β -Lactamase and the Resistance of *Pseudomonas aeruginosa* to Various Penicillins and Cephalosporins

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SUMMARY

Eight strains of *Pseudomonas aeruginosa*, induced by penicillin G, produced different amounts of β -lactamase. Significant enzyme activity appeared within 5 min. and its highest value was obtained 3 h. after the addition of penicillin G. The production of the enzyme was arrested shortly after removal of the inducer. High concentrations of penicillin G, other penicillins or cephalosporins were necessary for optimal induction. 6-Aminopenicillanic acid (6-APA) was the best inducer tested, while cephalothin was the worst. Most of the enzyme was intracellular after conversion of the cells to spheroplasts in the presence of carbenicillin.

The structure of both the nucleus and side chain of the penicillins and cephalosporins determined the rate of their hydrolysis by the β -lactamase. Generally, the enzyme was more active against 7-aminocephalosporanic acid (7-ACA) derivatives than against 6-APA derivatives, except cephalixin and cephaloglycin, which were relatively resistant to hydrolysis.

No relation was found between the sensitivity of the pseudomonads to penicillin G and the amount of the enzyme in their cell-free preparations. The sensitivity of the different antibiotics to hydrolysis by the enzyme was not a major determinant in the resistance of *Pseudomonas aeruginosa* 1978 to them. These results indicate that the resistance of the bacteria to the β -lactamase-sensitive penicillins and to cephalosporins is dependent on a combined effect of β -lactamase and on an intrinsic resistance, while the resistance of the bacteria to the β -lactamase-resistant penicillins depends on the intrinsic resistance alone.

INTRODUCTION

Infections by *Pseudomonas aeruginosa* are extremely refractory to chemotherapy due to the high resistance of these bacteria to most of the widely used antibiotics. The biochemical basis underlying this resistance may be either a resistance of each individual bacterium (the intrinsic resistance) or the capacity of the culture as a whole to produce an enzyme which inactivates the drug. For a long time, the resistance of *Pseudomonas* species to penicillin has been attributed to tolerance, with poor capacity to destroy the drug (Sutherland, 1964). β -Lactamase (penicillin amidohydrolase, EC 3.5.2.6) activity in some strains of *Pseudomonas aeruginosa* was reported by Percival, Brumfitt & de Louvois (1963) and in other species of *Pseudomonas* by Desai & Goldner (1968). The β -lactamases of *P. aeruginosa* were found to be either constitutive (Newsom, Sykes & Richmond, 1970), or inducible enzymes formed only in the presence of high concentrations of penicillin G (Jago, Migliacci & Abraham, 1963; Sabath, Jago & Abraham, 1965; Sabath & Finland, 1967).

In the present investigation eight strains of *Pseudomonas aeruginosa* were examined for β -lactamase activity. One of them, which produced the highest amount of the enzyme, was used in detailed studies on production of the enzyme, its specificity and its contribution to the resistance of the bacterium towards various penicillins and cephalosporins. A preliminary report of this research has appeared (Garber, Friedman & Rozansky, 1969).

METHODS

Organisms and their drug sensitivity. The eight strains of *Pseudomonas aeruginosa* (Table 1) used in this study were a gift of the late Professor R. Rozansky (Institute of Microbiology, Tel-Aviv-Jaffa Municipality Hospitals). Their sensitivity to antibiotics was determined on Mueller-Hinton agar plates according to the zones of inhibition around drug-containing discs (A/S Rosco, Farmaceutisk Industri, Taastrup, Denmark).

Table 1. *The strains of Pseudomonas aeruginosa and their sensitivity to antibiotics*

Sensitivity to antibiotics was determined on Mueller-Hinton agar plates according to the zones of inhibition around drug containing discs (A/S Rosco).

Strain no.	Origin	Sensitivity to					
		Ampicillin	Carbenicillin	Cephaloridine	Polymyxin	Streptomycin	Chloramphenicol
1316	Unknown	R	MS	R	R	MS	RR
1705	Unknown	R	RR	R	R	R	R
1978	Knee fluid	R	S	R	R	RR	S
1989	Eye	R	—	—	S	S	S
2064	Ear	R	S	R	S	R	S
2094	Sputum	S	S	—	S	S	S
2320	Trachea	S	S	R	R	R	S
2331	Ear	R	S	—	S	—	R

S, sensitive; MS, moderately sensitive; RR, relatively resistant; R, resistant.

Media. Nutrient broth (Difco) supplemented with 20 % sucrose and 0.2 % MgSO_4 (NBS medium) adjusted to pH 7. Stock cultures were maintained on brain heart agar slopes (Difco) and stored at 4°. Lederberg & Clair (1958) medium (LC medium) composed of: 1 % casein digest, 10 % sucrose, 1 % meat extract, 0.35 % NaCl, 0.1 % glucose and 0.2 % MgSO_4 , pH 7, was used for the conversion of *Pseudomonas aeruginosa* to spheroplasts.

Penicillins and cephalosporins. The penicillins and cephalosporins used are listed in Table 2.

Determination of minimal inhibitory concentration. The minimal inhibitory concentration (MIC) was determined by the tube dilution method (2 ml. NBS medium/tube). Each tube was inoculated with 10^6 bacteria/ml. (high inoculum) or 10^2 bacteria/ml. (low inoculum). The MIC was determined by measuring the turbidity after incubation for 18 h. at 37°.

β -Lactamase assay. The manometric method (Henry & Housewright, 1947; Pollock, 1950) was usually used for measuring β -lactamase activity. Twenty μ moles substrate/3 ml. experimental system were used to saturate the enzyme. The iodometric method (Perret, 1954) was used for comparing enzymic activities of the various strains

of *P. aeruginosa*. The micro-iodometric method (Novick, 1962*b*) was used for the determination of 6-APA hydrolysis. One unit of β -lactamase activity was defined as the amount of enzyme which hydrolysed 1 μ mole benzylpenicillin in 60 min. at 30° and pH 7 (Pollock & Torriani, 1953). Protein determinations were done by the method of Lowry, Rosebrough, Farr & Randall (1951).

Table 2. *Antibiotics used*

Penicillins			Cephalosporins		
Name	Purity (%)	Origin	Name	Purity (%)	Origin
6-Amino penicillanic acid (6-APA)	100	B.R.L.	Cephalothin	90	Lilly
Penicillin G (Na salt)	100	Rafa	Cephaloridine	90	Glaxo
Carbenicillin (di Na salt)	77	B.R.L.	Cephalexin	96	Lilly
Penicillin V (K salt)	100	Glaxo	Cephaloglycin	96	Lilly
Phenethicillin (K salt)	99	B.R.L.	—	—	—
Propicillin (K salt)	98	B.R.L.	—	—	—
Methicillin (Na salt)	100	B.R.L.	—	—	—
Cloxacillin	90	B.R.L.	—	—	—
Dicloxacillin (Na salt)	90	B.R.L.	—	—	—

Beecham Research Laboratories (B.R.L.), Clarendon Road, Worthing, Sussex, England; Rafa Laboratories, Jerusalem, Israel; Glaxo Laboratories Ltd, Greenford, Middlesex, England; Eli Lilly and Co., Indianapolis, Indiana 46202, U.S.A.

Determination of the inductive ability of various penicillins and cephalosporin.

Bacteria were grown in 100 ml. NBS medium with shaking in a water bath at 37° for 12 to 17 h. and then transferred to fresh broth with initial bacterial concentration of 7.2×10^7 bacteria/ml. The inducer was added after shaking for 2 to 3 h. (logarithmic phase) and 8-hydroxyquinoline sulphate (8-HQ, Fluka) at final concentration of 1 mM was added 1 to 3 h. later to stop the induction. The cultures were harvested (10 min. at 16,300*g*), the pellets washed twice and suspended in 0.001 M-phosphate buffer pH 7 (5 to 10 g. wet wt bacteria/100 ml.). Part of this suspension (8 ml.) was subjected to three 20 sec. pulses of ultrasonic treatment (Branson, ultrasonic disintegrator Model No. S-125, 125 W) at 0°. The disrupted suspension was centrifuged for 10 min. at 20,850*g* and the supernatant fluid used as the source of intracellular enzyme.

Determination of the kinetics of enzyme production and its dependence on the presence of penicillin. Four cultures were induced with penicillin G (5 mg./ml.). The induction was stopped by 8-HQ (1 mM) after 0, 15, 30 and 60 min. Another four cultures, similarly induced, were centrifuged for 10 min. at 16,300*g* at +2° after growth for 15 min. Their supernatant fluids were removed and the sediments washed twice and resuspended in NBS medium. Two of them were reinduced with penicillin G (5 mg./ml.) and one was shaken for 15 min. and the other for 45 min. at 37°. No more penicillin

was added to the other two cultures which were also shaken for 15 and 45 min., respectively. In each case induction was terminated by adding 8-HQ and the intracellular β -lactamase activity of the eight samples measured by the manometric method.

Conversion of Pseudomonas aeruginosa 1978 bacteria to spheroplasts. Carbenicillin was used for the induction of β -lactamase and for the production of spheroplasts in cultures of *Pseudomonas aeruginosa* 1978 according to Watanakunakorn & Hamburger (1969). Bacteria were grown in LC medium at 37° with shaking. After 2 h. carbenicillin (1 mg./ml.) was added and the incubation continued for 8 h. Complete conversion to spheroplasts was confirmed by a phase-contrast microscope. Enzyme production was stopped by adding 8-HQ (1 mM). The spheroplasts were collected by centrifugation but were not washed in order to avoid their lysis. They were suspended in phosphate buffer 0.001 M pH 7 and subjected to three 20 sec. pulses with the Branson sonifier at 0°. β -Lactamase activity was determined in the disintegrated spheroplast suspension as well as in the supernatant medium, by the manometric method.

RESULTS

β -Lactamase production and its contribution to the resistance of the bacteria to penicillin G

The eight strains of *Pseudomonas aeruginosa* tested, induced by penicillin G for 3 h., produced different amounts of β -lactamase (Table 3). Generally, the enzyme was more active on cephaloridine than on penicillin G. The ratio of activity on cephaloridine to that on penicillin G was 3.56 by the manometric method and 3.33 by Perret's method.

No correlation was found between the value of β -lactamase production by these bacteria and the degree of their resistance to penicillin G. The MIC of *Pseudomonas aeruginosa* 1978, which produced the highest amount of β -lactamase (15.35 units/mg. protein by Perret's method) was 2.5 mg. penicillin G/ml., while that of strain 1316 whose β -lactamase activity was lower (6.3 units/mg. protein) was 10 mg. penicillin G/ml.

Table 3. *Substrate specificity of the β -lactamase produced by strains of Pseudomonas aeruginosa*

Young log-phase culture of each *Pseudomonas* strain in NBS medium was induced for 3 h. in the presence of 5 mg. penicillin G/ml.; the bacteria were then harvested, washed twice and suspended in 0.001 M-phosphate buffer pH 7. The bacteria were disrupted by ultrasonic treatment and β -lactamase activity of the extracts determined.

Strain no.	Activity against penicillin G (units/mg. protein)		Activity against cephaloridine (units/mg. protein)		Ratio of activities on cephaloridine: penicillin G	
	Manometric method	Perret method	Manometric method	Perret method	Manometric method	Perret method
1316	2.83	6.30	16.95	32.48	6.00	5.12
1705	7.80	11.56	21.60	41.80	2.77	3.62
1978	10.00	15.35	39.60	73.00	3.96	4.76
1989	0	1.84	3.90	6.16	—	3.35
2064	4.97	5.05	15.90	16.00	3.20	3.15
2094	0	3.00	0	4.00	—	1.34
2320	0.84	1.29	0.99	1.29	1.18	1.00
2331	2.46	5.81	11.05	25.40	4.50	4.36
				Average	3.56	3.33

The kinetics of β -lactamase production

No β -lactamase activity was detected in the absence of penicillin G. Optimal induction was obtained in the presence of 4 to 5 mg. penicillin G/ml. (Fig. 1). The rate of production of the enzyme induced by penicillin G (5 mg./ml.) and its activities towards penicillin G and cephaloridine are presented in Fig. 2. Detectable amounts of the enzyme appeared within 5 min. of addition of the inducer. Optimal β -lactamase activities on penicillin G and on cephaloridine were obtained after induction for 3 h. A ratio of about 3.5 between the activity on cephaloridine and that on penicillin G was maintained throughout the induction period.

Fig. 1

Fig. 2

Fig. 3

Fig. 1. Effect of concentration of penicillin G on the formation of β -lactamase by *Pseudomonas aeruginosa* 1978 grown in NBS medium to the logarithmic phase and then induced with penicillin G. After 1 h., 8-hydroxyquinoline (1 mM) was added and the β -lactamase activity of the disrupted bacteria measured by the manometric method.

Fig. 2. The kinetics of β -lactamase formation by *Pseudomonas aeruginosa* 1978 induced by penicillin G. β -lactamase activity determined with cephaloridine (●) and penicillin G (○) as substrates.

Fig. 3. The effect of removal of inducer on β -lactamase formation by *Pseudomonas aeruginosa* 1978. Young log-phase cultures of *P. aeruginosa* 1978 were divided into three groups. The cultures of the first group were grown for different periods in penicillin G (5 mg./ml.) containing NBS medium (●). The cultures of the second and third groups were grown for 15 min. in the same medium, harvested, washed and resuspended in a new medium containing the inducer (○), while the cultures of the third group were resuspended in a penicillin G-free NBS medium (▲). β -Lactamase activity was determined in the cell-free extracts by the manometric method with penicillin G as substrate. In each case, the induction was terminated by adding 8-hydroxyquinoline.

The effect of removal of the inducer on β -lactamase production

The presence of penicillin G (5 mg./ml.) was a prerequisite for β -lactamase production. The formation of the enzyme was stopped shortly after removal of the inducer (Fig. 3).

Fig. 4. Induced β -lactamase formation by *Pseudomonas aeruginosa* 1978 in the presence of various penicillins and cephalosporins. Young log-phase cultures of *P. aeruginosa* 1978 grown in NBS medium were induced for 1 h. with several concentrations of various penicillins or cephalosporins. After adding 8-hydroxyquinoline, the bacteria were washed, resuspended in 0.001 M-phosphate buffer pH 7, and disrupted. The cell-free extract was assayed for β -lactamase activity on penicillin G by the manometric method.

Table 4. *The effect of EDTA on the induced formation of β -lactamase by Pseudomonas aeruginosa 1978*

Young log-phase cultures of *Pseudomonas aeruginosa* 1978 in NBS medium were induced for 1 h. with penicillin G in presence of various concentrations of EDTA. The bacteria were harvested, suspended, washed twice and resuspended in 0.001 M-phosphate buffer pH 7. The bacteria were disrupted by ultrasonic treatment and β -lactamase activity determined in the cell-free extracts.

EDTA* ($\times 10^{-3}$ M)	Penicillin G (mg./ml.)	β -Lactamase activity (units/mg. protein)
1.75	0.5	4.40
0.87	0.5	5.96
0.43	0.5	6.17
0.21	0.5	6.25
—	0.5	8.40
—	5.0	19.80

* The MIC of EDTA for *Pseudomonas aeruginosa* 1978 was 7×10^{-3} M.

The effect of EDTA on β -lactamase induction in the presence of penicillin G

The intensity of the induction of β -lactamase by penicillin G (0.5 mg./ml.) was not increased by the addition of subinhibitory concentrations of EDTA (Table 4).

Penicillins and cephalosporins as inducers for β -lactamase formation

About 5 mg./ml. was the optimal concentration for most of the penicillins and cephalosporins tested for the induction of β -lactamase, but the optimal concentration of 6-APA and cephaloridine was 1 mg./ml. 6-Aminopenicillanic acid was the best inducer; cephalothin was the worst (Fig. 4).

Fig. 5. The rate of hydrolysis of various penicillins and cephalosporins by the β -lactamase of *Pseudomonas aeruginosa* 1978 and their antibacterial activity on this bacterium. The MIC determined at low inoculum is represented by the hatched areas. The MIC determined at high inoculum is represented by the hatched plus unshaded areas. Black areas represent the hydrolysis (%) of the antibiotics by the β -lactamase of the bacterium determined by the manometric method (compared to the hydrolysis of penicillin G). The MIC values for carbenicillin and 6-APA were too low to be accurately represented in the above figure. Values for carbenicillin were 12.5 μ g./ml. at high inoculum and 4 μ g./ml. at low. Values for 6-APA were 250 and 80 μ g./ml., respectively.

*Relative rates of hydrolysis of various penicillins and cephalosporins by β -lactamase of *Pseudomonas aeruginosa* 1978: relation to antibacterial effect*

The rate of hydrolysis of the penicillins and cephalosporins was measured by the manometric method and calculated relative to that for penicillin G (taken as 100 %).

The rate of hydrolysis of the antibiotics by the β -lactamase and their antibacterial activity at high and low inoculum are shown in Fig. 5. No relationship was found between the MIC (at high inoculum) and the rate at which the antibiotics were hydrolysed by the enzyme. The ratio of the MIC with high inoculum (10^8 bacteria/ml.) to MIC with a low one (10^2 bacteria/ml.) was used as a criterion for estimating the role of β -lactamase in the resistance of the bacteria to the penicillins and cephalosporins tested. A small inoculum effect (two- to tenfold) was observed with all the antibiotics tested, including those which are resistant to the β -lactamase (Fig. 5).

Localization of the β -lactamase of Pseudomonas aeruginosa 1978

On conversion of the cells of *Pseudomonas aeruginosa* 1978 to spheroplasts in the presence of carbenicillin (1 mg./ml.), 71 % of the induced β -lactamase activity was associated with the spheroplasts while 29 % was found in the medium.

DISCUSSION

Five out of eight strains of *Pseudomonas aeruginosa* isolated from clinical sources produced an inducible β -lactamase of the type described by Jago *et al.* (1963) and Sabath *et al.* (1965), with a cephalosporinase specificity. The strains were all carbenicillin-sensitive in contrast to the carbenicillin-resistant strain recently described by Newsom *et al.* (1970) which produces a constitutive β -lactamase of a carbenicillinase type. No correlation was found between the sensitivity of the strains to penicillin G and the amount of the enzyme in cell-free preparations. Strain 1978, which was chosen for the detailed investigation due to its high production of the enzyme, was no more resistant to penicillin G than strains with a lower enzyme amounts.

In requiring a high concentration of penicillin G for induction of β -lactamase, the pseudomonads are similar to some *Proteus* strains (Hamilton-Miller, 1963*a*; Ayliffe, 1964) but differ from *Bacillus cereus* 569 (Pollock, 1950) and *Staphylococcus aureus* (Novick, 1962*a*) which require a very low concentration of inducer for optimal induction. The reason for this concentration difference is not understood, but it may be a general trait of *Pseudomonas*, since the amidase of *Pseudomonas* is also produced only in the presence of a high concentration of inducer (Kelly & Clarke, 1962). A slow rate of penetration of the penicillin through the cell wall and membrane is a possible explanation, and is consistent with the fact that 6-APA, which penetrates easily into Gram-negative bacteria (Hamilton-Miller, 1963*b*), was also found to be the best inducer of *Pseudomonas aeruginosa* β -lactamase. On the other hand, attempts to increase the permeability of the cell envelope to the inducer by using colistin (Sabath *et al.* 1965) and EDTA treatment in the present study were ineffective, indicating that permeability was not the sole factor. The appearance of the enzyme within 5 min. of contact with inducer, and the strict dependence of enzyme production on the presence of inducer, differ from those reported for *Bacillus cereus* 569 where the lag interval for the appearance of the enzyme is somewhat longer (Pollock, 1952) and the production of the enzyme continues for some generations after removal of the inducer (Pollock, 1950). In both of these traits the β -lactamase system of the pseudomonads is similar to the β -galactosidase system of *Escherichia coli* (Pardee & Prestidge, 1961). The strict dependence of enzyme production on the presence of inducer may indicate the existence of the very labile m-RNA which is produced or stabilized only in the presence of the inducer.

A correlation was found between stability of the antibiotics to the action of β -lactamase and their inducing power. 6-Aminopenicillanic acid, the best inducer, and other good inducers like cloxacillin, dicloxacillin and carbenicillin, were resistant to the action of the enzyme, in contrast to the cephalosporins which were weak inducers and easily hydrolysed by the enzyme. The fact that the rate of hydrolysis of the different penicillins and cephalosporins was not correlated with their inhibitory effect on the *Pseudomonas aeruginosa* 1978 and that a low inoculum effect was observed, indicates that the β -lactamase is not the sole factor contributing to the resistance of *Pseudomonas* species to penicillins. It seems therefore that the contribution of the β -lactamase to the resistance of pseudomonads to penicillins is a minor factor while the intrinsic resistance is the major one.

This research is dedicated to the memory of the late Professor R. Rozansky, who encouraged us in our work. Grateful thanks are due to Beecham Research Laboratories, to Eli Lilly and Co. and to Glaxo Laboratories for their generous supplies of antibiotics. We also wish to thank Chemicals and Technical Supplies Ltd, Israel, for their generous help in acquiring various antibiotics.

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