NOTES

ISOLATION AND PROPERTIES OF β-LACTAMASE-LESS MUTANTS FROM CLINICALLY IMPORTANT GRAM-NEGATIVE BACTERIA

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Gram-negative bacteria are becoming more important as the opportunistic pathogens in the hospital, and it is significant to investigate the mechanism of drug resistance in these organisms. It has been suggested by some authors that intracellular β-lactamase plays a vital role in the resistance to β-lactam antibiotics in Gram-negative bacteria.[1,2,3]

We attempted to isolate β-lactamase-less mutants from some clinically important Gram-negative bacteria by an improved method in order to demonstrate the role of β-lactamase more clearly. By comparing the MIC value of a β-lactam antibiotic against the β-lactamase-less mutant with that of the parent, we would be able to estimate readily the β-lactamase susceptibility and the potential antibacterial activity of the β-lactam antibiotic.

The present paper describes the efficient procedure for the isolation of β-lactamase-less mutants and their properties.

As shown in Table 1, β-lactamase production was found to be inducible with four strains of Gram-negative bacteria but not with *Klebsiella pneumoniae* H-2. In order to isolate β-lactamase-less mutants (L-mutant) from the strains possesing inducible β-lactamase, the parents were at first converted to β-lactamase-constitutive mutants (C-mutant), which were subsequently mutated to β-lactamase-less strains. N-Methyl-N'-nitro-N-nitrosoguanidine (NTG) was employed as the mutagen. This procedure would give the β-lactamase-less mutant in a real sense instead of a mutant impaired in the inducibility of β-lactamase as reported by ROSSELET and ZIMMERMAN.[4]

Bacterial cells in the logarithmic phase were treated with 300 μg/ml NTG as described by ADELBERG *et al.*[5] After appropriate dilution, 0.05 ml of the cell suspension was spread on a Difco nutrient agar plate (DN-plate) and incubated overnight at 37°C.

The detection of β-lactamase activity of the resulting colonies was based on the fact that the hydrolytic product of penicillin G decolorizes the purple color of agar-iodine complex. The DN-plate with 100~150 colonies was covered with 7 ml of agar which was prepared just before use by mixing 5 ml of 1.2% agar, 1.5 ml of 16 mM iodine containing 60 mM KI and 0.5 ml of 300 mM penicillin G. After standing for about 5 minutes at room temperature, the decolorized halo formation was observed around the colonies if they possessed β-lactamase activity. On the other hand, β-lactamase-less colonies did not show haloes around them.

C-Mutants were obtained by isolating the colonies which showed halo formation in the absence of the inducer. Only the colonies which still formed haloes when grown at 25°C were selected to eliminate temperature-sensitive type of C-mutants.

In the case of L-mutants, colonies of C-mutants treated with NTG were first replicated onto other DN-plates, and β-lactamase activity was detected as described above using the original plate.

The colonies without halo formation were picked up from the replicated plates and subsequently purified. The replica was necessary because the complete L-mutants were easily killed at the concentration of penicillin G employed in the assay conditions. Finally, the β-lactamase activities of the mutants were measured with the cell-free extracts to ensure the loss of the enzyme activities according to modified SARGENT's method as described by SAWAI *et al.*[6]

MIC values for each strain were determined by serial agar twofold dilution tests in heart infusion agar medium (Nissan). The cell suspensions used for inocula contained 10^6 (bacterial) cells/ml of Trypto-soy broth (Eiken). A loopful of the cell suspensions (0.005 ml) was transferred onto 10 ml agar plates containing the drugs tested.

By employing the procedure stated above, we have isolated β-lactamase-less mutants efficiently from the five strains of gram-negative bacteria

Table 1. β-Lactamase activities and sensitivity to various β-lactam antibiotics of some Gram-negative bacteria and their mutants.

Microorganism	β-Lactamase activity[a]		MIC (μg/ml)						
	− induction	+ induction[b]	PCG*	ABPC	CBPC	CER	CET	CEZ	KM
Pseudomonas aeruginosa									
IFO 3080	<0.03	4.8	1,600	400	25	>1,600	>1,600	>1,600	100
IFO 3080-C	6.4	6.6	>1,600	1,600	25	>1,600	>1,600	>1,600	50
IFO 3080-L	<0.03	<0.03	25	6.25	6.25	3.13	200	800	100
Pseudomonas aeruginosa									
Tid-53	<0.03	2.6	>1,600	>1,600	200	>1,600	>1,600	>1,600	>200
Tid-53-C	3.6	4.9	>1,600	>1,600	200	>1,600	>1,600	>1,600	>200
Tid-53-L	<0.03	<0.03	200	50	200	50	1,600	800	>200
Aerobacter cloacae									
IFO 12937	<0.03	6.2	1,600	200	3.13	400	400	800	3.13
IFO 12937-C	44.3	50.2	>1,600	>1,600	800	>1,600	>1,600	>1,600	6.25
IFO 12937-L	<0.03	<0.03	50	3.13	6.25	3.13	50	12.5	6.25
Serratia									
G-18	0.20	4.9	>1,600	200	12.5	>1,600	>1,600	>1,600	6.25
G-18-L	<0.03	<0.03	400	6.25	3.13	50	800	400	3.13
Klebsiella pneumoniae									
H-2	1.5[c]	1.6[c]	>1,600	>1,600	>1,600	100	50	25	3.13
H-2-L	<0.03[c]	<0.03[c]	50	12.5	200	1.56	3.13	1.56	1.56

* PCG: Penicillin G. ABPC: Ampicillin. CBPC: Carbenicillin. CER: Cephaloridine.
CET: Cephalothin. CEZ: Cefazolin. KM: Kanamycin.

a) β-Lactamase activity was expressed as μmoles of PCG or CET hydrolyzed/min per mg of protein.

b) The induction was carried out with 2 mg/ml of PCG at 37°C for 3 hours.

c) PCG was used as the substrate for the assay of β-lactamase activity. In other cases, CET was employed.

listed in Table 1.

In general, it was observed that the lack of β-lactamase resulted in a dramatic decrease in the resistance to most of the β-lactam antibiotics tested. The MIC value for a non-β-lactam antibiotic namely kanamycin, however, did not change significantly.

K. pneumoniae H–2 which produces β-lactamase (penicillinase) constitutively was highly resistant to penicillins but not to cephalosporins. The L-mutant from this strain became sensitive to penicillins, thus indicating that the constitutive penicillinase is mainly responsible for the resistance.

Similarly the inducible β-lactamases (cephalosporinases) are considered to play a significant role in the resistance to both penicillins and cephalosporins in *Pseudomonas aeruginosa, A. cloacae* and *Serratia*. Even *P. aeruginosa* which is known to be highly resistant to most of the β-lactam antibiotics became strikingly sensitive to certain penicillins and cephalosporins when devoid of the inducible β-lactamase. However, CET and CEZ were still inactive against the L-mutants from both strains of *P. aeruginosa* and from *Serratia*. These antibiotics were not inactivated by the intact cells of *P. aeruginosa* IFO 3080-L. (Data not shown) It remains to be elucidated whether CET and CEZ penetrate the cell less readily or whether they are essentially inactive against the target enzymes of this organism.

P. aeruginosa Tid 53–L was generally more resistant than *P. aeruginosa* IFO 3080-L. In particular, the high resistance to CBPC was not affected by the loss of β-lactamase activity in the former strain and thus the resistance is assumed to be independent on the β-lactamase. We have obtained a preliminary evidence that the strain carries an R-factor which harbors the resistance to gentamicin, kanamycin, tetracycline, sulfonamides, chloramphenicol and mercury. The experiments are in progress to investigate the possibility that the R-factor could be involved in the β-lactam antibiotic resistance through the mechanism other than β-lactamase as described by CURTIS *et al.*[7]

A. cloacae IFO 12937–C produced about 8-fold more β-lactamase constitutively than the parent in which the enzyme was fully induced by PCG. This mutant showed high resistance to CBPC although the enzyme from this strain is incapable of inactivating the antibiotic significantly. The resistance to CBPC, however, appears to be absolutely dependent on the high level of β-lactamase because the L-mutant showed complete loss of resistance.

As stated above, β-lactamases, whether they are constitutive or inducible, are considered to play a major role in the β-lactam antibiotic resistance in Gram-negative bacteria including *P. aeruginosa*.

In addition, the evidence for β-lactamase-independent resistance for example with *P. aeruginosa* Tid 53 to CBPC, CET and CEZ should be noted and further investigations are in progress.

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References

1) RICHMOND, M. H. & R. B. SYKES: The β-lactamase of gram-negative bacteria and their possible physiological role. A. H. ROSE & D. W. TEMPEST (ed.): Advances in Microbial Physiology, Vol. 9, pp. 31~88, Academic Press, London & New York, 1973
2) SYKES, R. B. & A. MORRIS: Resistance of *Pseudomonas aeruginosa* to antimicrobial drugs. G. P. ELLIS & G. B. WEST (ed.): Progress in Medicinal Chemistry, Vol. 12. pp. 333~393, North-Holland, Amsterdum, 1975
3) SAWAI, T.; S. NAKAJIMA, S. YAMAGISHI & S. MITSUHASHI: Temperature-sensitive induction for cephalosporinase synthesis in *Escherichia freundii*. S. MITSUHASHI, L. ROSIVAL & V. KRCMERY (ed.): Drug-inactivating enzymes and antibiotic resistance, pp. 59~66, Springer-Verlag, Berlin Heiderberg New York, 1975
4) ROSSELET, A. & W. ZIMMERMAN: Mutants of *Pseudomonas aeruginosa* with impaired β-lactamase inducibility and increased sensitivity to β-lactam antibiotics. J. Gen. Microbiol. 76: 455~457, 1973
5) ADELBERG, E. A.; M. MANDEL & G. C. C. CHEN: Optimal conditions for mutagenesis by N-methyl-N′-nitro-N-nitrosoguanidine in *Escherichia coli* K–12. Biochem. Biophys. Res. Comm. 18: 788~795, 1965
6) SAWAI, T.; I. TAKAHASHI & S. YAMAGISHI: Assay method for β-lactamase; application of SARGENT's method to various β-lactam antibiotics (in Japanese). Jap. J. Bacteriol. 31: 161, 1976
7) CURTIS, N. A. C.; M. H. RICHMOND & V. STANISICH: R-Factor mediated resistance to penicillins which does not involve a β-lactamase. J. Gen. Microbiol. 79: 163~166, 1973