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Molecular Aspects of High-Level Resistance to Sulbactam-Cefoperazone in *Klebsiella oxytoca* Clinical Isolates

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Nine *Klebsiella oxytoca* strains which demonstrated resistance to the combination of sulbactam and cefoperazone were isolated from geographically separate hospitals in Japan in 1995. Among them, *K. oxytoca* SB23 showed high-level resistance to sulbactam-cefoperazone (MIC, >128 μg/ml) and aztreonam (MIC, 128 μg/ml). The sulbactam-cefoperazone resistance was not transferred from strain SB23 to *Escherichia coli* CSH2 by conjugation. β-Lactamase RbiA, produced by strain SB23, was purified, and the molecular mass was estimated to be 29 kDa by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Kinetic parameters for RbiA revealed that cefoperazone and aztreonam were hydrolyzed efficiently by this enzyme. Moreover, ceftazidime and imipenem were also hydrolyzed weakly by RbiA, although strain SB23 did not show any resistance to these agents. Clavulanate, sulbactam, and tazobactam failed to block the hydrolysis of cefoperazone by RbiA. The structural gene of RbiA (bla_{RBI}) was cloned and sequenced, and the deduced amino acid sequence of RbiA demonstrated high-level similarities to those of the β-lactamases found in *K. oxytoca* D488, E23004, and plasmid-mediated MEN-1, which have been classified into Bush functional group 2be. Although RbiA demonstrates high-level molecular similarity to the enzymes in group 2be, from an enzymological point of view, this enzyme might be differentiated from the enzymes in that group. Hybridization analysis revealed that β-lactamase genes highly similar to bla_{RBI} were generally encoded on the chromosome of the sulbactam-cefoperazone-resistant clinical isolates of *K. oxytoca* tested in the study, despite their different derivations. This observation suggests that sulbactam-cefoperazone-resistant *K. oxytoca* strains which produce RbiA-type β-lactamases have been proliferating in many hospitals in Japan.

Clavulanate, sulbactam, and tazobactam are potent suicide inhibitors of serine β-lactamases, most of which belong to Ambler class A or C (1). These inhibitors block β-lactamases through the initial formation of stable acylated compound-enzyme intermediates. Therefore, several combinations of these inhibitors and β-lactams, such as clavulanate-amoxicillin, sulbactam-ampicillin, sulbactam-cefoperazone, and tazobactam-piperacillin, have been developed for clinical use, and they have opened a new era in the chemotherapy of infectious diseases caused by gram-negative bacteria producing β-lactamases. However, several clinical isolates demonstrating resistance to these combinations have emerged (29, 32, 33), and these were found to produce mainly TEM-related β-lactamases such as TEM-30 to TEM-41 (6, 7, 9, 13, 20, 32), which have been classified into the functional group 2br by Bush et al. (10). *Klebsiella oxytoca* strains producing large amounts of chromosomally encoded K1-type β-lactamase were also reported to show resistance to these β-lactamase inhibitors (33), although the resistance level was not as high as those of β-lactamases belonging to group 2br. On the other hand, these β-lactamase inhibitors were also reported to be ineffectual against the clinical isolates producing metallo-β-lactamases which belong to functional group 3 or molecular class B (18, 21, 23–25). Thus, the mechanisms of resistance to β-lactamase inhibitors have been getting more complicated. Under these circumstances, we recently isolated several strains of *K. oxytoca* demonstrating high-level resistance to sulbactam-cefoperazone from separate hospitals in Japan. In the present study, we investigated the mechanism underlying the high-level sulbactam-cefoperazone resistance found in clinical isolates of *K. oxytoca*.

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MATERIALS AND METHODS

Bacterial strains and plasmids. *K. oxytoca* SB23 was isolated from a patient who suffered from a urinary tract infection. All other cefoperazone-sulbactam-resistant *K. oxytoca* strains tested were also isolated from urine samples from different patients. *Escherichia coli* CSH2 was used as the recipient of a transferable large plasmid in the conjugation analysis. *E. coli* HB101 was used as the cloning host for a β-lactamase gene, and *E. coli* JM109 was used to amplify M13 phage for DNA sequencing. A plasmid vector, pMK16, that carries tetracycline and kanamycin resistance genes was used as a vector for DNA cloning, and M13 mp18 phage (33) was used for DNA sequencing by Sanger's method. The bacterial strains, plasmids, and bacteriophages used in the study are listed in Table 1. The clinical associations of nine sulbactam-cefoperazone-resistant *K. oxytoca* isolates tested by Southern hybridization analysis are also listed in Table 2, together with the MICs of five β-lactams for these isolates.

Reagents and medium components. The restriction endonucleases, exonuclease III, mung bean nuclease, Klenow's fragment, and T4 DNA ligase used in the study were purchased from Nippon Gene Co. Ltd., Toyama, Japan. A nucleotide sequencing kit was from Applied Biosystems Inc., Foster City, Calif. The following antibiotics were purchased from the indicated companies: ampicillin and kanamycin, Meijiseika Kaishya Ltd., Tokyo, Japan; cephaloridine and ceftazidime, Glaxo Group Research, Greenford, United Kingdom; cefazolin and ceftizoxime, Fujisawa Pharmaceutical Co., Ltd., Osaka, Japan; cefmenoxime, Takeda Pharmaceutical Co., Ltd., Osaka, Japan; cefotetan, Yamanouchi Pharmaceutical Co., Ltd., Tokyo, Japan; cefoperazone, Toyama Chemical Co., Tokyo, Japan; cefotaxime, Farbwerke Hoechst AG, Frankfurt, Germany; aztreonam, Eizai Co., Ltd., Tokyo, Japan; moxalactam, Shionogi and Co., Ltd., Osaka, Japan; sulbac-

TABLE 1. Bacterial strains, phages, and plasmids used in the study

Bacterial strain, phage, or plasmid	Characteristics	Source orreference
Strains		
Klebsiella oxytoca SB23, SB43, SB48, SB64, SB79, SB82, SB90, SB92, and SB95	Sulbactam-cefoperazone-resistant strains isolated from urine samples in Japan in 1995	This study
K. oxytoca E23004	A cefoperazone-resistant laboratory strain	4
E. coli HB101	F^- *hsdS20* (r^-, m^-) *recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1 supE44* λ^-	30
E. coli JM109	*recA1* Δ(*lac pro*) *endA1 gyrA96 thi-1 hsdR*$^-$ *supE44 relA1* F′(*traD36 proA*$^+$*B*$^+$ *lacI*^{q} *Z*Δ*M15*)	30
E. coli CSH2	*metB* F^- NA^r, Rif^r	T. Sawai, Chiba University
Plasmids and phages		
pMK16	Cloning vector; Km^r Tc^r	4
M13 mp18	Vector for DNA sequencing	34
pKOB23	Recombinant plasmid encoding bla_{RBI} of *K. oxytoca* SB23	This study
pKOBMP602, pKOBMP606	Recombinant phages carrying the bla_{RBI} used for DNA sequencing	This study

tam-cefoperazone and sulbactam, Pfizer Pharmaceuticals Inc., Tokyo, Japan; imipenem, Banyu Pharmaceutical Co., Ltd., Tokyo, Japan; meropenem, Sumitomo Pharmaceuticals Ltd., Osaka, Japan; clavulanate, SmithKline Beecham Pharmaceuticals, Surrey, United Kingdom; and tazobactam, Taiho Pharmaceutical Co., Ltd., Tokyo, Japan.

Susceptibility testing. MICs were determined according to a guideline of the National Committee for Clinical Laboratory Standards (22) by the agar dilution method with Mueller-Hinton II agar (BBL Microbiology Systems, Cockeysville, Md.) plates containing serially diluted antibiotics. A 5-μl aliquot of bacterial culture (approximately 10^6 CFU/ml) grown to the logarithmic phase in Mueller-Hinton broth (BBL) was inoculated onto the agar plates, and the plates were incubated at 37°C for 18 h for determination of the MIC of each agent.

DNA manipulation and cloning of β-lactamase gene. Manipulation of DNA was carried out by the method outlined by Sambrook et al. (30). Chromosomal DNA from *K. oxytoca* SB23 was digested with the restriction endonuclease *Eco*RI, and the fragments were ligated into the *Eco*RI site of the plasmid vector pMK16 with T4 DNA ligase. The ligated DNAs were then introduced into competent cells of *E. coli* HB101. Several transformants that were resistant to both kanamycin (50 μg/ml) and ampicillin (50 μg/ml) were isolated, and it was found that these transformants harbored a recombinant plasmid, pKOB23, carrying the gene responsible for sulbactam-cefoperazone resistance. For nucleotide sequencing, plasmid pKOB23 was partially digested with the restriction endonuclease *Sau*3AI and was then ligated into the *Bam*HI site of the M13 bacteriophage mp18. Two phage subclones, pKOBMP602 and pKOBMP606, were used for DNA sequencing, since they carried the same insert, but in opposite orientations. The M13 phage subclones were cleaved with *Sph*I and *Sal*I, and the fragments were deleted with exonuclease III and mung bean nuclease. Then the deleted fragments were ligated with T4 DNA ligase after trimming both terminals with Klenow's fragment. DNA sequencing was performed by Sanger's method with a dye primer (−21M13 Dye Primer) and a DNA sequencer (model 373A; Applied Biosystems, Inc.).

Purification of β-lactamase. For purification of β-lactamase, *E. coli* HB101 harboring plasmid pKOB23 carrying bla_{RBI} was cultured for 18 h in 1 liter of Luria-Bertani (LB) broth supplemented with 50 μg of kanamycin per ml. The bacterial cells were harvested by centrifugation (5,000 × *g*, 15 min, 4°C) and were suspended in 10 ml of 100 mM phosphate buffer (pH 7.0). The bacterial suspension was frozen at −80°C for 30 min and thawed at 30°C. This step was repeated twice, and then the suspension was subjected to ultracentrifugation (50,000 × *g*, 1 h, 4°C). The clear supernatant was chromatographed through DEAE-Sephacel equilibrated with 100 mM phosphate buffer (pH 7.0) to remove intermixed proteins, since β-lactamase molecules were not trapped by the DEAE-Sephacel under this condition. For further purification, 20 ml of crude enzyme preparation was condensed to 2 ml by ultrafiltration with Centricut (Kurabou Co., Ltd., Tokyo, Japan) and was then diluted with 15 ml of 50 mM MOPS (morpholine propanesulfonic acid) buffer (pH 6.0). The enzyme solution was chromatographed again through a HiTrap SP column with high-pressure liquid chromatography system (Pharmacia Biotechnology, Uppsala, Sweden). Under these conditions, the β-lactamase molecules were trapped by the column. Elution of the β-lactamase molecules was carried out with 50 mM MOPS buffer (pH 6.0) with 110 mM NaCl.

Enzymological analyses. The crude RbiA preparation was used for enzymological analyses. Enzymatic activities against various β-lactam substrates were detected spectrophotometrically at respective wavelengths in 100 mM sodium phosphate buffer (pH 7.0) at 30°C. The absorption maxima of the substrates used were as follows: ampicillin, 235 nm; cephaloridine, 295 nm; cefoperazone, 276 nm; cefotaxime, 264 nm; ceftazidime, 272 nm; cefotetan, 285 nm; moxalactam, 270 nm; aztreonam, 315 nm; and imipenem, 297 nm. Relative V_{max} and relative V_{max}/K_m values were calculated as recommended by Bush and Sykes (11). The K_i value of each inhibitor was determined with cefoperazone as the substrate. The enzyme and increasing concentrations of inhibitors were preincubated for 10 min at 30°C before the addition of cefoperazone to a final concentration of 100 μM. Standard Dixon plots (1/*vi* versus *I*, where *vi* is reaction velocity under coexistence of inhibitor and *I* is concentration of inhibitor) were prepared, and the apparent K_i was calculated.

Hybridization analyses. Total DNA preparations of 10 sulbactam-cefoperazone-resistant strains were digested with *Eco*RI, and the digests were blotted

TABLE 2. MICs of β-lactams for cefoperazone-sulbactam-resistant clinical *K. oxytoca* isolates from urine

K. oxytoca strains	Hospital[a]	Source	MIC (μg/ml)[b]				
			ABPC	CER	CPZ-SBT[c]	MOX	ATM
SB23	Kanagawa 3	Inpatient	>128	128	>128	<0.5	128
SB43	Tokyo 3	Inpatient	>128	>128	128	<0.5	2
SB48	Saitama C	Inpatient	>128	>128	>128	<0.5	16
SB64	Hokkaidou A	Outpatient	>128	>128	>128	<0.5	>128
SB79	Chiba F	Inpatient	>128	>128	>128	<0.5	128
SB82	Kanagawa 44	Inpatient	>128	>128	>128	<0.5	64
SB90	Nagano D	Outpatient	>128	>128	>128	<0.5	16
SB92	Kanagawa 39	Inpatient	>128	>128	>128	<0.5	128
SB95	Kanagawa 39	Outpatient	>128	>128	>128	<0.5	128

[a] Giving our careful consideration for each hospital from which the sulbactam-cefoperazone-resistant strains were isolated, the hospital names were substituted by their locations and a letter or number designation.

[b] Abbreviations: ABPC, ampicillin; CER, cephaloridine; CPZ, cefoperazone; SBT, sulbactam; MOX, moxalactam; ATM, aztreonam.

[c] CPZ-SBT (128 μg/ml) corresponds to the combination of 64 μg of cefoperazone per ml and 64 μg of sulbactam per ml.

TABLE 3. MICs of β-lactams for *K. oxytoca* SB23, *E. coli* HB101(pKOB23), *E. coli* HB101, and *K. oxytoca* E23004

Strain	MIC (μg/ml)[a]													
	ABPC	PIPC	CER	CEZ	CPZ	CTX	CZX	CMX	CAZ	MOX	CTT	ATM	IMP	MEPM
K. oxytoca SB23	>128	>128	128	>128	>128	4	<0.5	8	<0.5	<0.5	<0.5	128	<0.5	<0.5
E. coli HB101(pKOB23)	>128	>128	>128	>128	>128	8	2	16	<0.5	<0.5	<0.5	>128	1	<0.5
E. coli HB101	4	<0.5	4	1	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
K. oxytoca E23004	>128	>128	>128	>128	128	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	2	<0.5	<0.5

[a] Abbreviations: PIPC, piperacillin; CEZ, cefazolin; CTX, cefotaxime; CZX, ceftizoxime; CMX, cefmenoxime; CAZ, ceftazidime; CTT, cefotetan; IMP, imipenem; MEPM, meropenem. The other abbreviations are defined in footnote *b* of Table 2.

onto a nylon membrane (Hybond-N; Amersham, Buckinghamshire, United Kingdom) after agarose gel electrophoresis. Southern hybridization was performed with an [α-^{32}P]dCTP-labeled *Nhe*I-*Bgl*I fragment of pKOB23 as a hybridization probe under the conditions described previously (3).

Database analyses. DNA and amino acid sequences were obtained from databases (EMBL, GenBank, and SWISS-PROT) and were analyzed by using the SDC-GENETYX system (Software Development Co., Tokyo, Japan).

Nucleotide sequence accession number. The nucleotide sequence data reported here will appear in the DNA Data Bank of Japan, EMBL, and GenBank nucleotide sequence databases under accession number D84548.

RESULTS

Cloning of bla_{RIB} and restriction map. An *E. coli* HB101 transformant grew on an LB agar plate supplemented with 50 μg of both ampicillin and kanamycin per ml and harbored a plasmid, pKOB23, that carried a 5-kb *Eco*RI fragment. This transformant demonstrated high-level resistance to sulbactam-cefoperazone (MIC, >512 μg/ml) (Table 3). By deletion analyses, the gene, bla_{RBI}, responsible for sulbactam-cefoperazone resistance was localized in the 5-kb *Eco*RI fragment, as shown in Fig. 1.

MICs of β-lactams. All the strains tested in the study demonstrated high-level resistance to sulbactam-cefoperazone (MICs, ≥128 μg/ml) (Table 2); however, their resistance levels to aztreonam were diverse. *K. oxytoca* SB23 demonstrated high-level resistance to both sulbactam-cefoperazone (MIC, >128 μg/ml) and aztreonam (MIC, 128 μg/ml), while this strain was susceptible to the other broad-spectrum β-lactams such as cefotaxime, ceftizoxime, and moxalactam (Table 4). Strain SB23 was able to grow on an LB agar plate containing both 16 μg of clavulanate per ml and 16 μg of cefoperazone per ml or on an agar plate containing both 64 μg of tazobactam per ml and 64 μg of cefoperazone per ml (Table 4). Although *K. oxytoca* E23004, which was formerly identified as a cefoperazone-resistant strain, also showed similar properties against the inhibitors mentioned above, *K. oxytoca* SB23 was able to grow in the presence of higher concentrations of these suicide inhibitors than strain E23004 (Table 4). Among the three inhibitors, clavulanate had the strongest inhibitory effects on the growth of strains SB23 and E23004. *E. coli* HB101(pKOB23) also showed β-lactam susceptibility profiles similar to those of strains SB23 (Table 3).

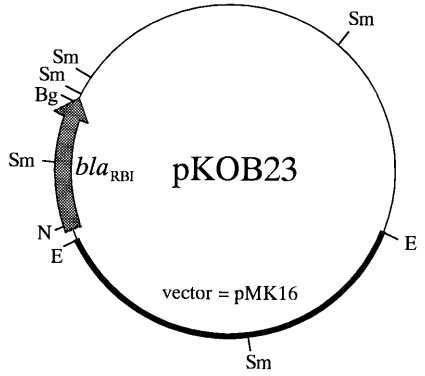

FIG. 1. Restriction map of pKOB23. The position of the bla_{RBI} gene responsible for sulbactam-cefoperazone resistance was localized near a *Sma*I restriction site in the 5-kb *Eco*RI insert. The thin line represents the 5-kb *Eco*RI insert, and the thick line indicates the cloning vector pMK16. Abbreviations: Bg, *Bgl*I; E, *Eco*RI; N, *Nhe*I; Sm, *Sma*I.

Purification of β-lactamase and its molecular mass. The β-lactamase RbiA was purified by the method described in Materials and Methods. The purity of this enzyme was estimated to be greater than 95%, since a single clear band appeared with Coomassie brilliant blue staining after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (data not shown). The molecular mass of the mature RbiA was estimated to be 29 kDa.

Kinetic parameters. RbiA hydrolyzed cefoperazone as efficiently as it hydrolyzed ampicillin and cephaloridine (Table 5). The hydrolysis of aztreonam was similar to that of cefotaxime, although the MIC of cefotaxime for strain SB23 was very low compared with that of aztreonam (Table 3). The K_m for aztreonam was relatively higher than that for cefoperazone, while the V_{max} for aztreonam was lower than that for cefoperazone. Hydrolysis rates (V_{max}/K_m) for ceftazidime and imipenem were lower than those for cefoperazone and aztreonam. In contrast, moxalactam and cefotetan were hardly hydrolyzed by RbiA. The K_m value for cefoperazone reveals that this agent binds well to RbiA, and it is hydrolyzed immediately when it is trapped by RbiA. The K_i of RbiA for sulbactam was 41.6 μM, and this value was relatively higher than those of the other β-lactamases belonging to group 2be. Among the three inhibitors, clavulanate and tazobactam behaved as the most effective inhibitors against RbiA. The K_i values for these inhibitors are also given in Table 5.

Nucleotide sequence and deduced amino acid sequence. A 1,020-bp nucleotide sequence determined in the present study is provided in Fig. 2, together with the deduced amino acid

TABLE 4. MICs of cefoperazone and β-lactamase inhibitor combinations

Strain	MIC (μg/ml) of β-lactam and β-lactamase inhibitor combinations[a]		
	CPZ + CA	CPZ + SBT	CPZ + TBT
K. oxytoca SB23	32 + 32	256 + 256	128 + 128
E. coli HB101 (pKOB23)	32 + 32	>256 + >256	>256 + >256
E. coli HB101	<0.25 + <0.25	<0.25 + <0.25	<0.25 + <0.25
K. oxytoca E23004	4 + 4	128 + 128	32 + 32

[a] CPZ, cefoperazone; CA, clavulanate; SBT, sulbactam; TBT, tazobactam.

TABLE 5. Kinetic parameters of RbiA for various β-lactams

Substrate	Relative V_{max}	K_m or K_i (μM)	Relative V_{max}/K_m
Ampicillin	100	143	100
Cephaloridine	51.8	120	61.8
Cefoperazone	0.475	0.422	161
Cefotaxime	6.69	516	1.85
Ceftazidime	1.72	118,000	0.0000208
Cefotetan	<0.01	NC[a]	NC
Moxalactam	<0.01	NC	NC
Aztreonam	33.3	967	4.93
Imipenem	0.0219	141	0.000221
Clavulanate		1.34[b]	
Sulbactam		41.6[b]	
Tazobactam		1.07[b]	

[a] NC, not calculated.
[b] Values are shown as K_i.

sequence of the gene product. A sequence similar to the −35 and −10 promoter of the *K. oxytoca* β-lactamase genes (4, 12) was found in the upstream region of the putative coding frame of bla_{RBI}. A possible Shine-Dalgarno sequence (AAGGA) corresponding to the 3′ terminus of *E. coli* 16S rRNA (3′-tAAG GAggtgatc---) was also identified adjacent to the putative initiation codon (ATG), and the coding frame was terminated by a TAA codon. The G+C content in the coding region was 56.33%. RbiA consisted of 289 amino acid residues, including the signal peptide, and its molecular mass was calculated to be 31,065 Da.

Southern hybridization analysis. The DNA probe that originated from the intergenic region of bla_{RBI} hybridized to the chromosomal positions on the blot in all the sulbactam-cefoperazone-resistant strains of *K. oxytoca* tested in the present study (Fig. 3A). Moreover, the same probe generally hybridized to the position of the 5-kb *Eco*RI fragment in eight of nine isolates, while the hybridization signal in SB48 was detected in a little higher position than the hybridization signals in the other eight strains (Fig. 3B). This observation suggests that the nucleotide sequences of β-lactamase genes and their flanking regions are highly conserved among the *K. oxytoca* strains tested except strain SB48.

Database analyses. The nucleotide sequence of bla_{RBI} determined in the present study was compared with those of various β-lactamase genes which have been deposited in the EMBL or GenBank database. The nucleotide sequence of bla_{RBI} showed significant homologies to the β-lactamase genes

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                                                    -35
TTATCAATATAGGAGCATAGAATTCCATCATCAATAAATTGCTTGTCAAAATAGCGGGAG   60
          -10
TCGCAGATAATCCGCTGCGACTTATCACTCTCAAGGAATCAGAAATGATAAAAAGTTCGT  120
                                S.D.      M  I  K  S  S  W
GGCGTAAAATTGCAATGCTAGCCGCCGTTCCGCTGCTGCTGGCGAGCGGCGCACTGTGGG  180
 R  K  I  A  M  L  A  A  V  P  L  L  L  A  S  G  A  L  W  A
CCAGTACCGATGCTATCCATCAGAAGCTGACAGATCTCGAGAAGCGTTCAGGCGGCAGGT  240
 S  T  D  A  I  H  Q  K  L  T  D  L  E  K  R  S  G  G  R  L
TGGGCGTGGCGCTAATCAACACGGCAGATAATTCTCAAATCTTATATCGCGGCGACGAGC  300
 G  V  A  L  I  N  T  A  D  N  S  Q  I  L  Y  R  G  D  E  R
GTTTTGCCATGTGCAGCACCAGTAAAGTGATGGCCGCCGCCGCGGTATTAAAACAGAGCG  360
 F  A  M  C  S  T  S  K  V  M  A  A  A  A  V  L  K  Q  S  E
AAAGCAATAAAGAGGTGGTAAATAAAAGGCTGGAGATTAACGCAGCCGATTTGGTGGTCT  420
 S  N  K  E  V  V  N  K  R  L  E  I  N  A  A  D  L  V  V  W
GGAGTCCGATTACCGAAAAACATCTCCAGAGCGGAATGACGCTGGCTGAGCTAAGCGCGG  480
 S  P  I  T  E  K  H  L  Q  S  G  M  T  L  A  E  L  S  A  A
CGACGCTGCAATATAGCGACAATACGGCGATGAATCTGATCATCGGCTACCTTGGCGGGC  540
 T  L  Q  Y  S  D  N  T  A  M  N  L  I  I  G  Y  L  G  G  P
CGGAAAAAGTCACCGCCTTCGCCCGCAGTATCGGCGATGCCACCTTTCGTCTCGATCGTA  600
 E  K  V  T  A  F  A  R  S  I  G  D  A  T  F  R  L  D  R  T
CGGAGCCCACGCTGAATACCGCCATCCCGGGCGATGAGCGTGATACCAGCACGCCGCTGG  660
 E  P  T  L  N  T  A  I  P  G  D  E  R  D  T  S  T  P  L  A
CGATGGCTGAAAGCCTACGCAAGCTGACGCTTGGCGATGCGCTGGGCGAACAGCAACGCG  720
 M  A  E  S  L  R  K  L  T  L  G  D  A  L  G  E  Q  Q  R  A
CCCAGTTAGTCACCTGGCTGAAAGGCAATACCACCGGCGGGCAAAGCATTCGCGCGGGCC  780
 Q  L  V  T  W  L  K  G  N  T  T  G  G  Q  S  I  R  A  G  L
TGCCTGAAAGCTGGGTGGTCGGCGATAAAACCGGCGCCGGAGATTACGGCACCACCAATG  840
 P  E  S  W  V  V  G  D  K  T  G  A  G  D  Y  G  T  T  N  D
ATATTGCGGTTATCTGGCCGGAAGATCACGCTCCGCTGGTATTAGTCACCTACTTTACCC  900
 I  A  V  I  W  P  E  D  H  A  P  L  V  L  V  T  Y  F  T  Q
AGCCGCAGCAGGATGCGAAAAACCGCAAAGAGGTGTTAGCCGCAGCGGCAAAAATCGTGA  960
 P  Q  Q  D  A  K  N  R  K  E  V  L  A  A  A  A  K  I  V  T
CCGAAGGGCTTTAAGCCGGGAACAGGCCCGTTCTGCAACACTCTGCACTCTGCTCCCGGG 1020
 E  G  L  ***
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FIG. 2. Nucleotide sequence of bla_{RBI} and its flanking regions. A 1,020-bp sequence determined in the present study is presented, and this sequence has been deposited in the EMBL and GenBank nucleotide sequence data banks through the DNA Data Bank of Japan (accession number D84548). The sequences 42-TTGTCA and 66-GATAAT), similar to that of the −35 and −10 promoter, are underlined, and the possible Shine-Dalgarno sequence (S.D.) is marked with a broken underline. The start and termination codons of bla_{RBI} are the ATG and TAA codons at positions 105 and 972, respectively. The deduced amino acid sequence of RbiA is indicated with the single-letter code below the nucleotide sequence.

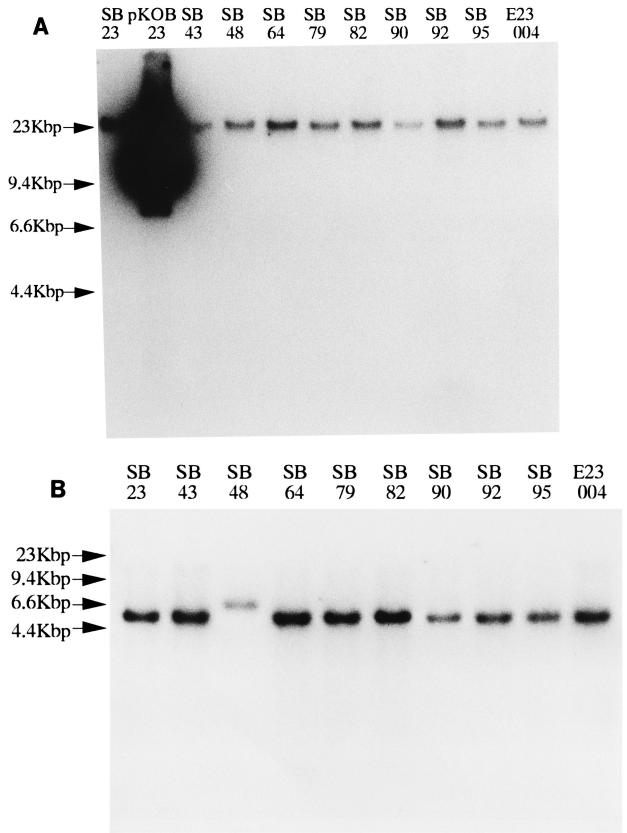

FIG. 3. Southern hybridization. Total DNA preparations from 10 sulbactam-cefoperazone-resistant *K. oxytoca* clinical isolates were subjected to agarose gel electrophoresis with (B) or without (A) digestion with *Eco*RI before blotting onto the membrane. The *Nhe*I-*Bgl*I fragment in the coding region of bla_{RBI} was used as a DNA probe. Hybridizations to the chromosomal positions detected in all strains tested are presented in panel A. Hybridizations to the 5-kb *Eco*RI fragments observed in 9 of 10 tested strains are presented in panel B. The hybridization signal to SB48 was detected at a position a little higher than those to the other strains.

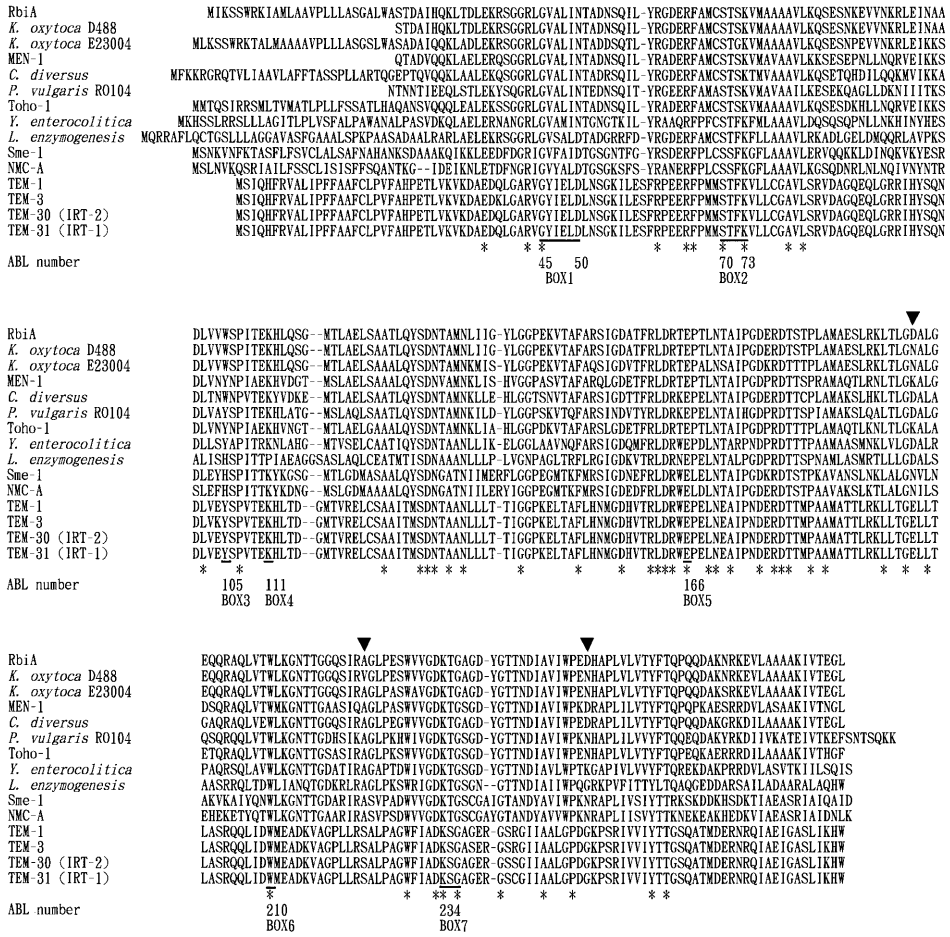

FIG. 4. Multiple alignment of amino acid sequences of 15 class A β-lactamases. Asterisks indicate the conserved amino acid residues among all 15 sequences compared. Dashes indicate gaps that were inserted to optimize the alignment. The positions of three amino acid substitutions observed between RbiA and the β-lactamase from *K. oxytoca* D488 are indicated by the arrowheads above the alignment. The seven boxes described by Joris et al. (19) are underlined. Amino acid residues are numbered according to a standard numbering scheme for class A β-lactamases recommended by Ambler et al. (2) (ABL). Boxes: BOX1, ABL 45 to 50; BOX2, ABL 70 to 73; BOX3, ABL 105; BOX4, ABL 111; BOX5, ABL 166; BOX6, ABL 210; BOX7, ABL 234 to 236. The following enzymes have been described previously: SCR-1 (this study), *K. oxytoca* D488 (28), *K. oxytoca* E23004 (4), MEN-1 (5), *Citrobacter diversus* (27), Toho-1 (17), *P. vulgaris* RO104 (26), *Yersinia enterocolitica* (31), *Lysobacter enzymogenesis* (8), Sme-1 (SWISS-PROT accession number Z28968); NMC-A (SWISS-PROT accession number Z21956), TEM-1 and TEM-3 (SWISS-PROT accession number P00810), TEM-30 (6), and TEM-31 (6).

found in *K. oxytoca* strains such as E23004 (4) and D488 (28) and those of plasmid-mediated MEN-1 (5) and Toho-1 (17), which were carried by clinical *E. coli* isolates. The multiple alignments of amino acid sequences among 15 class A β-lactamases are provided in Fig. 4, and the overall amino acid sequence of RbiA was highly similar to those of the β-lactamases mentioned above (Fig. 4). In particular, only three amino acid substitutions (a D to an N at position 191 [D_{191} to N_{191}], A_{223} to V_{223}, and D_{254} to N_{254}) were found between RbiA and the β-lactamase found in *K. oxytoca* D488, which has been reported to be blocked by clavulanate (28). Interestingly, all these substitutions were located outside of the domains constituting the active centers of these enzymes (14).

DISCUSSION

β-Lactamases which are not inhibited by clavulanate are classified into the functional group 2br, and these are mostly TEM-related enzymes such as TEM-30 to TEM-41 (10, 32). It was also reported that K1-type β-lactamase hyperproducers of *K. oxytoca* tend to demonstrate low-level resistance to these β-lactamase inhibitors (33). In the present study, we found that RbiA does not belong to group 2br but demonstrates remarkable similarity to the K1 type β-lactamases from *K. oxytoca* and plasmid-mediated MEN-1 belonging to group 2be, although they were reported to be blocked by clavulanate. Recently, a plasmid-mediated β-lactamase, Toho-1, demonstrating a high-level similarity to the β-lactamases from *K. oxytoca* (4, 28) and *Proteus vulgaris* (26) was also reported to be blocked by clavulanate (K_i, 0.6 μM) (17). In the present study, however, *K. oxytoca* E23004, which produces β-lactamase KOXY, was able to grow on the agar plate containing β-lactamase inhibitors (Table 4). Indeed, it was reported that several *K. oxytoca* strains with high-level K1 β-lactamase production were resistant to cefoperazone in the presence of a low concentration of sulbactam (2 μg/ml) (33), but in the present study SB23 was able to grow in the presence of high concentrations of both sulbactam (128 μg/ml) and cefoperazone (128 μg/ml). The K_i value of RbiA for sulbactam (41.6 μM) was relatively higher than those of the other β-lactamases belonging to group 2be. This reveals that both low binding affinities of sulbactam to RbiA and the intrinsic high hydrolytic activity of RbiA against cefoperazone are implicated in the high-level resistance to

sulbactam-cefoperazone of SB23. The K_i of RbiA for clavulanate was calculated to be 1.34 μM, while those of the other K1-type β-lactamases were much lower than that. From the viewpoint of the kinetics of RbiA for β-lactamase inhibitors, this enzyme might be differentiated from the functional group 2be β-lactamases, despite the considerable similarities of their amino acid sequences. Further comparative analyses of enzymatic properties among related β-lactamases should be undertaken.

The kinetic constant of RbiA for sulbactam reveals that this inhibitor has a low binding affinity to RbiA (K_i, 41.6 μM). The K_i of RbiA for clavulanate was determined to be 1.34 μM, while that of the β-lactamase from D488 was 0.2 μM (28). Interestingly, only three amino acid substitutions were observed between the RbiA and the β-lactamase produced by strain D488. All three substitutions were located close to critical and mostly conserved regions of β-lactamases, although these regions do not constitute the active site of the enzyme. It is not clear so far whether these substitutions affect the kinetics of RbiA. In contrast, the sequences of amino acid residues implicated in the construction of the active center of the enzyme were completely conserved between RbiA and D488 β-lactamases (Fig. 4). Similar observations have also been reported for the amino acid sequences between the group 2b β-lactamases such as TEM-1 and the group 2br β-lactamases, including TEM-30 to TEM-41, which were hardly blocked by clavulanate (13, 32). These observations suggest that the tertiary structure of the active center of β-lactamases might be affected through distortion of the overall conformation of the enzyme caused by the amino acid substitutions located close to critical and mostly conserved residues. The amino acid residues responsible for the high-level sulbactam-cefoperazone resistance in RbiA will be specified by point mutagenesis of bla_{RBI}.

Among the 10 strains of sulbactam-cefoperazone-resistant *K. oxytoca* tested, including strain E23004, the aztreonam susceptibility profiles of each strain appeared to be divergent. For instance, strains SB23, SB64, SB79, SB92, and SB95 were highly resistant to aztreonam, whereas strains E23004 and SB43 were susceptible to this agent. The susceptibility profiles of these strains for moxalactam lacked consistency with those for aztreonam and sulbactam-cefoperazone. This may be chiefly due to the level of β-lactamase production by each strain. Moreover, the substrate specificity of the β-lactamase produced by each strain may also reflect upon the MICs of the β-lactams, although these isolates carry β-lactamase genes highly similar to bla_{RBI}. For the β-lactamase from D488, the 50% inhibitory concentration of aztreonam was reported to be high (28). Further characterization of the mechanisms underlying the different levels of aztreonam resistance among the *K. oxytoca* strains will be continued.

K. oxytoca E23004 was isolated as a cefoperazone-resistant strain in 1980. After that, strains with similar susceptibility profiles were rarely isolated in Japan. This may be because of the development and clinical use of broad-spectrum cephems and carbapenems. Recently, however, the frequency of isolation of sulbactam-cefoperazone-resistant *K. oxytoca* strains has been increasing. Decreased use of broad-spectrum cephems in Japan for the prevention of nosocomial outbreaks of methicillin-resistant *Staphylococcus aureus* infections might have something to do with this phenomenon. Southern hybridization analysis suggests that the nucleotide sequences of β-lactamase genes and their flanking sequences are highly conserved among the *K. oxytoca* strains tested. This observation reveals the clonal proliferation of strains with similar genetic backgrounds in geographically separate hospitals in Japan. Moreover, we speculate that chromosomally encoded bla_{RBI} will be able to be translocated onto some plasmids in the near future, because several plasmid-mediated β-lactamase genes which probably originated from the chromosome of unknown microorganisms have been identified worldwide (5, 15–18). Thus, we should give careful consideration to the trend of the greater frequency of isolation of sulbactam-cefoperazone-resistant clinical isolates.

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