

Close evolutionary relationship between the chromosomally encoded β -lactamase gene of *Klebsiella pneumoniae* and the TEM β -lactamase gene mediated by R plasmids

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Sixty-three percent homology of nucleotide sequence and 67% homology of deduced amino acid sequence were found between the chromosomally encoded β -lactamase gene of *Klebsiella pneumoniae* and the TEM β -lactamase of transposon Tn3. Moreover, 22 out of 24 amino acid residues are identical around the predicted active site. It is therefore suggested that these two kinds of β -lactamases share a common evolutionary origin. The 0.5 kb DNA fragment of the cloned gene hybridized specifically with the chromosomal DNA of all the *K. pneumoniae* strains tested which had been isolated in Japan, USA and Europe.

β -Lactamase Nucleotide sequence Amino acid sequence (*Klebsiella pneumoniae*)

1. INTRODUCTION

Among various R plasmid-mediated β -lactamases, TEM-type β -lactamases have been found most frequently in many R plasmids [1] and have played an essential role in the prevalence of β -lactam antibiotic resistant bacteria. The TEM β -lactamase was classified into class A β -lactamase based on amino acid sequencing and enzymatic analysis [2,3] and was carried by the group of the transposon Tn3 [4]. The origin of the TEM β -lactamase gene has long been a mystery, although some similarity of amino acid sequences was found between the TEM β -lactamase and chromosomally encoded class A β -lactamases from *Bacillus* species [2]. *Klebsiella pneumoniae* was the only Gram-negative enteric bacterial species which carried a chromosomally encoded penicillinase type β -lactamase [5] and antigenic cross-reactivity was found between the penicillinase of *K. pneumoniae* and the TEM β -lactamase [6]. We have found high similarity of base sequences between the chromosomally encoded β -lactamase gene of *K.*

pneumoniae and the TEM β -lactamase gene of Tn3.

2. MATERIALS AND METHODS

2.1. Bacterial strains and plasmids

K. pneumoniae LEN-1 (03:K1-) [7], against which the minimal inhibitory concentration (MIC) of ampicillin was 7.8 μ g/ml, was harboring no plasmid. *E. coli* HB101 was used as a cloning host. Other strains used are listed in the legend of fig.2. Plasmid pMK16 and pBR322 were obtained from A. Ohta, Saitama University, Japan.

2.2. Media and reagents

Media for routine cultivation and detection were purchased from Difco. Restriction endonucleases, T₄ ligase, DNA sequencing kit and nick-translation kit were purchased from Takara Syuzo and Amersham. [α -³²P]dCTP was also purchased from Amersham.

2.3. DNA preparation

Plasmid DNA was prepared by the rapid alkaline extraction method and was purified by agarose gel electrophoresis [8]. Bacterial chromosomal DNA was extracted by the method of Stauffer et al. [9].

2.4. Recombinant DNA techniques and DNA sequencing

Recombinant DNA techniques were performed as described by Maniatis et al. [8]. DNA sequencing was carried out by the dideoxy chain termination method of Sanger et al. [10].

2.5. Hybridization

The transfer of DNA to nitrocellulose membranes was achieved by following the method of Southern [8]. The DNA probes were made with [α - 32 P]dCTP. The hybridization analysis was performed according to Yasue et al. [11].

3. RESULTS AND DISCUSSION

3.1. Cloning of the β -lactamase gene of *K. pneumoniae*

The *Eco*R1 digested chromosomal DNA of *K. pneumoniae* LEN-1 was ligated with *Eco*R1-cleaved plasmid pMK16 and transformed into HB101 and then ampicillin-resistant transformants were isolated. A 3 kb *Eco*R1-*Bam*H1 fragment of the first isolated 9 kb *Eco*R1 fragment was subcloned into pMK16 and the β -lactamase gene was expressed in *E. coli* HB101. The produced β -lactamase in HB101 had the same substrate profile as the β -lactamase of the parent strain of *K. pneumoniae* and rendered the host strain ampicillin-resistant (MIC > 100 μ g).

3.2. Nucleotide sequences of the β -lactamase gene

Fig.1 shows the entire base sequence of the cloned β -lactamase gene of *K. pneumoniae* LEN-1. The base sequence and the deduced amino acid sequence were compared with those of the TEM β -lactamase of Tn3 [12,13]. A 837 nucleotide coding sequence (nucleotides 286–1122) preceded by a possible –10 region TATTCT (223–228) and –35 region TTGTGA (199–204) of a promoter, encodes a polypeptide of 279 amino acids. The calculated molecular mass of the putative pre- β -lactamase is 30234 Da. The molecular mass of the

mature protein estimated from the gel electrophoresis of Maxicell is 28000 Da. Therefore, the first 15–20 amino acid sequence from the N-terminal methionine is supposed to be the putative signal sequence. Sixty-three percent nucleotide sequence homology and 67% amino acid sequence homology, respectively, were observed in the coding sequence, except the putative signal sequence, between the β -lactamase of *K. pneumoniae* and the Tn3 β -lactamase. Moreover, 22 out of 24 amino acid residues are identical around the predicted active site (fig.1). These values are strikingly high compared with the similarity values between TEM β -lactamase gene and the other class A β -lactamases (table 1). The high homology of nucleotide sequences between the β -lactamase gene of *K. pneumoniae* LEN-1 and the TEM β -lactamase gene of Tn3 clearly shows that these two kinds of β -lactamases originate from a common ancestor and belong to class A. Our results based on the nucleotide sequence analysis are consistent with the recent finding by Emanuel et al. [14] that the β -lactamase of *K. aerogenes* (*K. pneumoniae*) related to class A β -lactamases on the basis of the limited amino acid sequence analysis of the peptide. The relatively low homology in the 60 amino acid sequence from N-terminal and 20 amino acid from C-terminal (36 and 43% homology, respectively) suggests that these portions do not play essential roles in their enzymatic activities. No homology was found in the sequence at the promoter region and also in the adjacent sequences between the β -lactamase gene of *K. pneumoniae* and the TEM β -lactamase gene of Tn3 (fig.1). Although over 500 nucleotides both before and after the β -lactamase gene were sequenced (partly shown in fig.1), no sequence related to either transposons or insertion sequences was detected.

3.3. Hybridization analysis

The 0.5 kb *Pvu*II-*Pst*I DNA fragment of the cloned gene was used as a probe to detect the same β -lactamase gene on the chromosome of other *K. pneumoniae* strains. Fig.2 shows that the probe hybridized with the chromosomal DNA of all the *K. pneumoniae* strains tested which had been isolated in Japan, USA and Europe over the past four decades and with the band of pBR322, whereas the probe did not hybridize with

1090 1100 1110 1120 1130 1140
GGCCCGGAGCGCAACCGSGAGCGCATTTGGTGTATCTATCTCGGGGATACCCCGCGAGT
*** **
GGGCCAGATGGTAAGCCCTCCGTATCGTAGTTATCTACACGACGGGGAGTCAGGCAACT

1090 1100 1110 1120 1130 1140
ATGCCCGAGCGTAAACAATATATCGCCGGGATCGGCACGCGCCTGATCAGSACCTGGCAAC
*** **
ATGGATGAACGAAATAGACAGATCGCTGAGATAGTGCCCTCACTGATTAAAGCATTGGTAA

1150 1160 1170 1180 1190 1200
GCTAAGCCCGCGGTACCGTCGCTTAGCGCGGCGCCGACGACCTGGCAGGCGTGGCCGCGG
*** **
CTGTCAGACCAAGTTTACTCATATATACTTTAGATTGATTAAAACTTCATTTTTAATTT

1210 1220 1230 1240 1250 1260
ATATGACTGGCGCGGCATCGGACAGATCCATCGGTAATAATAGTTGTAACCGGGTCAAG
*
AAAAGGATCTAGGTGAAGATCCTTTTTTGATAATCTCATGACC AAAATCCCTTAACTGTAG

1270 1280 1290 1300 1310 1320
GGTAACGCCATAAAGTCGCTCCTGATTGTATTTCGAACCTGTCGACACGAGGATGCCT
*
TTTTCGTTCCA CTGAGCGTCAGACCCC

1330 1340 1350 1360 1370 1380
CTCGCGCTGACCTGGCTGACGGTCTCCTTGACGGTAACTTTGTTCTCATCAGGGGTGAAA

1390 1400 1410 1420 1430 1440
ATAGCTGTCCCAGCGCTGGCGGAGATAAAGGCCGTATCGATAGCCAGGTGGAGTAACGTT

1450 1460 1470 1480 1490 1500
CGCGCGCGCGATTTCGCCCACGAGGAGCGATTCTCCGCACAGTCCCGCGCGGTGTGGATC

1510 1520 1530 1540 1550 1560
ACGCCGCACTGGCTGGCATCGATCAGCAGTTGGGTTATCTCAAATCATTGGTCACCACC

1570
PstI
TGCAG

1 10 20 30 40 50 60
MRYVFLCVTSLSLITLLFHMVNAGFPDEQKIDSESLDSGRVGVHVDLDRNTDIAKADFA
MSIQHFVALDFFFNAFLPYFAHEETLVKVMDAEQLGARVGVTLDLNSCKILESTPEE
1 10 20 30 40 50 60

70 80 90 100 110 120
RFFPMSTFKVLLCGAVLARVDAGLEFLRRRIHYHQDLVDYSPVSEKHIMDGMTILGELQ
RFFPMSTFKVLLCGAVLSRDVAGOEFLRRRIHYSDQLVDYSPVTKIKHLTDGMTIVRELCS
70 80 90 100 110 120

130 140 150 160 170 180
AAITTSNDAQNLLIATMGGHAGLTAFIRIQIGINVTRLDRWBETALNEALPCDARDTTTPA
AAITTSNDAQNLLIATMGGHAKLTAFIRHNMGINVTRLDRWEPENEAETNDROTTPMA
130 140 150 160 170 180

190 200 210 220 230 240
SMATFLRKLLTAQHLSARSQQOOLLGNVDDRVAGPLLRVLPHGWFIADNLGAGERGARG
AMATFLRKLLTGELLTLASROOUIDMEADNVAGPLLESALHAGWFIADNSGAGERGARG
190 200 210 220 230 240

250 260 270 279
TVALLGPDGKKPRIVVIYLRTDTFASAGRNNOHIALGQR(K. pneumoniae LEN-1)
TVALLGPDGKKPRIVVIYTGSQVLDLDERNRQIAFTGLSIKH(Tn3)
250 260 270 280 286

Fig.1. Nucleotide sequence and predicted amino acid sequence of the β -lactamase gene of *K. pneumoniae* and their sequence homology to the TEM β -lactamase gene of Tn3. (A) The β -lactamase gene of *K. pneumoniae*. (B) The TEM β -lactamase gene of Tn3 [4]. Homologous nucleotide sequence (*) and amino acid sequence (boxed) are marked. The -35 and -10 regions of the β -lactamase promoter are also boxed. The region of dyad symmetry in the possible terminator is marked by horizontal arrows. The *PvuII* (388)-*PstI* (900) fragment was used as the probe for DNA hybridization.

Table 1
Similarity matrix among β -lactamases from different sources

β -Lactamase from	β -Lactamase from			
	<i>K. pneumoniae</i>	Tn3	<i>B. cereus</i> (type I)	<i>S. aureus</i>
<i>K. pneumoniae</i>	100 (100)	67 (63)	28 (37)	21
Tn3		100 (100)	34 (42)	30
<i>B. cereus</i> (type I)			100 (100)	38
<i>S. aureus</i>				100

Values were expressed as % homology of amino acid sequence, and those in parentheses show % homology of nucleotide sequence. These values were obtained using the data from reports about Tn3 β -lactamase [4], *B. cereus* β -lactamase (type I) [15] and *S. aureus* β -lactamase [16]. The nucleotide sequence of *S. aureus* β -lactamase has not yet been reported

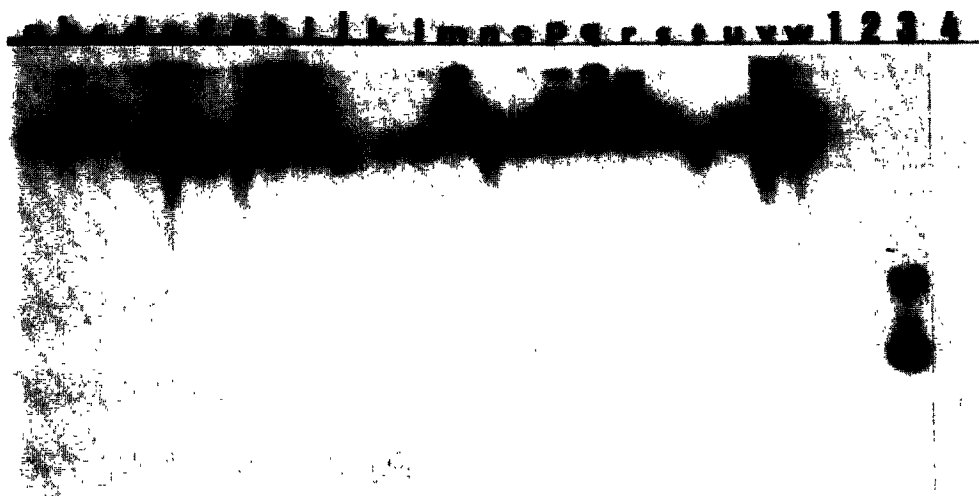


Fig.2. Southern blotting analysis of the chromosomal DNA from various strains of *K. pneumoniae*, *E. coli* and *B. cereus*. The *Pvu*II (388)-*Pst*I (900) fragment shown in fig.1 was labeled with [α - 32 P]dCTP by nick-translation and used as the probe. The sources of DNA specimens used are listed below.

Lane	Species	Strain	Serotype	Comment	Reference
a	<i>K. pneumoniae</i>	C5046	(O2:K3)	*	19
b	"	E5051	(O2:K5)	*	19
c	"	F5052	(O2:K6)	*	19
d	"	Aerogenes 4140	(O1:K7)	*	19
e	"	Klebs. 919	(O1:K10)	*	19
f	"	Klebs. 313	(O1:K12)	*	19
g	"	Mich. 61	(O4:K15)	*	20
h	"	5758	(O2:K28)	*	21
i	"	6258	(O3:K31)	*	21
j	"	NTCT8172	(O1:K64)	*	22

Lane	Species	Strain	Serotype	Comment	Reference
k	"	123	(K46)	**	
l	"	164	(K10)	**	
m	"	171	(K68)	**	
n	"	183	(K1)	**	
o	"	247	(K28)	**	
p	"	375	(K15)	**	
q	"	395	(K29)	**	
r	"	422	(K18)	**	
s	"	447		**	
t	"	1085		***	
u	"	1098		***	
v	"	Chedid	(O1:K2)	****	
w	"	LEN-1	(O3:K1-)	+	7
1	<i>E. coli</i>	K12(D21)		++	
2	"	B993-4413	(O9:K:-H-)	+++	
3	Plasmid	pBR322			
4	<i>B. cereus</i>	61		+++	

* Reference strains for the capsular types supplied by I. Orskov, International *Escherichia* and *Klebsiella* Center, Copenhagen, Denmark

** Clinical isolates supplied by Central Clinical Laboratory, Nagoya University Hospital, Nagoya, Japan

*** Isolates from foods supplied by Municipal Institute of Health, Nagoya, Japan

**** Laboratory strain

+ Parent strain of the cloned β -lactamase gene

++ Laboratory strain (ampicillin-resistant)

+++ Wild strain isolated in our laboratory

chromosomal DNA of ampicillin-resistant *E. coli* K12 strain D21 or *Bacillus cereus*. It is therefore confirmed that the DNA sequences homologous to the cloned β -lactamase gene are generally carried on the chromosomal DNA of *K. pneumoniae*. By contrast, this probe did not hybridize with the chromosomal DNA of the strains of *K. oxytoca* (manuscript in preparation).

Klebsiella is one of the most commonly distributed bacteria all over the world. Most clinical isolates of *K. pneumoniae* are naturally ampicillin-resistant. It is unlikely that the β -lactamase gene of all strains of *Klebsiella* used in fig.2 and of other ampicillin-resistant clinical isolates originated by the insertion of transposon. It is not surprising, therefore, to assume the TEM β -lactamase gene on R plasmids originated from *K. pneumoniae*. The fact that there is no sequence related to transposon in the sequences flanking the cloned β -lactamase gene also supports this

hypothesis. Many third position substitutions which do not alter coding amino acids and other alterations in the sequences surrounding the coding sequence, found between the cloned β -lactamase gene of *K. pneumoniae* and the TEM β -lactamase of Tn3, suggest that these genes have arisen from a common ancestor under the pressure of antibiotics use. There is no sequence homology between the cloned β -lactamase gene of *K. pneumoniae* and *E. coli amp C* gene [15]. Therefore these β -lactamases have different evolutionary origins in their chromosomes. Although it is widely accepted that there are similarities of amino acid sequences at the active sites among β -lactamases, penicillin-binding proteins of *E. coli* and D-alanine carboxypeptidases of *Bacillus* species [2,16], no overall sequence homology was observed between the cloned β -lactamase of *K. pneumoniae* and the penicillin-binding proteins or carboxypeptidases except the amino acid sequences

at the active site (-Phe---SerThr-Lys-).

Unlike many other enzymes, bacterial β -lactamases are extremely heterogeneous in both structure and substrate profiles. The determination of their primary structure enables us to understand in more detail the relationship between the substrate profiles and the active site of these enzymes, and it will facilitate the development of new β -lactam antibiotics.

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