. Bush.** 1989. Novel plasmid-mediated β-lactamase (TEM-10) conferring selective resistance to ceftazidime and aztreonam in clinical isolates of *Klebsiella pneumoniae*. Antimicrob. Agents Chemother. **33**:1451–1456.
31. **Seeberg, A. H., R. M. Tolxdorff-Neutzling, and B. Wiedemann.** 1983. Chromosomal β-lactamases of *Enterobacter cloacae* are responsible for resistance to third-generation cephalosporins. Antimicrob. Agents Chemother. **23**:918–925.
32. **Shlaes, D. M., A. A. Medeiros, M. A. Kron, C. Currie-McCumber, E. Papa, and C. V. Vartian.** 1986. Novel plasmid-mediated β-lactamase in members of the family *Enterobacteriaceae* from Ohio. Antimicrob. Agents Chemother. **30**:220–224.
33. **Sirot, D., J. Sirot, R. Labia, A. Morand, P. Courvalin, A. Darfeuille-Michaud, R. Perroux, and R. Cluzel.** 1987. Transferable resistance to third generation cephalosporins in clinical isolates of *K. pneumoniae*: identification of CTX-1 a novel beta-lactamase. J. Antimicrob. Chemother. **20**:323–334.
34. **Sirot, J., R. Labia, and A. Thabaut.** 1987. *K. pneumoniae* strains more resistant to ceftazidime than other third generation cephalosporins. J. Antimicrob. Chemother. **20**:611–612.
35. **Sougakoff, W., S. Goussard, G. Gerband, and P. Courvalin.** 1988. Plasmid-mediated resistance to third generation cephalosporin caused by point mutations in TEM-type penicillinase genes. Rev. Infect. Dis. **10**:879–884.
36. **Spencer, R. C., P. F. Wheat, T. G. Winstanley, and D. M. Cox.** 1987. Novel beta-lactamase in a clinical isolate of *K. pneumoniae* conferring unusual resistance to beta-lactam antibiotics. J. Antimicrob. Chemother. **20**:919–921.
37. **Sutcliffe, J. C.** 1978. Nucleotide sequence of ampicillin resistance gene of *Escherichia coli* plasmid pBR322. Proc. Natl. Acad. Sci. USA **75**:3737–3741.
38. **Sykes, R. B., and K. Bush.** 1982. Physiology, biochemistry and inactivation of beta-lactamases, p. 155–207. *In* R. B. Morin and M. Gorman (ed.), Chemistry and biology of beta-lactam antibiotics, vol. 3. Academic Press, Inc., New York.
39. **Vecoli, C., F. E. Prevost, J. J. Ververis, A. A. Medeiros, and G. P. O'Leary, Jr.** 1983. Comparison of polyacrylamide and agarose gel thin-layer isoelectric focusing for the characterization of β-lactamases. Antimicrob. Agents Chemother. **24**:186–189.