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-lactamase 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[9]. By contrast, the main mechanism of antibiotic resistance in Gram-negative rods is the production of β-lactamase, which hydrolyses the β-lactam ring[9]. 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.

MATERIALS 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[9]. 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\textsuperscript{10,12}. One drop of 0.01 mol phosphate buffer (pH 7.0) was added to the $\beta$-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 $\beta$-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 $\beta$-lactamase\textsuperscript{15}:

- 10 $\mu$g/ml of cefmetazole (CMZ, Sankyo Pharmaceutical Co. Ltd., Tokyo, Japan) for \textit{P. aeruginosa},
- 10 $\mu$g/ml of CEZ for \textit{E. cloacae}, \textit{C. freundii} and \textit{S. marcescens}, and
- 10 $\mu$g/ml of PCG for \textit{E. coli} and \textit{K. pneumoniae}.

Clinical isolates were cultured with an inducer at 37°C for 24 hours before $\beta$-lactamase detection was performed.

The $\beta$-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 $\beta$-lactamase producing strains of each organism (Fig. 1)

The percentages of $\beta$-lactamase producing strains of \textit{E. cloacae} (100%) and \textit{C. freundii} (96.7%) were higher than those of the other species. The most common $\beta$-lactamase was type $C$ in \textit{E. coli}, \textit{P. aeruginosa}, and \textit{C. freundii}, type $P$ in \textit{K. pneumoniae}, and type $C/P$ in \textit{S. marcescens} and \textit{E. cloacae}.

\begin{table}
\centering
\begin{tabular}{|c|c|c|c|}
\hline
Organism & $P$- & $C$- & $C/P$- \\
\hline
\textit{P. aeruginosa} & 100 & 50 & 0 \\
\hline
\textit{E. cloacae} & 100 & 50 & 0 \\
\hline
\textit{C. freundii} & 96.7 & 4.3 & 0 \\
\hline
\textit{S. marcescens} & 75.8 & 24.2 & 0 \\
\hline
\textit{E. coli} & 50 & 50 & 0 \\
\hline
\textit{K. pneumoniae} & 0 & 100 & 0 \\
\hline
\end{tabular}
\caption{Isolation rates of $\beta$-lactamase producing strains of 6 different organisms}
\end{table}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{isolation_rates.png}
\caption{Isolation rates of $\beta$-lactamase producing strains of 6 different organisms.}
\end{figure}

The $\beta$-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 $\beta$-lactamase was type $C$ in \textit{E. coli}, \textit{P. aeruginosa}, and \textit{C. freundii}, type $P$ in \textit{K. pneumoniae}, and type $C/P$ in \textit{S. marcescens} and \textit{E. cloacae}.
common $\beta$-lactamase was type $C$ in $E. coli$, $P. aeruginosa$, and $C. freundii$, type $P$ in $K. pneumoniae$, and type $C/P$ in $S. marcescens$ and $E. cloacae$.

2) Antibiotic susceptibility of each $\beta$-lactamase type (Fig. 2)

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

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

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

**DISCUSSION**

It now appears probable that all bacteria produce at least one chromosomally-mediated $\beta$-lactamase, and that these enzymes are specific for genus, species, and subspecies. In addition, bacteria can carry R plasmids (resistance factors), which specify $\beta$-lactamase that is different from the chro-
mosomally mediated enzymes\(^5\). R plasmid-
determined \(\beta\)-lactam is not specific to any bac-
terial species or strain, and this is produced in a
constitutive manner\(^6\), while the chromosomally
determined \(\beta\)-lactam is inducible.

We distinguished strains producing chromosomally
mediated \(\beta\)-lactamase and strains producing both
chromosomally and R plasmid-mediated enzymes in
the following manner. The chromosomally medi­
ated \(\beta\)-lactamase of almost all Gram-negative rods
hydrolyses cephalosporins at a much greater rate
than penicillins. We defined this type of \(\beta\)-lactamase
as type C. The exceptions to this rule are \(K.\) pneu­
moniae and \(P.\) mirabilis, which have been
reported to produce chromosomally mediated \(\beta-
\)lactamases with predominantly penicillinase
activity\(^7\). This type of \(\beta\)-lactamase was defined as
type P. Although R plasmid-mediated \(\beta\)-lactamase
has been grouped with TEM-type penicillinases, this
broad spectrum enzyme is actually almost equally
active against both penicillins and cephalosporins\(^8\).
This type of \(\beta\)-lactamase was defined as type C/P. Since the \(\beta\)-lactamase that was grouped chro­
osomally as type C effectively hydrolysed both
penicillins and cephalosporins, we hypothesized
that this enzyme might have also acquired
resistance by an R plasmid. Therefore, we consi­
dered that type C/P \(\beta\)-lactamases were mediated by
both chromosome and R plasmids.

The classification of \(\beta\)-lactamase by Richmond &
Sykes\(^1\) has been widely employed. The chro­
osomal enzymes have been grouped further into
those that are primarily cephalosporinases or
primarily penicillinases. These correspond to clas­
es I, II or IV of Richmond & Sykes. The R plasmid-
mediated \(\beta\)-lactamases have been grouped into the
broad spectrum TEM type of enzymes and enzymes
that hydrolyse isoxazoyl \(\beta\)-lactam substrates. These
correspond to classes III and V of Richmond &
Sykes.

Along with the extensive use of antibiotics, mul­
tiple drug-resistant bacterial strains have been in­
creasing, and in most cases the resistance is medi­
dated by R plasmids\(^4,15\). Therefore, it seems
reasonable to expect in this study that the strains
producing type C/P \(\beta\)-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 \(\beta\)-lactamase does not inacti­
vate PIPC, the Gram-negative rods including \(E.\) coli
that produced type C/P \(\beta\)-lactamase had acquired
resistance to this antibiotic.

\(\beta\)-lactamase inhibitors have recently come into
clinical use. Clavanic acid is said to be effective
against both penicillins and cephalosporins, and
causes irreversible inactivation of R plasmid-
mediated \(\beta\)-lactam (type C/P)\(^9\). In combination
with PIPC, clavlanic acid could extend the effec-
tive range of this antibiotic against resistant
strains.

The majority of organisms produce relatively
small amounts of chromosomally mediated \(\beta-
\)lactamase, and production may be induced to higher
levels in the presence of \(\beta\)-lactams\(^9\). However,
multiple strains of \(E.\) cloacae producing chro­
osomally mediated \(\beta\)-lactamase constitutively show
a high degree of resistance to most of the third-
generation \(\beta\)-lactams\(^10\). Several laboratories have
proposed that the tight binding of non-hydrolyzable
\(\beta\)-lactams by periplasmic \(\beta\)-lactamase molecules, i.
.e., trapping of \(\beta\)-lactams, might create resistance
to third-generation agents seen in \(\beta\)-lactamase-
constitutive mutants of \(E.\) cloacae\(^6\).

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