VOL. 52 NO. 12, DEC. 1999 THE JOURNAL OF ANTIBIOTICS pp. 1135 — 1139

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Presence of Genes for β-Lactamases of Two Different Classes on a Single Plasmid from a Clinical Isolate of *Serratia marcescens*

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(Received for publication September 21,1999)

The majority of β-lactam antibiotics have become increasingly ineffective therapeutic agents due to the rapid development of resistant strains[1]. Mechanisms responsible for the development of resistance to β-lactams include (i) hydrolysis by β-lactamases, (ii) creation of nonhydrolytic barriers by β-lactamases, (iii) reduction in affinity of target proteins, and (iv) reduction in permeability[2]. For Gram-negative enteric bacteria, the emergence of resistance to broad-spectrum penicillins and β-lactamase-stable cephalosporins occurs frequently[3]. The complexity of β-lactamase epidemiology has greatly increased with the emergence of extended-spectrum β-lactamases (ESBLs) derived from plasmid-encoded TEM and SHV-type enzymes[4]. These β-lactamases show greater specificity for β-lactam antibiotics.

β-Lactamases are divided by their substrates into classes A, B, C and D. When an isolate produces two different classes of β-lactamases, the genes coding for each β-lactamase are located separately on a plasmid and on a chromosome. For example, plasmid-encoded class A enzymes and chromosome-encoded class C enzymes are produced in Gram-negative bacteria. On the other hand, plasmid-encoded class B enzymes have been reported from Japan[5]. However, there are no reports of plasmids carrying the genes of two different classes of β-lactamases, such as class A and class B or class A and class D.

In this report, we describe a novel plasmid carrying the genes for both class A and class B β-lactamases in a clinical isolate of *Serratia marcescens* in Japan.

Materials and Methods

Test Organisms

The *S. marcescens* strain described here was isolated from the urine of a patient with a urinary tract infection at a hospital in northern Japan in 1996. The organism was identified by standard methods. *Escherichia coli* K12 ML4901 and ML4947 strains, plasmid-regulated β-lactamase-producing bacteria, and plasmids of various incompatibility groups (R9-5, F*olac-tet*, R621a, N3, RP4-1, S-a, R27, R446b and R14) were stocked at the Department of Microbiology, Kitasato University School of Medicine.

Culture Medium

The test strains were incubated in sensitivity assay liquid medium (MH broth, Nissui, Tokyo) for 18 hours at 35°C. Minimum inhibitory concentrations (MICs) were determined using sensitivity assay agar medium (MH agar medium, Nissui)[6]. BTB lactose agar medium (Nissui) was used for the identification of incompatibility group plasmids and for pure culture of transconjugants, while L broth was used for assay of β-lactamases[7].

Test Drugs

The following antibacterial agents were used in this study and reference powder of different drugs of known potency were provided by the respective manufacturers. Benzylpenicillin (PCG, Banyu, Tokyo) and piperacillin (PIPC, Toyama Chemical, Tokyo) were used as representative penicillins, while cephalotin (CET, Shionogi, Osaka), cefotiam (CTM, Takeda Chemical Industries, Osaka), cefmetazole (CMZ, Sankyo, Tokyo) and cefotaxime (CTX, Hoechst Japan, Tokyo) were used as representative cephalosporins. Other β-lactams, including imipenem (IPM, Banyu, Tokyo), meropenem (MEPM, Sumitomo, Osaka), panipenem (PAPM, Sankyo, Tokyo) and aztreonam (AZT, Eisai, Tokyo), as well as kanamycin (KM, Meiji Seika, Tokyo), chloramphenicol (CM, Sankyo) and tetracycline (TC, Lederle Japan, Tokyo) were used. Other reagents, including sulfonamide (SA), streptomycin

(SM), nalidixic acid (NA, Daiichi, Tokyo), various restriction enzymes, and marker DNAs were purchased from commercial sources.

Drug Susceptibility Assay

The susceptibility of the test strains to each drug was assayed in accordance with the specified drug sensitivity assay methods of the Japan Society of Chemotherapy except for the antibiotic concentrations used[6]. After overnight incubation at 35°C, medium containing organisms was diluted in buffered saline with gelatin to a concentration of $5\sim6\times10^6$ cfu/ml. Using a microplanter (Sakuma Seisakusho, Tokyo), aliquots of this suspension (about $5\sim6\times10^4$ cfu/spot) were inoculated onto plates containing the test drug at various concentrations, and each plate was incubated for 18 hours at 35°C. The lowest concentration at which no bacterial growth was observed macroscopically was determined to be the minimum inhibitory concentration (MIC).

Characterization of Plasmids

For determination of incompatibility groups, the plasmid under study was introduced into *E. coli* K12 ML4901 together with a standard plasmid (previously grouped by conjugal transfer or transformation), and the incompatibility group of the test plasmid was determined as follows[7]. Organisms containing both the test plasmid and a standard plasmid of each incompatibility group were again incubated overnight in MH broth at 35°C. This broth was then smeared on MH agar medium and again incubated overnight at 35°C. The resulting colonies were collected and streaked onto plates containing a drug corresponding to the resistance pattern of the two plasmids, followed by further incubation overnight at 35°C. When organisms proliferated on both drug-containing plates, the test plasmid was considered to be of a different group from that of the standard plasmid. When organisms proliferated on only one of the two plates, the test plasmid was considered to belong to the same incompatibility group as the standard plasmid.

Preparation and Assay of β-Lactamase

Test strains were incubated overnight in L broth, which was then diluted 20-fold with fresh L broth. A 10-ml aliquot of this diluted culture broth was incubated at 35°C with shaking. Organisms in late logarithmic growth phase were collected by centrifugation at $18,000\times g$ for 10 minutes, washed with 0.05 M phosphate buffer (pH 7.0) with 10 μM $ZnCl_2$, and suspended in 3 ml of the same buffer. This bacterial suspension was sonicated and then centrifuged at $18,000\times g$ for 20 minutes at 4°C. The supernatant was used as the crude enzyme preparation[8]. Enzyme activity was determined by spectrophotometry (UV2000, Shimadzu Corp., Tokyo) at 30°C in 50 mM phosphate buffer (pH 7.0) with or without 10 μM $ZnCl_2$, using antibiotics as the substrate.

Analytical Isoelectric Focusing

Isoelectric focusing was carried out with a Phast system (Pharmacia Biotech, Uppsala, Sweden) and a Phast Gel IEF 3-9 (Pharmacia Biotech). The enzyme protein on the gel plate was detected by staining with Coomassie brilliant blue G-250, and β-lactamase activity was confirmed with nitrocefin (100 μg/ml).

Isolation of DNA

The procedures described in the manuals of Takara Suzo Co., Otsu and in previous reports were followed for the isolation of plasmid DNA[9].

Gene amplification was performed by a polymerase chain reaction (PCR) method using a commercially available PCR kit (Gene Amp PCR Reaction Kit with Ampli TaqDNA Polymerase, Takara) and a DNA Thermal

Table 1. PCR primers.

Type of β-lactamase	Size of PCR product (bp)	Sequence
TEM	108	5'-AAGCCATACCAAACGACGAG-3'
		5'-ATTGTTGCCGGGAAGCTAGA-3'
Class B	448	5'-CATGGTTTGGTGGTTCTTGT-3'
		5'-ATAATTTGGCGGACTTTGGC-3'
SHV-1	593	5'-TCTCCCTGTAAGCCACCCTG-3'
		5'-CCACTGCAGCAGCTGC(A/C)GTT-3'
Toho-1	164	5'-TGGAAGCCCTGGAGAAAAGT-3'
		5'-CTTATCGCTCTCGCTCTGTT-3'

bp, base pair

Cycler PH2000 (Perkin-Elmer Cetus Instruments, Emeryville, Calif). PCR primers were chosen on the basis of the published TEM-1[11], bla_{IPM}[12], SHV-1[13] and Toho-1[14] sequences and are listed in Table 1. Bacterial strains of KU2013, 1917, 2017 and 3522, which were stocked at the Department of Microbiology at Kitasato University School of Medicine, were used as positive controls for TEM, bla_{IPM}, SHV-1 and Toho-1. The reaction mixture contained 61.5 μl of H_2O, 10 μl of 10×buffer, 2 μl each of 10 mM dATP, dCTP, dGTP, and dTTP, 0.5 μl of Taq polymerase (5 units/μl), 0.1 μl each of primers 1 and 2 (2 μM), and 10 μl of template DNA. The PCR involved 25 cycles of denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute, and elongation at 72°C for 1 minute, followed by heating at 72°C for 7 minutes. Five μl of the PCR product were subjected to electrophoresis on 1.2% agarose gel to identify the amplified DNA fragment.

Results and Discussion

The *S. marcescens* strain KU3838 that carries the plasmid described here was isolated from a patient with a urinary tract infection in November 1996 in Japan. *E. coli* KU3999 was a transconjugant that served to transfer the conjugative plasmid from *S. marcescens* KU3838 to *E. coli* K12 ML4901. The frequency of transfer by conjugation to *E. coli* K12 ML4901 was about 10^{-5} to 10^{-6}, and resistance to PIPC and IPM was transferred simultaneously as a single genetic unit, indicating that the plasmid-encoded class A and B β-lactamases investigated in this study were transferable. This plasmid was designated pKU501. The incompatibility group of this plasmid was identified as previously described[7]. When a donor pKU501 culture was mixed with a recipient carrying plasmid S-a, which belongs to incompatibility group W, the resistance conferred by S-a was eliminated from the transconjugants by the newly introduced pKU501. In this case, resistance to both PIPC and IPM was expressed in the recipient strain but the TC resistance mediated by S-a was lost. Therefore, class A and B β-lactamase genes were confirmed to be located on the same plasmid, which belonged to incompatibility group W.

The plasmid R9-5, which belongs to the incompatibility group FIV, is transferable to *E. coli* K12 ML4947 by conjugation at a frequency of 10^{-1}. When pKU501 coexisted with R9-5, the frequency of transfer by conjugation of pKU501 was 10 times higher than that of pKU501 alone. These results suggested the possibility of clinical spread of the plasmid-encoded class A and B β-lactamases by conjugation.

Drug resistance of *S. marcescens* KU3838 and the transconjugant strain of *E. coli* KU3999 was measured by determining the minimum inhibitory concentration (MIC) of various drugs as previously described[6] (Table 2). *S. marcescens* KU3838 and *E. coli* KU3999 were resistant to PIPC, cephalosporins and carbapenems. The MICs of PIPC and PIPC/CVA (clavulanic acid) against *E. coli* KU3999 were >128 and 32 μg/ml, respectively, indicating that the MIC was slightly decreased in the presence of CVA, which inhibits class A β-lactamase activity. Recently, clinical isolates possessing class A ESBLs that differ by a few point mutations have been described[15]. These enzymes hydrolyze cephalosporins and monobactam, including CAZ, CTX and AZT, in addition to ABPC and PIPC, but not cefmetazole and cefminox, which belongs to the cephamycin group, or IPM, PAPM and MEPM. The MICs of IPM, PAPM and

Table 2. Minimum inhibitory concentrations (mg/ml) of different antibiotics for *S. marcescens* KU3838, *E. coli* KU3999 and *E. coli* ML4901.

Antibiotic	*S. marcecsens* KU3838	*E. coli* KU3999	*E. coli* ML4901
Piperacillin	>128	>128	4
Piperacillin/ CVA	>128	32	0.5
Cephalotin	>128	>128	0.13
Cefmetazole	>128	>128	0.25
Cefotiam	>128	128	0.03
Cefotaxime	>128	>128	0.13
Cefepime	>128	16	0.13
Imipenem	32	8	0.25
Panipenem	>128	64	0.25
Meropenem	>128	64	0.5
Aztreonam	128	0.25	0.06

CVA, clavulanic acid

Table 3. Hydrolyzing activities of β-lactamases mediated by the pKU501 plasmid.

Substrate	β-Lactamase activity (U/ mg of protein)		
	KU3999	KU3999/ CVA	KU3991
Penicillin G	9.7 (3590)	0.17(68)	0.15 (88)
Piperacillin	6.6 (2440)	<0.01	0.02 (12)
Cephalothin	4.7 (1740)	3.09(1240)	0.10 (58)
Cefotaxime	0.65(240)	0.96(384)	0.05 (29)
Aztreonam	<0.01	<0.01	0.09 (52)
Imipenem	*0.19(100)	*0.20(100)	0.17(100)
Panipenem	*0.26(136)	*0.33(173)	0.07 (41)
Meropenem	*0.35(184)	*0.28(147)	0.03 (17)

( ) indicates the relative rate of enzyme activity, normalized to activity against IPM as 100.
* indicates the average of two separate experiments.
CVA; Clavulanic acid,

MEPM for *E. coli* KU3999 were 8, 64 and 64 μg/ml, respectively. *S. marcescens* KU3838 showed an MIC for AZT of 128 μg/ml, while *E. coli* KU3999 was inhibited at an AZT concentration of 0.25 μg/ml as was the recipient *E. coli* strain ML4901. These MICs suggested that pKU501 carries genes encoding class A and B β-lactamases.

PCR was performed to determine whether pKU501 does carry class A and B β-lactamase genes. PCR primers were chosen on the basis of published sequences and were obtained from Takara Suzo. As the results of PCR, 108 bp and 448 bp PCR products, which were consistent with expected length, were obtained (data not shown). In addition, the enzyme isolated from pKU501 yielded two bands in SDS-polyacrylamide gel electrophoresis, simultaneously. We estimated isoelectric points of 5.4 and 9.2, which were identical to that of TEM type and bla_{IMP} type β-lactamase. These results confirmed that pKU501 carries both TEM type and bla_{IMP} type β-lactamase genes.

Enzyme activity was determined by spectrophotometry (UV2000, Shimadzu Corp.) at 30°C in 50 mM phosphate buffer (pH 7.0) with or without 10 μM $ZnCl_2$, using antibiotics as the substrate. As a positive control, the enzyme activity of *E. coli* KU3991, which produces a plasmid-mediated class B β-lactamase, was also determined. The activities of these β-lactamases are shown in Table 3. The enzyme from *E. coli* KU3999 had a broad substrate profile, hydrolyzed PCG, PIPC and CET frequently, and was inhibited by CVA. Moreover, when enzyme activity was assayed in the presence of CVA, hydrolysis of CTX, IPM, PAPM and MEPM but not of AZT was still observed. These results suggested that pKU501 encodes both class A and B β-lactamases. In addition, the enzyme activities against PAPM and MEPM were higher than against IPM, and substrate profiles of the enzyme encoded by pKU501 were quite different from that of the enzyme produced by *E. coli* KU3991. These results reflected the MICs of IPM, PAPM and MEPM. The results showed that the activity of the enzyme encoded by pKU501 differed from that of the class B β-lactamase reported in another study[5]. From these results, the class B β-lactamase encoded by pKU501 appears to be an extended-spectrum β-lactamase. We are now studying the genetic and molecular properties of pKU501. The DNA sequence analysis will be described elsewhere.

Bacteria have developed many ways to counteract antibiotics, but knowledge of the mechanisms involved yields a variety of strategies to counter antimicrobial resistance. Recently, there have been increasing reports of Gram-negative bacteria carrying the transferable carbapenem resistance gene bla_{IMP}, including *Pseudomonas aeruginosa* and *S. marcescens*, with close genetic relationships of strains demonstrated by pulsed-field gel electrophoresis (PFGE)[16]. This suggested that rates of nosocomial spread may differ between hospitals. The incidence of Gram-negative bacteria carrying bla_{IMP} was high in one hospital[17], but low in another[18]. More rational and appropriate use of antibiotics can reduce the selective pressure for resistance mutations and the spread of resistance plasmids. In addition, controlling nosocomial infection is also very effective to limit to spread of resistance genes in these organisms.

In conclusion, we report a transferable plasmid carrying

genes encoding both class A and B β-lactamases found in a *S. marcescens* clinical isolate. It is important to monitor such strains closely and prevent their spread.

Acknowledgements

We thank SHIZUKO IYOBE, Laboratory of Drug Resistance in Bacteria, Gunma University School of Medicine, for her generous gift of bla_{IMP}.

This work was supported in part by grant-in-aid 09670296 from the Ministry of Science, Education and Culture of Japan.

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