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Nucleotide Sequence and Characterization of a Carbenicillin-Hydrolyzing Penicillinase Gene from *Proteus mirabilis*

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The structural gene of a carbenicillinase was cloned from the chromosomal DNA of *Proteus mirabilis* GN79. This gene codes for a protein of 270 amino acids. Alignment of the amino acid sequence with those of known β-lactamases revealed that the enzyme is a novel class A β-lactamase with a unique conserved triad, RTG. By using a DNA fragment of the structural gene, a lack of cross hybridization was confirmed between the DNA probe and total DNAs from natural isolates of *P. mirabilis*, suggesting that the carbenicillinase may not be a species-specific β-lactamase of *P. mirabilis*.

Proteus mirabilis GN79 was isolated in Japan from a patient in 1965 (25) prior to the discovery of the PSE enzymes in *Pseudomonas aeruginosa* (12). Strain GN79 produces a constitutive β-lactamase with the characteristics of a carbenicillinase, and the enzyme could be distinguished from the PSE-1, -3, and -4 enzymes by its substrate profile and its nonreactivity with the antiserum against a plasmid-mediated carbenicillinase (30). The enzyme production by the GN79 strain is hereditarily stable, and progeny lacking the enzyme could not be isolated even though many possible treatments for plasmid elimination were tried, suggesting that the enzyme may be mediated by a chromosomal gene. To our knowledge, a carbenicillinase mediated by a chromosomal gene in a gram-negative enteric bacterium has not been found previously. Therefore, the enzyme was expected to be a species-specific β-lactamase of *P. mirabilis*. Besides, the *P. mirabilis* enzyme is interesting as a possible insight into the origin of carbenicillinase genes on plasmids. In order to examine the genetical background of the carbenicillinase, the enzyme gene was cloned and sequenced.

The chromosomal DNA fraction of *P. mirabilis* GN79 was partially digested with a restriction endonuclease, *Sau*3AI. The digested DNA fragments were ligated into the *Bam*HI site of a vector plasmid, pHSG398 (31). *Escherichia coli* TG1 (6) was transformed with the recombinant plasmid DNAs by the calcium chloride method of Mandel and Higa (16), and then colonies resistant to ampicillin (25 μg/ml) were selected. A recombinant plasmid with an insertion of 2.6 kb was obtained and designated pPM79P1. This 2.6-kb fragment on pPM79P1 was reduced in length to 2.3 kb by digestion with a restriction endonuclease, *Hin*cII, and the plasmid carrying the 2.3-kb fragment was termed pPM79P2. The two recombinant plasmids were introduced into *E. coli* AS226-51 cells. AS226-51 is an *ampD* mutant of C600 and also a deletion mutant of *ampC* (33). As shown in Table 1, the *E. coli* strains carrying the recombinant plasmids showed high resistance to ampicillin and carbenicillin but were susceptible to cephalosporins. This susceptibility pattern is similar to that found for *E. coli* cells producing an R-plasmid-mediated carbenicillinase (30). The cephalosporin resistance of *P. mirabilis* GN79 is believed to be intrinsic, because we did not find detectable cephalosporin hydrolysis activity in the *P. mirabilis* cells. The specific enzyme activity in the *E. coli* cells was about two times that in the *P. mirabilis* cells, indicating that the structural gene of the enzyme lies in the 2.3-kb fragment. However, taking into account that the vector is a multicopy plasmid, the promoter activity of the enzyme gene in *E. coli* cells should be lower than that in *P. mirabilis* cells.

The pPM79P1 enzyme and the GN79 enzyme showed identical isoelectric point values, 6.6, this value being obviously different from those of carbenicillinases mediated by naturally occurring R plasmids in *P. mirabilis*, pCS203 (30) and pCS229 (30) (Fig. 1). It was also confirmed that the substrate profiles of the pPM79P1 and pPM79P2 enzymes are identical to that of the GN79 enzyme.

A 1,247-bp DNA fragment containing the carbenicillinase gene was sequenced by the dideoxynucleotide chain termination method of Sanger et al. (23). The nucleotide sequence presented in Fig. 2 contains only one open reading frame of 894 nucleotides. The ATG initiation codon was assumed to be located at nucleotide positions 312 to 314, and the open reading frame was terminated by a stop codon, TGA, at positions 1206 to 1208. A putative −35 region, Pribnow box, and Shine-Dalgarno sequence are also indicated in Fig. 2. The N-terminal amino acid sequence of the mature enzyme

TABLE 1. β-Lactamase activity and levels of resistance to β-lactams of *E. coli* strains carrying the cloned carbenicillinase gene and *P. mirabilis* GN79

Bacterial strain	MIC (μg/ml) of[a]:				β-Lactamase activity (U/mg of protein)[b]
	APC	CPC	CER	CEZ	
P. mirabilis GN79	3,200	3,200	25	100	0.30
E. coli AS226-51/pPM79P1	3,200	3,200	3.2	1.6	0.72
E. coli AS226-51/pPM79P2	3,200	3,200	1.6	1.6	0.58
E. coli AS226-51	1.6	6.3	0.8	0.8	$<10^{-4}$

[a] MICs were measured by the serial agar dilution method (27). The β-lactams used were as follows: APC, ampicillin; CPC, carbenicillin; CER, cephaloridine; CEZ, cefazolin.

[b] β-Lactamase activity in sonically disrupted bacterial cells was measured by the microiodometric method (19), with benzylpenicillin as the substrate. One unit of the enzyme was defined as the amount of enzyme which hydrolyzed 1 μmol of benzylpenicillin in 1 min at pH 7.0 and 30°C.

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TABLE 2. Amino acid sequences of the conserved triad regions in β-lactamases and D,D-peptidase

Enzyme	Class	Amino acid sequence[a]
β-Lactamases[b]		
TEM-1	A	WFIAD**KSG**AGERGSR
SHV-1	A	WFIAD**KTG**AGERGAR
LEN-1	A	WFIAD**KTG**AGERGAR
PSE-3	A	WQIAD**KSG**AGGHGSR
PES-4	A	WNIAD**RSG**AGGFGAR
PM	A	WIVAD**RTG**AGGYGSR
PV	A	WVVGD**KTG**SGDYGTT
SA	A	YKVAD**KSG**QAITYAS
EC	C	ASWVH**KTG**ATGGFGS
CF	C	ASWVH**KTG**STGGFGS
OXA-1	D	TKLYG**KTG**AGFTANR
OXA-2	D	WILRA**KTG**WEGRMGW
PSE-2	D	YLVHS**KTG**FGSGVGTE
D,D-peptidase		ISVYG**HTG**TVQGYYT

[a] The conserved triad residues are indicated by boldface type.

[b] The abbreviations for the enzymes and the references for their amino acid sequences are as follows: TEM-1, TEM-1 penicillinase (29); SHV-1, SHV-1 penicillinase (2); LEN-1, *K. pneumoniae* LEN-1 penicillinase (1); PSE-3, PSE-3 carbenicillinase (5); PSE-4, PSE-4 carbenicillinase (4); PM, *P. mirabilis* GN79 carbenicillinase (this paper); PV, *P. vulgaris* 5E/78-1 β-lactamase (21); SA, *S. aureus* PC1 penicillinase (7); EC, *E. coli* K-12 *ampC* cephalosporinase (13); CF, *C. freundii* GN346 cephalosporinase (33); OXA-1, OXA-1 penicillinase (22); OXA-2, OXA-2 penicillinase (8); PSE-2, PSE-2 penicillinase (11); D,D-peptidase, D,D-peptidase of *Streptomyces* sp. strain R61 (9).

was determined by using an Applied Biosystems Gas-Phase Sequencer (Applied Biosystems, Foster City, Calif.) to be NH_2-Thr-Asp-Ser-Val-Leu-Glu-Ala-Val-Thr-Asn, coinciding with nucleotide positions 396 to 425. The mature enzyme is composed of 270 amino acids and has a molecular mass of 29,657 Da.

The seven conserved boxes described by Joris et al. (14) for β-lactamases and penicillin-binding proteins were assigned on the basis of the alignment of the sequence of the enzyme to those of known β-lactamases and R61 D-alanyl-D-alanine peptidase (D,D-peptidase). A Ser-Thr-Phe-Lys tetrad characteristic of the active-site region of most class A β-lactamases was found at amino acid positions 44 to 47 (box II), and Ser-44 was determined to be the active-site serine. Box I, including Gly-20 and Asp-25, box III (Tyr-79), box IV (Lys-85), box V (Glu-141), and box VI (Trp-185) in the carbenicillinase are common to most class A β-lactamases. Box VII is composed of the conserved triad residues. A KTG or KSG triad is quite common in class A, C, and D β-lactamases (14). The basic amino acid at the first position is assumed to be necessary for the enzymes to interact with substrate and to accelerate the deacylation of the acyl intermediate (20, 32). This study revealed that the conserved triad in the *P. mirabilis* carbenicillinase is RTG (Arg-209, Thr-210, and Gly-211), which is a novel motif in the known β-lactamases (Table 2). Recently, Boissinot and Levesque (4) reported the motif of box VII in the PSE-4 carbenicillinase and that the triad is RSG. This agreement in the first basic residue, arginine, may suggest a significant role of the first basic residue in the characteristic substrate spectrum.

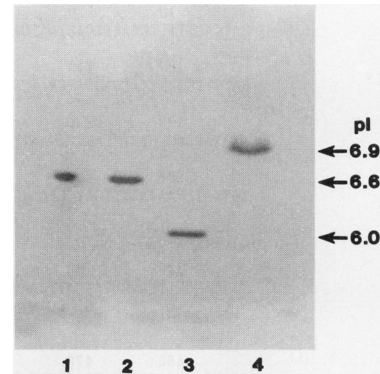

FIG. 1. Comparison of the carbenicillinase from *E. coli* AS226-51/pPM79P1 (lane 1) with those from *P. mirabilis* GN79 (lane 2), *P. mirabilis* N3/pCS203 (lane 3), and *P. mirabilis* N29/pCS229 (lane 4) on an isoelectric focusing gel. Extracts of the bacterial cells were subjected to isoelectric focusing. The β-lactamase activity on the gel was detected by spraying a chromogenic cephalosporin, FR18417. The isoelectric pH values of the carbenicillinases are indicated at the right of the photograph.

The amino acid sequences of the *P. mirabilis* carbenicillinase and other known β-lactamases and D,D-peptidase were compared (Table 3). The alignment was carried out with a computer program (Genetyx; Software Development Co., Tokyo, Japan) with some modification with reference to the tertiary structures of the *Staphylococcus aureus* β-lactamase (10) and the *Citrobacter freundii* β-lactamase (20). The

TABLE 3. Similarity of the amino acid sequence of the *P. mirabilis* carbenicillinase to those of other β-lactamases and D,D-peptidase[a]

Enzyme	Class	Amino acid sequence similarity (%) with:												
		TEM-1	SHV-1	LEN-1	PSE-3	PSE-4	PM	PV	SA	EC	CF	OXA-1	OXA-2	PSE-2
β-Lactamases														
SHV-1	A	67.6												
LEN-1	A	63.2	89.1											
PSE-3	A	45.7	49.0	49.0										
PSE-4	A	43.4	46.5	46.1	46.9									
PM	A	46.2	47.3	47.3	48.8	47.3								
PV	A	37.1	38.3	38.3	36.7	35.9	40.1							
SA	A	30.0	29.8	29.8	31.0	34.0	33.0	33.6						
EC	C	<1	<1	<1	<1	<1	<1	<1	<1					
CF	C	<1	<1	<1	<1	<1	<1	<1	<1	77.0				
OXA-1	D	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1			
OXA-2	D	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	27.8		
PSE-2	D	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	26.4	35.2	
D,D-peptidase		<1	<1	<1	<1	<1	<1	<1	<1	22.5	22.3	<1	<1	<1

[a] β-Lactamase abbreviations and the references are the same as in Table 2, footnote *b*.

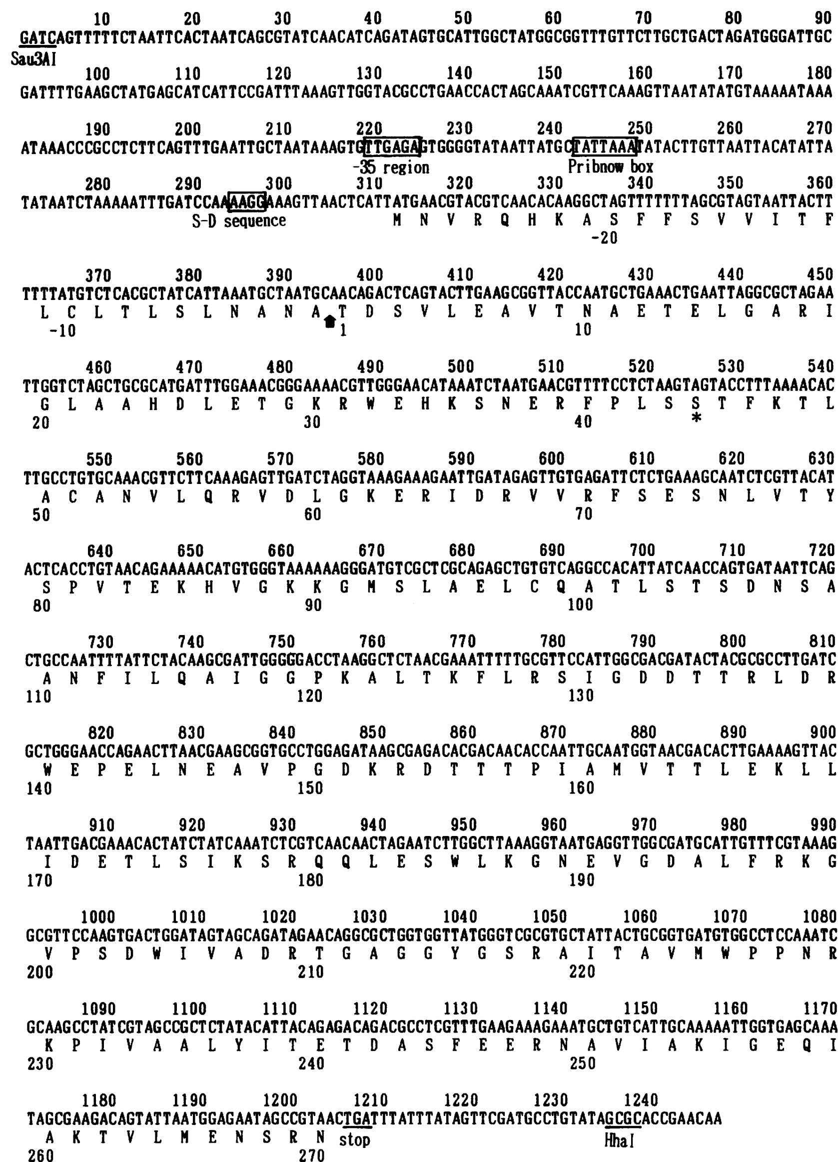

FIG. 2. DNA sequences of carbenicillinase and flanking regions and the predicted amino acid sequence for the enzyme. The nucleotides are numbered from the *Sau*3AI site. The −35 region, Pribnow box, and Shine-Dalgarno (S-D) sequence are boxed. The position of the N-terminal amino acid of the mature enzyme is designated as position 1 of the amino acid sequence. The amino acid sequence from −28 to −1 is assumed to be that of the signal peptide. The active-site serine is indicated by an asterisk.

comparative method revealed that the *P. mirabilis* carbenicillinase is a typical class A β-lactamase, the enzyme showing 46.2 to 48.9% similarity to four class A β-lactamases mediated by R plasmids. On the other hand, the *P. mirabilis* enzyme showed a somewhat lower relationship to two other class A β-lactamases, a chromosomal β-lactamase of *Proteus vulgaris* and the *S. aureus* β-lactamase. It should be noted that *P. vulgaris* is one of the two species composing the genus *Proteus*, the other being *P. mirabilis*. Contrary to expectation, a significantly higher homology to the PSE β-lactamases than to other class A β-lactamases could be not observed. To clarify chromosomal localization of the carbenicillinase gene, we used a 2.6-kb *Hin*dIII fragment of pPM79P1 as a probe for Southern blot analysis (28). This probe hybridized only to the chromosomal DNA from the GN79 cells, which was definitely separated from three kinds of plasmids inhabiting the GN79 cells on agarose gel electrophoresis. The plasmid DNA in *P. mirabilis* GN79 was extracted from the cells by the alkaline lysis method (3) or the Kado-Liu method (15). DNA hybridization could not be detected at the migration positions of the plasmid DNAs on the gel plate even under conditions of lower stringency. The reactivities of total DNAs from the following strains with the carbenicillinase gene probe were examined by Southern blot analysis: *P. aeruginosa* 1937E/RPL11 (PSE-1 carbenicillinase) (18), *P. aeruginosa* 1973E/R151 (PSE-2, OXA-type penicillinase) (17), *P. aeruginosa* 1920E/Rms149 (PSE-3 carbenicillinase) (24), *P. aeruginosa* 1559E/pMG19 (PSE-4 carbenicillinase) (12), *P. mirabilis* N-3/pCS203 (a carbenicillinase) (30), *P. mirabilis* N-29/pCS229 (a carbenicillinase) (30), *E. coli* ML1410/RGN14 (TEM-1-type penicillinase) (34), *E. coli* ML1410/RGN238 (OXA-1-type penicillinase)

(34), *Klebsiella pneumoniae* GN69 (a chromosomal penicillinase similar to a TEM-type penicillinase) (26), and 11 natural isolates of *P. mirabilis*. As a result, the DNA probe was found to hybridize only with the DNA of the *P. mirabilis* GN79 cells. Negative results were also obtained in the tests, even though the experiments were performed under lower-stringency conditions. It may be concluded that the carbenicillinase mediated by the gene on the *P. mirabilis* GN79 chromosome is a novel carbenicillinase and that the carbenicillinase gene originated outside of this species.

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