ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, May 1977, p. 785-790
Vol. 11, No. 5

Printed in U.S.A.

Beta-Lactamases Produced by a *Pseudomonas aeruginosa* Strain Highly Resistant to Carbenicillin

ROGER LABIA,* MARLÈNE GUIONIE, JEAN-MICHEL MASSON, ALAIN PHILIPPON, AND MICHEL BARTHELEMY

CERCOA-CNRS, 94320 Thiais, France, and Service de Bactériologie CHU Cochin, 75674 Paris, cédex 14, France*

Received for publication 18 November 1976

A *Pseudomonas aeruginosa* strain isolated at Besançon Hospital, France, proved to be highly resistant to carbenicillin and showed a high hydrolytic activity toward this antibiotic. We clearly demonstrated that two β-lactamases were synthetized: one of them, constitutive, has its enzymatic activity directed mainly toward penicillins, and carbenicillin appears to be its best substrate (higher V_{max}); thus, this β-lactamase is a "carbenicillinase" that differs from the well-known "TEM-like" enzymes. The isoelectric point of this carbenicillinase is 5.30 ± 0.03. The other one is an inducible cephalosporinase, very similar to the cephalosporinases usually found in these organisms. Its isoelectric point is 8.66 ± 0.04. These two enzymes have been separated by affinity chromatography and isoelectric focusing. The kinetic constants were measured by computerized microacidimetry.

Pseudomonas aeruginosa resistance to cephalosporins is usually interpreted as the result of the biosynthesis by the bacteria of β-lactamase active toward these antibiotics, an inducible cephalosporinase (29) that seems to be chromosally mediated. As carbenicillin is not a substrate for this enzyme (28, 29), usually *P. aeruginosa* strains are susceptible to this antibiotic, but very early some strains appear to be resistant (2, 9).

P. aeruginosa resistance to carbenicillin is a complex problem: bacterial strains resistant to this antibiotic are frequently encountered, but they do not necessarily show any hydrolytic activity toward this molecule. In these cases, carbenicillin resistance might be related to permeability modifications of the cell wall (1, 14).

Some strains were described where a low hydrolytic activity toward carbenicillin had been shown. This activity did not seem to have much physiological meaning (29).

In 1970 (22), for the first time *P. aeruginosa* strain (Dalgleish) was described that showed an unusually high hydrolytic activity against carbenicillin. A few years later, the enzyme produced by this strain was subjected to more sophisticated investigations (6).

Then the presence of R-factor-determined β-lactamases, active toward carbenicillin, was proved by transfer from *P. aeruginosa* to *Escherichia coli* (5) (31). Transfer occurred with a higher frequency when the recipient strain was another *P. aeruginosa* (3, 8). Some plasmids were isolated (4, 7).

In France (17) a TEM β-lactamase with pI = 5.4 was identified in a *P. aeruginosa* strain. This enzyme is similar to the type I described by Pitton (26).

In this paper, we study a new *P. aeruginosa* strain presenting a high hydrolytic activity toward carbenicillin. This strain has already been subjected to preliminary studies (21).

MATERIALS AND METHODS

Bacterial strain. *P. aeruginosa* strain HL was isolated by Michel Briand at the bacteriology service of Besancon Hospital, France. The carbenicillin minimal inhibitory concentration was approximately 4,000 μg/ml.

Enzymatic extract. To detect the presence of constitutive and/or inducible β-lactamases, uninduced cultures and cultures induced by up to 5 mg of penicillin G per ml added at the last 3 h of exponential phase were used, as described previously (14).

Determination of kinetic constants. Kinetic constants (K_m and V_{max}) were measured by the computerized microacidimetric method (11, 16) with a Mettler pH meter and a Wang 600 computer. Affinity constants (K_m) are given in micromolar concentrations, whereas the V_{max} values are expressed as a percentage of penicillin G maximum rate of hydrolysis.

Enzymatic stability $\tau = K_m/V_{max}$, which reflects the half-life of antibiotics at a low concentration, was also determined for each substrate and expressed as a percentage of enzymatic stability for penicillin G (10).

Enzymatic units. It is convenient to define the β-lactamase unit as the quantity of the enzyme that hydrolyzes 1 μmol of penicillin G per min at pH 7 and 37°C. This unit is almost similar, but not identical, to the one defined by Novick (23). An estimate of total protein concentration was measured either directly by ultraviolet spectrophotometry at 280 nm for concentrated solutions or by the classical Lowry method for diluted solutions. In each case bovine serum albumin was the standard. The specific activity of a bacterial extract is expressed as the number of β-lactamase units per milligram of total protein.

Preparative isoelectric focusing. Preparative isoelectric focusing in sucrose gradients was performed with an LKB 8100 system according to the instructions of the manufacturer, with ampholines of pH range 3.5 to 10 or 4 to 6.

Analytical isoelectric focusing. Analytical isoelectric focusing on polyacrylamide gel sheets was performed with a procedure similar to that of Matthew et al. (19). β-Lactamase revelation was performed with an agar overlay gel containing starch-iodine and penicillin G, as described previously (12). Thus, Fig. 3 is shown as a negative. The samples (20 μl) were applied near the cathode as drops of liquid on the surface of the gel.

Affinity chromatography. For affinity chromatography, Indubiose 4A (Industrie Biologique Française) (100 ml of hydrated form) activated by cyanogen bromide was used, and then cephalosporin C was bound to this activated resin (17). After exhaustive washing of the column with distilled water, the crude enzymatic extract (2 ml) was eluted with a sodium chloride gradient from 0.01 to 2 M.

RESULTS

Enzymatic extracts obtained from uninduced cultures showed a high β-lactamase activity, i.e., 800 to 900 mU/mg of total protein (crude extracts), when measured with penicillin G as substrate. Isoelectric focusing with ampholines of a pH range of 3.5 to 10 gave a single peak of β-lactamase, with a pI of 5.4. This pI can be measured more accurately with ampholines of a pH range of 4 to 6 and was finally found at pH 5.30 ± 0.03.

Table 1 is a summary of the kinetic constants of the enzyme, K_m, V_{max} and τ, at pH 7 and 37°C for 13 β-lactam antibiotics, including carbenicillin and two "carbenicillin-like" compounds, ticarcillin and sulbenicillin. This β-lactamase showed a very low hydrolytic activity toward all the cephalosporins tested, i.e., a very low V_{max} and a high K_m (bad affinity). We therefore looked for a possible inducible cephalosporinase.

Enzymatic extracts prepared from cultures induced with 500 μg of penicillin G per ml showed no significant difference as compared with the first extracts. It was only with penicillin G concentrations as high as 5,000 μg/ml that we induced a good cephalosporinase activity: the specific activity of that cephalosporinase being about 15% of the penicillinase activity (both measured with penicillin G as substrate). We then undertook the separation of these two enzymes by affinity chromatography and isoelectric focusing.

Affinity chromatography. Elution of extracts obtained with highly induced cultures (5 mg of penicillin G per ml) gave two peaks of enzymatic activity (Fig. 1); the enzyme eluted first showed little affinity for the column and is therefore little purified.

Elution with NaCl >0.5 M was needed to obtain the second peak of enzymatic activity, and the enzyme purification was approximately a 100-fold.

Isoelectric focusing of the fractions corresponding to the two peaks gave, respectively,

TABLE 1. *Kinetic constants of the constitutive carbenicillinase from P. aeruginosa strain HL*

Antibiotic	K_m (μM)	V_{max} (relative)[a]	τ (relative)[a]
Penicillin G	11.1	100	100
Penicillin V	9.7	98	89
Ampicillin	20.1	148	122
Amoxicillin	17.0	54	285
Epicillin	33	187	159
Carbenicillin	196	282	626
Ticarcillin	34	104	295
Sulbenicillin	23	88	235
Cephalothin	46	1.1	37,700
Cephaloridine	395	28	12,700
Cephalexin	Very low hydrolysis, if any		
Cefazolin	222	4.5	42,500

[a] Values are expressed as a percentage of the corresponding values for penicillin G.

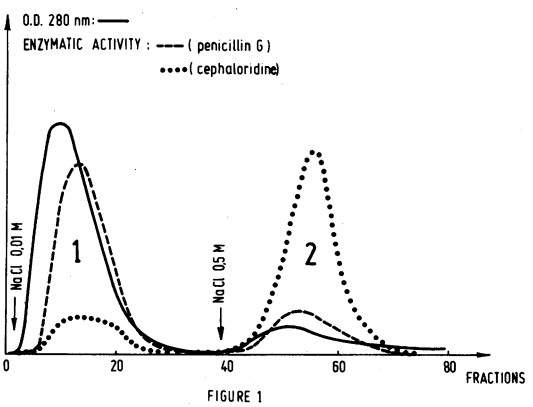

FIGURE 1

FIG. 1. *Affinity chromatography of the mixture of β-lactamases from P. aeruginosa HL (culture induced by penicillin G). Peak 1 is the carbenicillinase, obtained with a poor purification. Peak 2, mainly active toward cephaloridine, is collected with a good purification. OD, Optical density.*

one band at pH 5.30 for peak 1 and one band at pH 8.66 for peak 2. The two fractions were, thus, not contaminated by each other.

Isoelectric focusing. Preparative isoelectric focusing in sucrose gradients of the crude enzymatic extract (induced by penicillin G) was in good agreement with the preceeding results; it also gave two peaks of enzymatic activity at pH 5.30 and 8.66 (Fig. 2). Analytical isoelectric focusing on polyacrylamide gels gave the same results (Fig. 3).

The fractions obtained from affinity chromatography or preparative isoelectric focusing were suitable for kinetic studies, particularly for the inducible cephalosporinase that must be separated from the constitutive carbenicillinase.

The kinetic constants of the inducible cephalosporinase are given in Table 2; these data and also analytical isoelectric focusing show that this cephalosporinase is very similar to type C (13). This enzyme gives very low, if any, hydrolysis of the ampicillin and carbenicillin groups. The K_i values were measured for ampicillin (K_i = 0.3 μM), carbenicillin (K_i = 0.02 μM), and cloxacillin (K_i = 0.002 μM).

DISCUSSION

P. aeruginosa strain HL produces two lactamases. One of them is a constitutive β-lactamase (pI = 5.30) biosynthetized at a fairly high level, with a specific activity reaching 800 to 900 mU/mg (crude preparation). Kinetic constants (Table 1) show a very good hydrolytic activity against the eight penicillins tested, but a very poor activity against the cephalosporins. Carbenicillin is the antibiotic hydrolyzed at the higher rate, and this enzyme could be named "carbenicillinase."

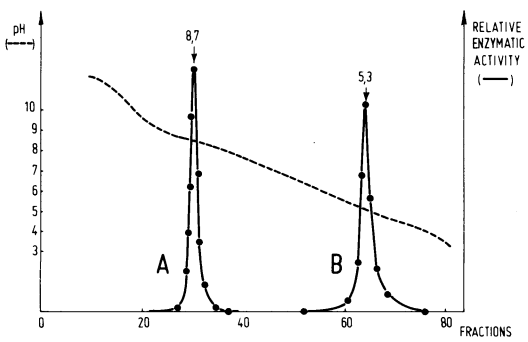

FIG. 2. *Isoelectric focusing of the β-lactamase mixture (crude extracts from culture induced by penicillin G) clearly shows two peaks, peak A (pI then precises at about 8.66) being the cephalosporinase and peak B (pI = 5.30) the carbenicillinase. Enzymatic activity was detected with cephaloridine.*

We previously studied in our laboratory *P. aeruginosa* strain MAR (17), which produced another constitutive penicillinase at a lower level (90 to 100 mU/mg), but showed a very similar isoelectric point of 5.40. This last enzyme had the same pI and kinetic constants as those of the extrachromosomic β-lactamase

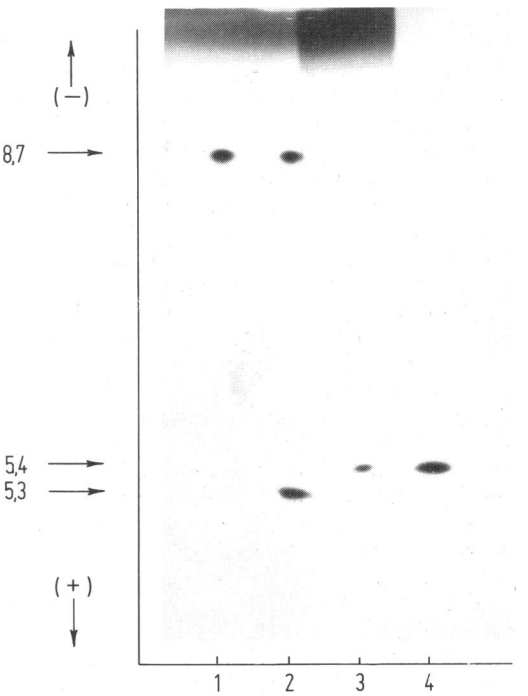

FIG. 3. *Analytical isoelectric focusing of (1) cephalosporinase type C from P. aeruginosa RL39, (2) the two β-lactamases from P. aeruginosa HL, (3) type I penicillinase from E. coli PIII, (4) type I penicillinase from P. aeruginosa MAR (uninduced culture). The figure shown is a negative.*

TABLE 2. *Kinetic constants of the inducible cephalosporinase from P. aeruginosa strain HL*[a]

Antibiotic	K_m (μM)	V_{max} (relative)	τ (relative)
Penicillin G	1.0	100	100
Penicillin V	0.9	60	150
Ampicillin	K_i = 0.3		
Amoxicillin	Low hydrolysis, if any		
Epicillin	Low hydrolysis, if any		
Carbenicillin	Low hydrolysis, if any	(K_i = 0.02 μM)	
Ticarcillin	Low hydrolysis, if any		
Sulbenicillin	Low hydrolysis, if any		
Cephalothin	40	800	500
Cephaloridine	60	540	1,100
Cephalexin	3	30	1,000
Cefazolin	400	6,500	615
Cefamandole	3	4	7,500

[a] Ampicillin and carbenicillin were tested as inhibitors, with penicillin G as substrate.

type I produced by *Escherichia coli* P111 (15, 26), which was also indistinguishable from the well-known R-TEM penicillinase. These are enzymes from class III of Richmond and Sykes (27).

It was possible to study the two β-lactamases from the two *P. aeruginosa* strains HL and MAR by the same techniques in our laboratory, and these two enzymes appear to be clearly different. First, analytical isoelectric focusing showed a clear reproducible difference between β-lactamases HL and MAR (Fig. 3). Another interesting difference was shown in the kinetic constants (K_m and V_{max}) (Tables 1 and 3, essentially with carbenicillin, ticarcillin, sulbenicillin, cephalothin, and cephaloridine. Despite these differences, we can see that the parameter $\tau = K_m/V_{max}$, corresponding to the half-life of the antibiotic at a "low" concentration, is very similar. For example, with HL carbenicillinase, carbenicillin gives $\tau = 626$, and with type I penicillinase $\tau = 450$.

The constitutive β-lactamase of the HL strain looks a little like the one of the Dalgleish strain (6), especially its "profile" defined with maximum rates. But it is very different when the K_m values are considered, which were measured with different techniques: computerized microacidimetry (11) for the HL strain and microiodometry (25) for the Dalgleish strain. The results of Furth (6) unfortunately lack precision concerning the pI of the enzyme she studied, but the value given (5.0 to 5.5) is of the same order as ours. These two enzymes could belong to class Vd of Richmond and Sykes (27).

Table 4 gives some characteristics of a few β-lactamases with high activity against penicillin G (and ampicillin) and low, if any, action against oxacillin and cloxacillin; all of them are constitutive. The TEM-1 β-lactamase (pI = 5.4) is produced by many bacteria, such as *P. aeruginosa* MAR (17) or *E. coli* Rms212 (= R-GN14) (30, 33), and is similar to type I of Pitton (15, 26). A common characteristic of the enzymes of Table 4 is the isoelectric point, which ranges from 5.0 to 5.7. Only one penicillinase, type II of Pitton (15, 26), shows a very different pI, 7.7. Most of these enzymes appear to be R-factor mediated.

Based on the relative rate of carbenicillin hydrolysis, the enzymes from Table 4 may be classified into two groups. One group gives high hydrolysis of carbenicillin (Rms139, Dalgleish, and HL), and the other gives a low hydrolysis (Rms165, RP4, TEM-1, and type II). These values for βL75 are not given (24).

P. aeruginosa HL extracts from cultures induced with 5,000 μg of penicillin G per ml show the presence of an inducible β-lactamase.

One needs to observe that the concentration of inducer needed in this case is much higher than the one used for the cephalosporinase from *P. aeruginosa* MAR (17). This might be related to the fact that the constitutive carbenicillinase of the HL strain is produced at a much higher level than the R-TEM β-lactamase and then destroys the inducer more rapidly.

The induced β-lactamase from the HL strain has a pI of 8.66. The kinetic constants show that it has a good activity toward cephalosporins and a lower one toward penicillins G and V (Table 2). It is therefore a cephalosporinase, with kinetic characteristics very similar to the ones of enzymes already described. To us, this

TABLE 3. *Kinetic constants of the extrachromosomic β-lactamase TEM-1 obtained from P. aeruginosa MAR*

Antibiotic	K_m (μM)	V_{max} (relative)	τ (relative)
Penicillin G	21	100	100
Penicillin V	11.5	59	93
Ampicillin	31.3	94	159
Amoxicillin	24.9	78	152
Epicillin	43.8	127	164
Carbenicillin	10.0	10.6	450
Ticarcillin	9.7	9.6	481
Sulbenicillin	7.4	4.5	783
Cephalothin	230	10	10,900
Cephaloridine	950	170	2,700
Cephalexin	170	0.33	2.5×10^5
Cephradine	115	0.26	2.1×10^5

TABLE 4. *Comparison of some β-lactamases from literature data and the constitutive carbenicillinase produced by P. aeruginosa HL*[a]

Enzyme, bacterial strain, or reference	pI	Penicillin G K_m (μM)	Carbenicillin	
			K_m (μM)	V_{max} (relative)
Dalgleish (6, 22)	5–5.5 (imprecise)	1	2	150
Rms139 (30)	5.7	15	40	110
HL (this paper)	5.30	11.1	196	282
Rms165 (30)	5.25	50	8	10
RP4 (= RP1) (TEM-2)	5.5 (reference 30) Corrected: 5.6 (references 19 and 20)	10	5	9
TEM-1 β-lactamase	5.4	21	10	10
βL75 (24)	5.26	42.8		
Type II (26) (15)	7.7	10.6	6	9

[a] In the case of βL75, the values for carbenicillin are not published. All of them are constitutive.

TABLE 5. *Comparison of a few cephalosporinases from literature data and the inducible cephalosporinase from P. aeruginosa HL*

Enzyme or strain (reference)	pI	Cephalothin K_m (μM)	Cephaloridine K_m (μM)	Penicillin G K_m (μM)	Cloxacillin K_i (μM)
Strain NCTC 8203 (1, 29)	7.5 (reference 18)	450	130	13	0.03
Bobrowski et al. (1)			400		0.18
Yaginuma et al. (32)	8.7	132	156	12	0.023
Type A (16)	9.2	41	51	2.4	0.015
Type B (16)	6.67	8.5	19.8	0.9	0.003
HL and type C (13)	8.66	40	60	1	0.002

cephalosporinase appears to be identical (pI, K_m, V_{max}) to our type C cephalosporinase (13).

Table 5 is a comparison of the five cephalosporinases already known with the one identified in the HL strain. The pI of the type C cephalosporinase is the same as the pI of the cephalosporinase produced by the GN918 strain (32). These two cephalosporinases might be identical; at any rate, they present strong analogies. The pI of the enzyme from strain NCTC 8203 has been measured recently and is different, i.e., 7.5 (18). It can also be observed in Table 2 that the pI values of cephalosporinases from *P. aeruginosa* are mainly basic (Table 4). All of these cephalosporinases belong to the Id group defined by Richmond and Sykes (27).

ACKNOWLEDGMENTS

We thank Y. Michel-Briand for generously providing the *P. aeruginosa* HL strain.

LITERATURE CITED

1. **Bobrowski, M., and E. Borowski.** 1971. Interaction between carbenicillin and β lactamases from Gram negative bacteria. J. Gen. Microbiol. 68:263-272.
2. **Brumfitt, W., A. Percival, and D. A. Leigh.** 1967. Clinical and laboratory studies with carbenicillin, a new penicillin active against *Ps. pyocyanea*. Lancet i:1289-1293.
3. **Bryan, L. E., H. M. Van Den Elzen, and Jui Teng-Tseng.** 1972. Transferable drug resistance in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. 1:22-29.
4. **Datta, N., R. W. Hedges, E. Shaw, R. B. Sykes, and M. H. Richmond.** 1972. Properties of an R factor from *Pseudomonas aeruginosa*. J. Bacteriol. 108:1244-1249.
5. **Fullbrook, P. D., S. W. Elson, and G. T. Slocombe.** 1970. R-factor mediated β-lactamase in *Pseudomonas aeruginosa*. Nature (London) 226:1054-1056.
6. **Furth, A. J.** 1975. Purification and properties of a constitutive β-lactamase from *Pseudomonas aeruginosa* strain Dalgleish. Biochim. Biophys. Acta 377:431-443.
7. **Ingram, L. C., M. H. Richmond, and R. B. Sykes.** 1973. Molecular characterization of the R factors implicated in the carbenicillin resistance of a sequence of *Pseudomonas aeruginosa* strains isolated from burns. Antimicrob. Agents Chemother. 3:279-288.
8. **Iyobe, S., K. Hasuda, A. Fuse, and S. Mitsuashi.** 1974. Demonstration of R factors from *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. 5:547-552.
9. **Jones, R. M., and E. J. L. Lowbury.** 1967. Prophylaxis and therapy for *Pseudomonas aeruginosa* with carbenicillin and with gentamicin. Br. Med. J. III: 79-92.
10. **Labia, R.** 1974. Comportement enzyme-substrat, introduction de la notion de stabilité enzymatique dans le case des β lactamases. C.R. Acad. Sci. Ser. D 279:109-112.
11. **Labia, R., J. Andrillon, and F. Le Goffic.** 1973. Computerized microacidimetric determination of a β lactamase Michaelis Menten constant. FEBS Lett. 33:42-44.
12. **Labia, R., M. Barthelemy, and J. M. Masson.** 1976. Multiplicité des β lactamases: un problème d'isoenzymes? C.R. Acad. Sci. Ser. D 283:1597-1600.
13. **Labia, R., C. Fabre, and M. Guionie.** 1976. Etude cinétique d'une nouvelle céphalosporinase de *Ps. aeruginosa*. Biochimie 58:913-915.
14. **Labia, R., A. Kazmierczak, A. Philippon, F. Le Goffic, J. C. Faye, F. W. Golstein, and J. F. Acar.** 1975. β lactamases de *Pseudomonas aeruginosa* et résistance à la carbenicilline. Ann. Microbiol. (Paris) 126A:449-459.
15. **Labia, R., F. Le Goffic, and J. Andrillon.** 1974. Etude cinétique de deux β lactamases responsables d'un même phenotype. Biochime 56:1025-1030.
16. **Labia, R., F. Le Goffic, J. C. Faye, and A. Philippon.** 1974. Deux cephalosporinases de *Pseudomonas aeruginosa*. Biochimie 56:1333-1337.
17. **Labia, R., A. Philippon, F. Le Goffic, and J. C. Faye.** 1975. Identification de la β lactamase R-TEM chez le *Pseudomonas aeruginosa*. Biochimie 57:139-143.
18. **McPhail, M., and A. J. Furth.** 1973. Purification and properties of an inducible β-lactamase from *Pseudomonas aeruginosa* NCTC 8203. Biochem. Soc. Trans. 1:260-263.
19. **Matthew, M., A. M. Harris, M. J. Marshall, and G. W. Ross.** 1975. The use of analytical isoelectric focusing for detection and identification of β lactamases. J. Gen. Microbiol. 88:169-178.
20. **Matthew, M., and R. W. Hedges.** 1976. Analytical isoelectric focusing of R factor-determined β-lactamases: correlation with plasmid compatibility. J. Bacteriol. 125:713-718.
21. **Michel-Briand, Y., and L. Bruant.** 1972. Etude de la production de β lactamase pour la carbenicilline chez trente souches de *Pseudomonas aeruginosa* (mise en évidence d'une souche hautement résistante). C.R. Acad. Sci. Ser. D. 275:503-506.
22. **Newson, S. W. B., R. B. Sykes, and M. H. Richmond.** 1970. Detection of a β-lactamase markedly active against carbenicillin in a strain of *Pseudomonas aeruginosa*. J. Bacteriol. 101:1079-1080.
23. **Novick, R. P.** 1962. Micro-iodometric assay for penicillinase. Biochem. J. 83:236-240.
24. **Ogawara, H., K. Maeda, and H. Umezawa.** 1972. A β-lactamase of *Escherichia coli*. Biochim. Biophys. Acta 289:203-211.

25. **Perret, J. C.** 1954. Iodometric assay of penicillinase. Nature (London) 174:1012–1013.
26. **Pitton, J. S.** 1973. β-lactamase à dépendance plasmidique chez les enterobactériacées. Pathol. Microbiol. 39:3.
27. **Richmond, M. H., and R. B. Sykes.** 1973. The β lactamases of gram-negative bacteria and their possible physiological role. Adv. Microb. Physiol. 9:31–88.
28. **Sabath, L. D., and M. Finland.** 1967. Resistance of penicillins and cephalosporins to beta lactamases from gram negative bacilli: some correlation with antibacterial activity. Ann. N.Y. Acad. Sci. 145:237–247.
29. **Sabath, L. D., M. Jago, and E. P. Abraham.** 1965. Cephalosporinase and penicillinase activities of a β lactamase from *Pseudomonas pyocyanea*. Biochem. J. 96:739–752.
30. **Sawada, Y., S. Yaginuma, M. Tai, S. Iyobe, and S. Mitsuhashi.** 1976. Plasmid-mediated penicillin beta-lactamases in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. 9:55–60.
31. **Sykes, R. B., and M. H. Richmond.** 1970. Intergeneric transfer of a β lactamase gene between *Ps. aeruginosa* and *E. coli*. Nature (London) 226:952–954.
32. **Yaginuma, S., T. Sawai, H. Ono, S. Yamagishi, and S. Mitsuhashi.** 1973. Biochemical properties of a cephalosporin β lactamase from *Pseudomonas aeruginosa*. Jpn. J. Microbiol. 17:141–149.
33. **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.