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β-lactamase in Gram-negative Rods: the relationship between penicillinase and R plasmids in Gram-negative rods

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ABSTRACT

Concomitant with the extensive use of antibiotics, the number of multiple antibiotic-resistant strains has been increasing. Since resistance is mainly mediated by R plasmids, we undertook to investigate the characteristics of R plasmid-determined β-lactamase in 6 Gram-negative rods.

The β-lactamase produced by each organism was classified by its substrate: type P which attacks penicillins, type C which attacks cephalosporins, and type C/P which attacks both penicillins and cephalosporins. Though the chromosomally mediated β-lactamase of almost all Gram-negative rods is classified as type C, R plasmid-mediated β-lactamase is almost equally active against both penicillins and cephalosporins. Therefore, we suggest that type C/P β-lactamase was mediated by R plasmids in Gram-negative rods which already produced chromosomally mediated type C β-lactamase.

The strains which produced type C/P β-lactamse tended to be more resistant to antibiotics than the other β-lactamase producing strains. Among type C/P strains, the sensitivity to cephalosporins varied with the bacterial species, whereas all these strains were highly resistant to penicillins. Even for piperacillin, which is stable to cephalosporinase, the MIC at which the cumulative percentage of strains inhibited was 50% (MIC50) was over 50 μg/ml in all strains tested.

Key words: *β-lactamase, R plasmid, Antibiotic resistance, Chemotherapeutic agents*

In recent years, Surgical departments have seen an increase in the frequency of isolation of bacteria with multiple drug resistance. With methicillin-resistant *Staphylococcus aureus* (MRSA) the suggested mechanism of resistance is an alteration of penicillin binding protein 2, a key enzyme in bacterial cell wall synthesis, the target of β-lactams[3]. By contrast, the main mechanism of antibiotic resistance in Gram-negative rods is the production of β-lactamase, which hydrolyses the β-lactam ring[15]. The β-lactamase of Gram-positive cocci is penicillinases only, but Gram-negative rods produce both cephalosporinases and penicillinases[5,8,11]. Therefore, classification of the β-lactamases of Gram-negative bacteria is a more complicated task than that of Gram-positive cocci. This study was undertaken to investigate the characteristics of the R plasmid-determined β-lactamase in Gram-negative rod.

METERIALS AND METHODS

1) Bacterial strains

We investigated the following bacterial strains isolated from clinical specimens obtained from the admitted patients at our department from 1983 to 1989: 267 strains of *Escherichia coli* (*E. coli*), 109 strains of *Klebsiella pneumoniae* (*K. pneumoniae*), 85 strains of *Enterobacter cloacae* (*E. cloacae*), 67 strains of *Citrobacter freundii* (*C. freundii*), 36 strains of *Serratia marcescens* (*S. marcescens*) and 201 strains of *Pseudomonas aeruginosa* (*P. aeruginosa*).

2) Determination of the minimum inhibitory concentration (MIC).

Antibiotic sensitivity was evaluated by MIC employing the standard method of the Japan Society of Chemotherapy[2]. We measured MICs for cefazolin (CEZ, Fujisawa Pharmaceutical Co. Ltd., Osaka, Japan), latamoxef (LMOX, Shionogi Pharmaceutical Co, Ltd., Osaka, Japan), cefoperazone (CPZ, Pfizer Inc., Tokyo, Japan), and piperacillin (PIPC, Toyama Pharmaceutical Co. Ltd., Tokyo, Japan).

3) β-lactamase detection

The sensitivity of the acidimetry disk method is lower than that of the nitrocefin method. Therefore, we evaluated the isolates for high levels of β-

lactamase production using the acidimetry disk method[10,12]. One drop of 0.01 mol phosphate buffer (pH 7.0) was added to the β-lactamase detection disk (Nippon Seibutsu Zairyou Center), which contained penicillin-G (PCG, Banyu Pharmaceutical Co. Ltd., Tokyo, Japan) and CEZ as the substrates. The extent of β-lactamase production was then determined by the alteration in disk color.

The following antibiotics were added to bacterial culture media as inducers to boost production of β-lactamase[15]: 10 μg/ml of cefmetazole (CMZ, Sankyo Pharmaceutical Co. Ltd., Tokyo, Japan) for *P. aeruginosa*, 10 μg/ml of CEZ for *E. cloacae, C. freundii* and *S. marsescens,* and 10 u/ml of PCG for *E. coli* and *K. pneumoniae.* Clinical isolates were cultured with an inducer at 37°C for 24 hours before β-lactamase detection was performed.

The β-lactamase was classified by their hydrolysed substrates, as type P attacking penicillins, type C attacking cephalosporins, and type C/P attacking both penicillins and cephalosporins.

RESULTS

1) Percentage of β-lactamase producing strains of each organism (Fig. 1)

The percentages of β-lactamase producing strains of *E. cloacae* (100%) and *C. freundii* (96.7%) were higher than those of the other species. The most

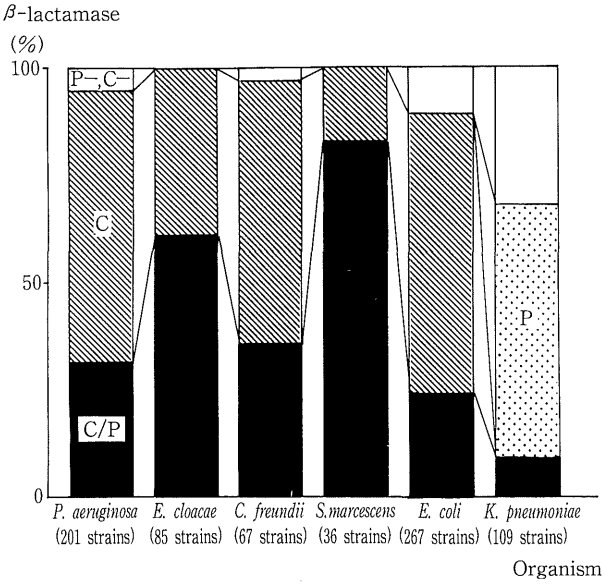
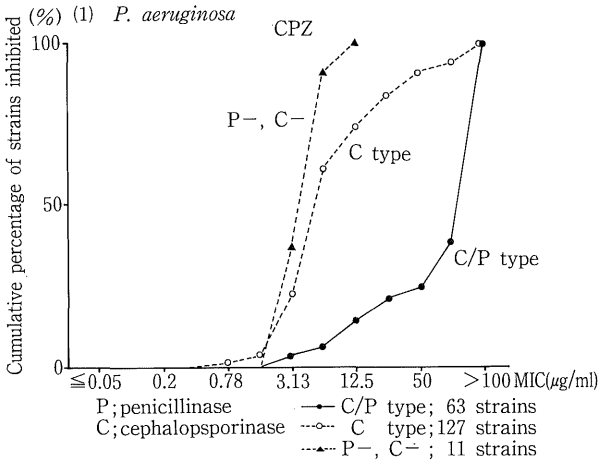
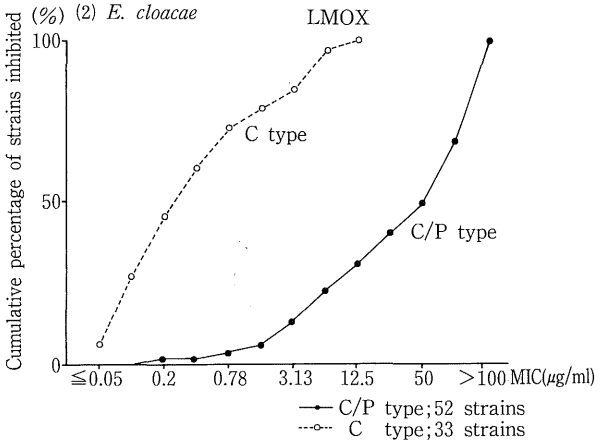
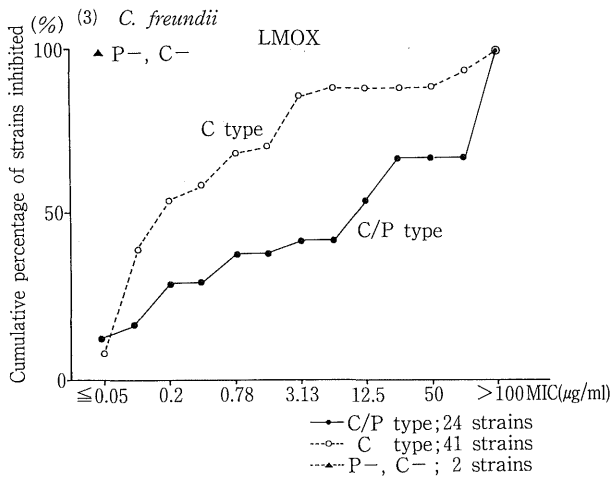
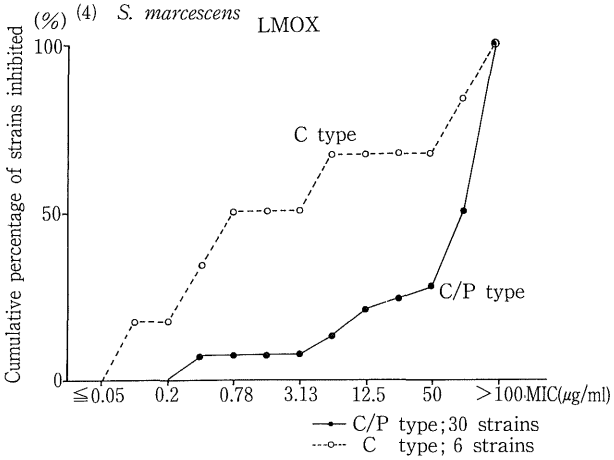


Fig. 1. Isolation rates of β-lactamase producing strains of 6 different organisms
The β-lactamase produced by each organism was classified by its substrate: type P which attacks penicillins, type P which attacks cephalosporins, and type C/P which attacks both penicillins and cephalosporins. The most common β-lactamase was type C in *E. coli, P. aeruginosa,* and *C. freundii,* type P in *K. pneumoniae,* and type C/P in *S. marsescence* and *E. cloacae.*









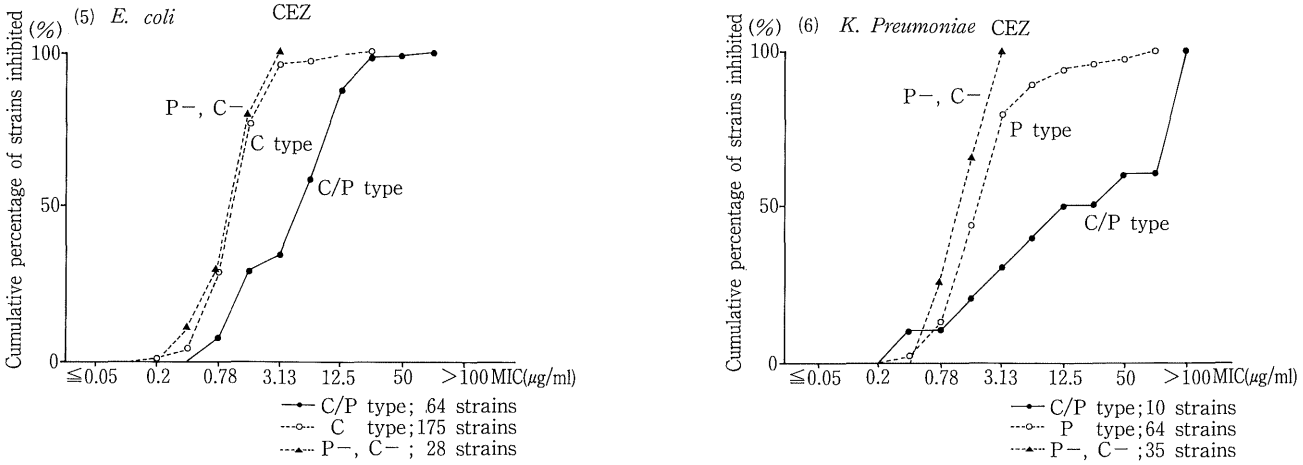


Fig. 2. Antibiotic susceptibility of 6 different organisms producing type C, type P, or type C/P β-lactamase. The β-lactamase produced by each organism was classified by its substrate: type P which attacks penicillins, type P which attacks cephalosporins, and type C/P which attacks both penicillins and cephalosporins. The Gram-negative strains which produced type C/P β-lactamase tended to be more resistant to antibiotics than the other β-lactamase producing strains. (CPZ: cefoperazon, LMOX: latamoxef, CEZ: cefazolin)

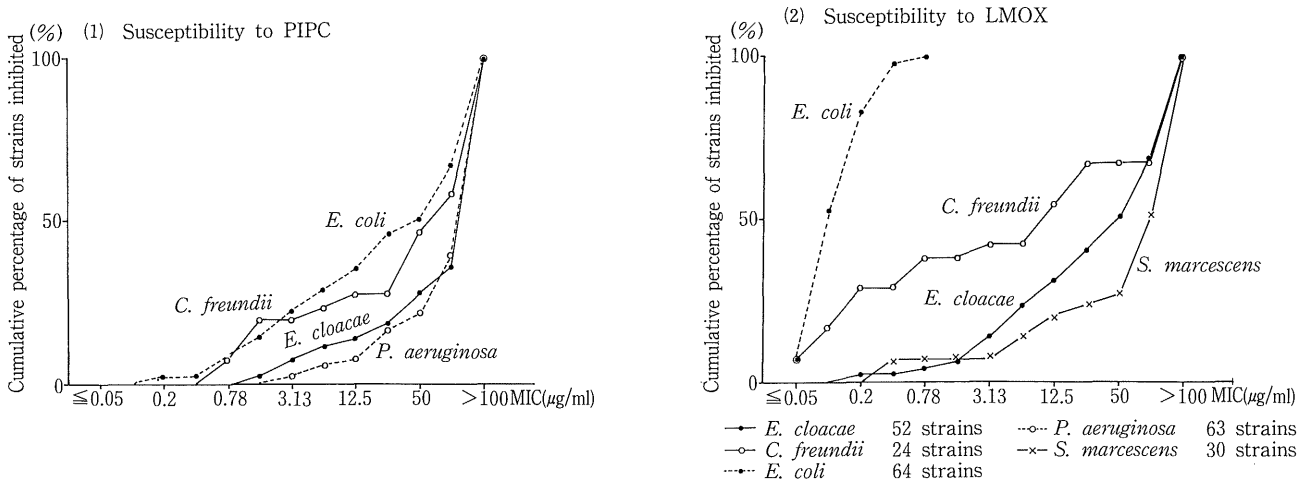


Fig. 3. Susceptibility of C/P type strains of 5 different organisms to PIPC and LMOX.
The β-lactamase which attacks both penicillins and cephalosporins was defined as type C/P. Among type C/P strains, the sensitivity to cephalosporin (LMOX) varied with the bacterial species, whereas all these bacteria were highly resistant to penicillin (PIPC). (LMOX: latamoxef, PIPC: piperacillin)

common β-lactamase was type C in *E. coli, P. aeruginosa,* and *C. freundii,* type P in *K. pneumoniae,* and type C/P in *S. marsescens* and *E. cloacae.*

2) Antibiotic susceptibility of each β-lactamase type (Fig. 2)

The bacterial strains producing type C/P β-lactamase tended to show greater antibiotic resistance than other types of bacteria producing β-lactamase. The MICs at which the cumulative percentage of strains inhibited was 80% (MIC 80) were, for type C/P β-lactamase-producing strains, as follows: *E. coli,* ABPC > 100 μg/ml and CEZ 12.5 μg/ml; *K. pneumoniae,* CEZ > 100 μg/ml; *P. aeruginosa,* CPZ > 100 μg/ml; *E. cloacae,* LMOX > 100 μg/ml; and *C. freundii* LMOX > 100 μg/ml.

3) Susceptibility to penicillins and cephalosporins of the strains which produce type C/P β-lactamase (Fig. 3)

All strains that produced type C/P β-lactamase tended to be highly resistant to penicillins. Even with PIPC (which is stable to cephalosporinase), the MIC50 was over 50 μg/ml for every strain tested. Sensitivity to cephalosporins varied widely, e. g., the MIC50 for LMOX ranged from 0.1 μg/ml to > 100 μg/ml.

DISCUSSION

It now appears probable that all bacteria produce at least one chromosomally-mediated β-lactamase, and that these enzymes are specific for genus, species, and subspecies[6]. In addition, bacteria can carry R plasmids (resistance factors), which specify β-lactamase that is different from the chro-

mosomally mediated enzymes[4]. R plasmid-determined β-lactamase is not specific to any bacterial species or strain, and this is produced in a constitutive manner[8], while the chromosomally determined β-lactamase is inducible.

We distinguished strains producing chromosomally mediated β-lactamase and strains producing both chromosomally and R plasmid-mediated enzymes in the following manner. The chromosomally mediated β-lactamase of almost all Gram-negative rods hydrolyses cephalosporins at a much greater rate than penicillins. We defined this type of β-lactamase as type C. The exceptions to this rule are *K. pneumoniae* and *Proteus mirabilis,* which have been reported to produce chromosomally mediated β-lactamases with predominantly penicillinase activity[5]. This type of β-lactamase was defined as type P. Although R plasmid-mediated β-lactamase has been grouped with TEM-type penicillinases, this broad spectrum enzyme is actually almost equally active against both penicillins and cephalosporins[7]. This type of β-lactamase was defined as type C/P. Since the β-lactamase that was grouped chromosomally as type C effectively hydrolysed both penicillins and cephalosporions, we hypothesized that this enzyme might have also acquired resistance by an R plasmid. Therefore, we considered that type C/P β-lactamases were mediated by both chromosome and R plasmids.

The classification of β-lactamase by Richmond & Sykes[11] has been widely employed. The chromosomal enzymes have been grouped further into those that are primarily cephalosporinases or primarily penicillinases. These correspond to classes I, II or IV of Richmond & Sykes. The R plasmid-mediated β-lactamases have been grouped into the broad spectrum TEM type of enzymes and enzymes that hydrolyse isoxazoyl β-lactam substrates. These correspond to classes III and V of Richmond & Sykes.

Along with the extensive use of antibiotics, multiple drug-resistant bacterial strains have been increasing, and in most cases the resistance is mediated by R plasmids[14,15]. Therefore, it seems reasonable to expect in this study that the strains producing type C/P β-lactamase would reveal high antibiotic resistance.

Among type C/P strains, sensitivity to cephalosporins varied between species, whereas all the bacteria were highly resistant to penicillins. Although most type C β-lactamase does not inactivate PIPC, the Gram-negative rods including *E. coli* that produced type C/P β-lactamase had acquired resistance to this antibiotic.

β-lactamase inhibitors have recently come into clinical use. Clavlanic acid is said to be effective against both penicillinase and cephalosporinase, and causes irreversible inactivation of R plasmid-mediated β-lactamse (type C/P)[1]. In combination with PIPC, clavlanic acid could extend the effective range of this antibiotic against resistant strains.

The majority of organisms produce relatively small amounts of chromosomally mediated β-lactamase, and production may be induced to higher levels in the presence of β-lactams[9]. However, mutant strains of *E. cloacae* producing chromosomally mediated β-lactamase constitutively show a high degree of resistance to most of the third-generation β-lactams[13]. Several laboratories have proposed that the tight binding of non-hydrolyzable β-lactams by periplasmic β-lactamase molecules, i. e., trapping of β-lactams, might create resistance to third-generation agents seen in β-lactamase-constitutive mutants of *E. cloacae*[16].

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