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Cloning and Sequencing of the Gene Encoding Toho-2, a Class A β-Lactamase Preferentially Inhibited by Tazobactam

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***Escherichia coli* TUM1083, which is resistant to ampicillin, carbenicillin, cephaloridine, cephalothin, piperacillin, cefuzonam, and aztreonam while being sensitive to cefoxitin, moxalactam, cefmetazole, ceftazidime, and imipenem, was isolated from the urine of a patient treated with β-lactam antibiotics. The β-lactamase (Toho-2) purified from the bacteria hydrolyzed β-lactam antibiotics such as penicillin G, carbenicillin, cephaloridine, cefoxitin, cefotaxime, ceftazidime, and aztreonam and especially had increased relative hydrolysis rates for cephalothin, cephaloridine, cefotaxime, and ceftizoxime. Different from other extended-spectrum β-lactamases, Toho-2 was inhibited 16-fold better by the β-lactamase inhibitor tazobactam than by clavulanic acid. Resistance to β-lactams was transferred by conjugation from *E. coli* TUM1083 to *E. coli* ML4909, and the transferred plasmid was about 54.4 kbp, belonging to the incompatibility group IncFII. The cefotaxime resistance gene for Toho-2 was subcloned from the 54.4-kbp plasmid. The sequence of the gene was determined, and the open reading frame of the gene was found to consist of 981 bases. The nucleotide sequence of the gene (DDBJ accession no. D89862) designated as bla_{toho} was found to have 76.3% identity to class A β-lactamase CTX-M-2 and 76.2% identity to Toho-1. It has 55.9% identity to SHV-1 β-lactamase and 47.5% identity to TEM-1 β-lactamase. Therefore, the newly isolated β-lactamase designated as Toho-2 produced by *E. coli* TUM1083 is categorized as an enzyme similar to Toho-1 group β-lactamases rather than to mutants of TEM or SHV enzymes. According to the amino acid sequence deduced from the DNA sequence, the precursor consisted of 327 amino acid residues. Comparison of Toho-2 with other β-lactamase (non-Toho-1 group) suggests that the substitutions of threonine for Arg-244 and arginine for Asn-276 are important for the extension of the substrate specificity.**

β-Lactam antibiotics are widely used as front line agents in the clinical field. In the early 1980s, expanded-spectrum β-lactams, with stability for β-lactamase and good activity against gram-negative bacteria, were first used in the clinical setting. Not long after the beginning of wide use of the expanded-spectrum β-lactams, extended-spectrum β-lactamases were isolated in Europe and the United States and now have become a serious problem in the clinical field (29). In the late 1980s and early 1990s, those enzymes hydrolyzing the expanded-spectrum β-lactams were generally derived from TEM- or SHV-type β-lactamases through several mutations (6, 24). The mutations of Glu-104, Arg-164, and Glu-240 have been suggested to be important for the spectrum expansion (16, 24). In more recent years, non-TEM- or non-SHV-type β-lactamases such as Toho-1 (13), CTX-M-2 (5), and MEN-1 (3) have been identified. Those β-lactamases have high homology to the chromosomally encoded β-lactamase of *Proteus vulgaris* or *Klebsiella oxytoca* (2, 9, 23). In most cases, the β-lactamase-producing organisms show resistance to expanded-spectrum β-lactams such as cefotaxime and ceftazidime (6, 24). On the other hand, they are susceptible to carbapenems such as imipenem (6, 24). The main characteristic of those class A β-lactamases, except TEM-30 to TEM-40, is that they are sensitive to β-lactamase inhibitors such as clavulanic acid, sulbactam, and tazobactam (6). The reaction mechanism and the amino acid residues associated with the spectrum expansion of β-lactamases are still under investigation. Ishii et al. (13) proposed that mutations at positions 244 and 276 are important for the substrate extension after performing sequence alignment of Toho-1 and other β-lactamases.

The expanded-spectrum β-lactam-resistant strains isolated from several hospitals were surveyed and collected. We investigated those strains by enzymological and molecular biological methods and focused upon *Escherichia coli* TUM1083, a cefotaxime-resistant clinical isolate.

In this report, we discuss a correlation between the mutation and the substrate specificity of the β-lactamase from *E. coli* TUM1083 based on the sequence alignment and a three-dimensional structure of a related β-lactamase of *Bacillus licheniformis* (17).

MATERIALS AND METHODS

Bacterial strains and plasmids. Table 1 shows the bacterial strains and plasmids used in this study. *E. coli* TUM1083 was isolated in March 1995 from the urine of a 69-year-old male who suffered from colon cancer. Before strain isolation, the patient had received an empiric antibiotic treatment consisting of a combination of piperacillin, cefuzonam, cefotiam, imipenem, levofloxacin, and tosulfloxacin. The strain did not ferment lactose but was identified as *E. coli* by tests with API 20E (Asuka, Tokyo, Japan) and the Vitek system (bioMerieux Vitek, Inc., Hazelwood, Mo.). *E. coli* ML4909, used for plasmid conjugation, was provided by Matsuhisa Inoue of Kitasato University. *E. coli* AS226 (13) was used for β-lactamase purification, and *E. coli* MV1184 (31) was used for transformation.

Conjugation. Conjugation was performed by the broth method (7). *E. coli* TUM1083 and *E. coli* ML4909 (recipient) were incubated for 30 min at 35°C before selection of transconjugant.

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TABLE 1. Bacterial strains and plasmids used

E. coli strain or plasmid	Characteristic(s)	Reference or source
Strains		
TUM1083	Clinical isolate, cefotaxime-resistant strain	This study
TUM1103	Transformant, *E. coli* AS226 harboring pMTY036	This study
ML4909	F$^-$ *galK2 galT22 hsdR metB1 relA supE44* Rif^r	M. Inoue
C600	F$^-$ *thi-1 thr-2 leuB6 lacY1 tonA21 supE44* λ$^-$ Rif^r	27
AS226	F$^-$ *thr-1 leuB6 thi-1 hsdS1 lacY1 tonA21 supE44 ampC*Δ λ$^-$	13
MV1184	*ara* Δ (*lac-proAB*) Δ (*srl-racA*) *306*::Tn*10* φ*80* d*lacZ* ΔM15 *rpsL thi* [F′ *lacI*^{q} *lacZ* ΔM15*proAB traD36*]	31
Plasmids		
pHSG397	*lac cat*	30
pMTY002	Cefotaxime resistant	This study
pMTY036	2.1-kb *Sau3*AI fragment from pMTY002 cloned into pHSG397	This study
R388	IncW	33
R386	IncFI	27
R100-1	IncFII	27
R124	IncFIV	27
R64-11	IncIα	27
R621α	IncIγ	10
R27	IncHI	27
N3	IncN	27
R751	IncP	15
R446B	IncM	27
R401	IncT	27
R6K	IncX	27

Antibacterial agents. Penicillin G and ampicillin (Meiji Seika, Ltd., Tokyo, Japan); oxacillin and imipenem (Banyu Pharmaceutical Co., Ltd., Tokyo, Japan); carbenicillin and ceftizoxime (Fujisawa Pharmaceutical Co., Ltd., Tokyo, Japan); piperacillin (Toyama Chemical Co., Ltd., Tokyo, Japan); cefuzonam (Lederle Japan Ltd., Tokyo, Japan); cephalothin, cephaloridine, and moxalactam (Shionogi & Co., Ltd., Osaka, Japan); ceftazidime (Nippon Glaxo Ltd., Tokyo, Japan); cefotaxime (Hoechst Marion Roussel, Ltd., Tokyo, Japan); aztreonam (Sankyo Co., Ltd., Tokyo, Japan); and tazobactam and YP-14, a combination of tazobactam and piperacillin at a ratio of 1 to 4, respectively (Taiho Pharmaceutical Co., Ltd., Tokyo, Japan), all with known potencies, were used.

Drug sensitivity tests. MICs were determined by the broth microdilution method with Mueller-Hinton broth (Difco, Detroit, Mich.). The organisms were inoculated at about 5×10^5 cells/well with MIC2000 (Dynatech, McLean, Va.). The MIC was defined as the lowest concentration preventing visible growth after incubation for 18 h at 35°C.

Incompatibility tests and β-lactamase assay. Incompatibility tests were carried out according to the method described in a previous report (7). The plasmids and strains used in this study were described previously (13).

Purification of β-lactamase. The β-lactamase was purified from *E. coli* TUM1103, in which pMTY036 was transformed. The organisms were incubated for 5 h in 2 liters of Luria-Bertani broth and centrifuged at 7,000 × *g* for 10 min at 4°C. The supernatant was discarded, and MES (morpholineethanesulfonic acid)-NaOH buffer (20 mM, pH 6.5) was added to the tube to suspend the sediments. The suspended solution was disrupted by sonication (100 W, 30 min) in a volume of 15 ml of MES-NaOH buffer (20 mM, pH 6.5). The lysates were centrifuged again at 45,000 × *g* for 30 min. The supernatant was dialyzed for 24 h against 10 mM MES-NaOH buffer (pH 6.5), was then applied to carboxymethyl–Bio-Gel A (column size, 2.5 by 10 cm; Bio-Rad, Richmond, Calif.), and washed overnight with 10 mM MES-NaOH buffer (pH 6.5). Elution was performed with 10 mM MES-NaOH buffer (pH 6.5) containing 0.05 M NaCl. The activities of the eluted fractions were checked with nitrocefin (Oxoid, Basingstoke, England), and the active fractions were pooled and concentrated with a Centriprep-10 concentrator (Amicon, Beverly, Mass.) and purified again by fast protein liquid chromatography (column; Mono S 5/5 [Pharmacia Biotech, Uppsala, Sweden]; binding buffer, 10 mM MES-NaOH [pH 6.5]; elution buffer, 10 mM MES-NaOH [pH 6.5]–25 mM NaCl; flow rate, 0.5 ml/min; detector; UV, 280 nm). The purified β-lactamase was concentrated with a Centriprep-10 concentrator (Amicon). Isoelectric focusing was carried out with a Multiphor II electrophoresis system (Pharmacia Biotech) and a gel plate containing 2% Ampholine (pH 6.0 to 8.0). The enzyme protein on the gel plate was detected by staining with Coomassie brilliant blue R-250. The molecular weight was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (18).

Assay of β-lactamase activity. The activity of the highly purified β-lactamase was measured by spectrophotometric assay (32) with a spectrophotometer (DU640; Beckman, Fullerton, Calif.) with a thermoregulator. The peak wavelength for each antibiotic used for the measurement was set according to that described in previous reports (13). K_m and k_{cat} values were derived by the linear regression analysis of Lineweaver-Burk plots (19) of initial velocity data that were obtained at different substrate concentrations ranging from 10 to 100 μM. The apparent K_i value was determined with nitrocefin as substrate at concentrations from 10 to 100 μM, after preincubation for 1 min with β-lactamase inhibitor concentrations from 1 to 25 μM, and data were analyzed by a Dixon plot (8).

Cloning and analysis of recombinant plasmids. Plasmid DNA was purified by extracting plasmid DNA by the large-scale alkaline method and the ethidium bromide-CsCl linear gradient method (26) at 80,000 rpm for 16 h with a Beckman TLA centrifuge (Beckman). Restriction enzymes and T4 DNA ligase were purchased from Takara Shuzo Co., Ltd. (Shiga, Japan). The plasmid size was calculated from the size of the fragments obtained by cleaving the plasmid with restriction enzymes with cleaving λ phage DNA cleaved with *Sty*I as a molecular marker. The cefotaxime resistance gene was cloned as follows. After plasmid DNA was cleaved partially by *Sau*3AI, the resultant fragments were ligated into the *Bam*HI site of pHSG397 (30). *E. coli* MV1184 (31) was transformed with the ligated DNA, and cefotaxime-resistant colonies were selected on an L agar plate (26) supplemented with 10 μg of cefotaxime per ml.

DNA sequencing analysis. After pMTY036 was double digested with *Kpn*I and *Xho*I, *Sph*I and *Sal*I deletion mutants were prepared by using a Takara deletion kit (Takara Shuzo). From these deletion mutants, five subclones were sequenced with the universal primer M13 pUC sequencing primer (Takara Shuzo), the Takara *Bca* BEST Dideoxy sequencing kit (Takara Shuzo), and a DSQ-1000 DNA sequencer (Shimadzu, Tokyo, Japan). Then, a 17-mer oligonucleotide reversed primer was prepared on the basis of the results obtained with the universal primer. The sequence was determined according to the scheme shown in Fig. 1.

Computer analysis. The DNA sequence data were analyzed primarily by using a UNIX computer and software from the DNA Data Bank of Japan (National Institute of Genetics, Mishima, Japan). The alignments of the DNA and peptide sequences were examined by using the Fasta mail server (22), and the multiple sequence alignment was examined by using the ODEN and Karashi programs (12). The sequences extracted from the database and used for examination of the multiple alignment were Toho-1 from *E. coli* TUH12191 (13), MEN-1 from *E. coli* MEN-1 (3), KLEOX from *K. oxytoca* E23004 (2), CITDI from *Citrobacter diversus* ULA 27 (25), STRAL from *Streptomyces albus* G (20), TEM-1 from *E. coli* TEM-1 (14), SHV-1 from *E. coli* SHV-1 (4), PC-1 from *Staphylococcus aureus* PC-1 (11), PROVU from *Proteus vulgaris* RO104 (23), and YEREN from *Yersinia enterocolitica* (28).

Nucleotide sequence accession number. The nucleotide sequence data for the Toho-2 gene appear in the EMBL-GenBank-DDBJ data libraries under accession no. D89862.

RESULTS

One hundred fifty strains of *E. coli* or *Klebsiella pneumoniae* which show resistance to expanded-spectrum β-lactams were examined by PCR with the specific primer sets of the TEM (14), SHV (4), KOXY (2), Sme-1 (21), and Toho-1 (13) types of β-lactamases. No positive strain was detected by PCR with all the primer sets. The cefotaxime-resistant *E. coli* TUM1083

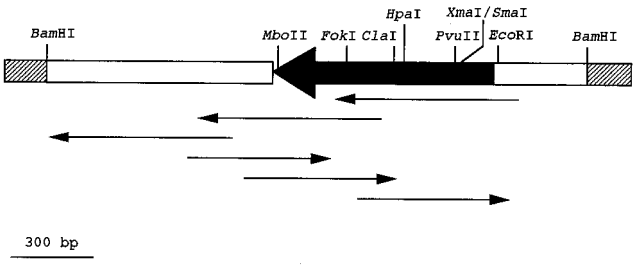

FIG. 1. Sequencing strategies for the bla_{toho} gene from pMTY036. The insert is shown as a dark, thick arrow, and *lacZ* is shown by open boxes. Sequence strategy is indicated by the arrows, which represent overlapping deletion mutants for sequencing of the bla_{toho} gene.

TABLE 2. MICs of various drugs for strains producing Toho-2 β-lactamase

Drug	MIC (μg/ml) for *E. coli* strain:		
	TUM1083	ML4909(pMTY002)	ML4909
Ampicillin	>512	>512	1.0
Carbenicillin	>512	>512	2.0
Piperacillin	>512	512	0.5
Cephalothin	>512	512	4.0
Cefoxitin	64	0.5	1.0
Ceftizoxime	128	1.0	≤0.3
Cefotaxime	>512	256	≤0.3
Ceftazidime	4.0	0.5	≤0.3
Cefuzonam	>512	512	≤0.3
Moxalactam	1.0	≤0.3	≤0.3
Aztreonam	256	4.0	≤0.3
Imipenem	≤0.3	0.5	0.5
Piperacillin-tazobactam[a]	16	2.0	0.5

[a] Tested at a ratio of 4:1, respectively.

strain, which produces β-lactamase Toho-2, was identified from those strains. *E. coli* TUM1083 was isolated in March 1995 from the urine of a 69-year-old male patient. The patient suffered from colon cancer and was administered piperacillin, cefuzonam, cefotiam, imipenem, levofloxacin, and tosulfloxacin. The strain produced a new β-lactamase with an isoelectric point of 7.7 named Toho-2.

Plasmid profile. Transconjugants which acquired cefotaxime resistance by conjugation appeared at a frequency of 10^{-4} to 10^{-6}. A plasmid profile of 20 transconjugants revealed the presence in each of a single 54.4-kbp plasmid. The plasmid, which is called pMTY002 (pMTY; registered with the Plasmid Reference Center), was cleaved into seven segments by *Eco*RI or *Hin*dIII. From the size of the fragments obtained, the size of pMTY002 was estimated to be about 54.4 kbp. The incompatibility of pMTY002 was examined by conjugation with *E. coli* C600 strains containing various different Inc plasmids listed in Table 1. In this way, pMTY002 was shown to be within incompatibility group FII.

Cloning of the β-lactamase gene. The fragments of pMTY002 generated by partial digestion with *Sau*3AI were inserted into pHSG397 and were transformed to *E. coli* MV1184. One plasmid, pMTY036, of about 4.3 kbp was isolated from a transformant selected on an L agar plate containing 10 μg of cefotaxime per ml. A restriction map and the position of the β-lactamase structural gene bla_{toho} are shown in Fig. 1.

TABLE 3. Kinetic parameters of Toho-2 β-lactamase

Antibiotic	k_{cat}[a] (s^{-1})	K_m or apparent K_i (μM)	k_{cat}/K_m ($\mu M^{-1}s^{-1}$)
Penicillin G	3.6	6	0.6
Ampicillin	1.2	12	0.1
Oxacillin	9.1	91	0.1
Carbenicillin	14	18	0.8
Piperacillin	130	84	1.6
Cephalothin	12,000	470	25
Cephaloridine	4,500	180	25
Cefoxitin	<0.1	4.8	<0.1
Ceftizoxime	30	150	0.2
Ceftazidime	1.3	160	<0.1
Cefotaxime	220	66	3.3
Aztreonam	0.1	140	<0.1
Imipenem		4.8	
Clavulanic acid		2.4	
Sulbactam		4.5	
Tazobactam		0.3	

[a] Absolute k_{cat} value.

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          10         20         30         40         50         60
AGAAGCAGTCTAAATTCTTCGTGAAATAGTGATTTTTGAAGCTAATAAAAAACACACGTG
                                        -35                -10
          70         80         90        100        110        120
GAATTTAGGGAATACTGATGTAACACGGATTGACCGTCTTGGGAGTTTGAGATGGTGACA
                                                   MetValThr
         130        140        150        160        170        180
AAGAGAGTGCAACGGATGATGTCCGCGGCGGCGGCGTGCATTCCGCTGCTGCTGGGCAGC
LysArgValGlnArgMetMetSerAlaAlaAlaAlaCysIleProLeuLeuLeuGlySer
         190        200        210        220        230        240
CCAACGCTTTATGCGCAGACGAGTGCGGTGCAGCAAAAGCTGGCGGCGCTGGAGAAAAGC
ProThrLeuTyrAlaGlnThrSerAlaValGlnGlnLysLeuAlaAlaLeuGluLysSer
         250        260        270        280        290        300
AGCGGAGGGCGGCTGGGCGTCGCGCTCATCGATACCGCAGATAATACGCAGGTGCTTTAT
SerGlyGlyArgLeuGlyValAlaLeuIleAspThrAlaAspAsnThrGlnValLeuTyr
         310        320        330        340        350        360
CGCGGTGATGAACGCTTTCCAATGTGCAGTACCAGTAAAGTTATGGCGGCCGCGGCGGTG
ArgGlyAspGluArgPheProMetCysSerThrSerLysValMetAlaAlaAlaAlaVal
         370        380        390        400        410        420
CTTAAGCAGAGTGAAACGCAAAAGCAGCTGCTTAATCAGCCTGTCGAGATCAAGCCTGCC
LeuLysGlnSerGluThrGlnLysGlnLeuLeuAsnGlnProValGluIleLysProAla
         430        440        450        460        470        480
GATCTGGTTAACTACAATCCGATTGCCGAAAAACACGTCAACGGCACAATGACGCTGGCA
AspLeuValAsnTyrAsnProIleAlaGluLysHisValAsnGlyThrMetThrLeuAla
         490        500        510        520        530        540
GAACTGAGCGCGGCCGCTTTGCAGTACAGCGACAATACCGCCATGAACAAATTGATTGCC
GluLeuSerAlaAlaAlaLeuGlnTyrSerAspAsnThrAlaMetAsnLysLeuIleAla
         550        560        570        580        590        600
CAGCTCGGTGGCCCGGGAGGCGTGACGGCTTTTGCCCGCGCGATCGGCGATGAGACGTTT
GlnLeuGlyGlyProGlyGlyValThrAlaPheAlaArgAlaIleGlyAspGluThrPhe
         610        620        630        640        650        660
CGTCTGGATCGCACTGAACCTACGCTGAATACCGCCATTCCCGGCGACCCGAGAGACACC
ArgLeuAspArgThrGluProThrLeuAsnThrAlaIleProGlyAspProArgAspThr
         670        680        690        700        710        720
ACCACGGCGCGGGCTGGCGCAGACGTTGCGTCATTACGCTGGGTCATGCGCTGGGCGAAA
ThrThrAlaArgAlaGlyAlaAspValAlaSerLeuArgTrpValMetArgTrpAlaLys
         730        740        750        760        770        780
CCCAGCGGCGCAGTTGGTGACGTGGCTCAAAGGCAATACGACCGCGCAGCCGGCATTCGG
ProSerGlyAlaValGlyAspValAlaGlnArgGlnTyrAspArgAlaAlaGlyIleArg
         790        800        810        820        830        840
GCCGGCTTACCGACGTCGTGGACTGTGGGTGATAAGACCGGCAGCGGCGACTACGGCACC
AlaGlyLeuProThrSerTrpThrValGlyAspLysThrGlySerGlyAspTyrGlyThr
         850        860        870        880        890        900
ACCAATGATATTGCGGTGATCTGGCCGCAGGGTCGTGCGCCGCTGGTTCTGGTGACCTAT
ThrAsnAspIleAlaValIleTrpProGlnGlyArgAlaProLeuValLeuValThrTyr
         910        920        930        940        950        960
TTTACCCAGCCGCAACAGAACGCAGAGAGCCGCCGCGATGTGCTGGCTTCAGCGGCGAGA
PheThrGlnProGlnGlnAsnAlaGluSerArgArgAspValLeuAlaSerAlaAlaArg
         970        980
ATCATCGCCGAAGGGCTGTAA
IleIleAlaGluGlyLeu***
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FIG. 2. Nucleotide sequence of the bla_{toho} gene. The conserved amino acid residues found in the active sites of class A β-lactamases are underlined. The deduced cleavage site of the signal peptide is shown by an arrow. Bold-face indicates −35 and −10 sequences.

Susceptibility to antibiotics. Table 2 shows MICs of β-lactam antibiotics against *E. coli* TUM1083 and *E. coli* ML4909 with and without pMTY002. Susceptibility tests were conducted with 10 clones of transconjugants. MICs of all penicillins, cephalothin, cefotaxime, and cefuzonam against *E. coli* TUM1083 and *E. coli* ML4909 (pMTY002) were ≥256 μg/ml. The MICs of inhibitors against *E. coli* TUM1083 and *E. coli* ML4909(pMTY002) were 512 μg/ml or more. However, MICs of piperacillin to these strains were markedly decreased to 16 and 2μg/ml in the presence of 4 and 0.5 μg/ml of tazobactam per ml. The MICs of aminoglycoside antibiotics against *E. coli* ML4909 were not changed in the presence of pMTY002.

Kinetic parameters. β-Lactamase was purified from *E. coli* cells harboring pMTY036, as described under Materials and

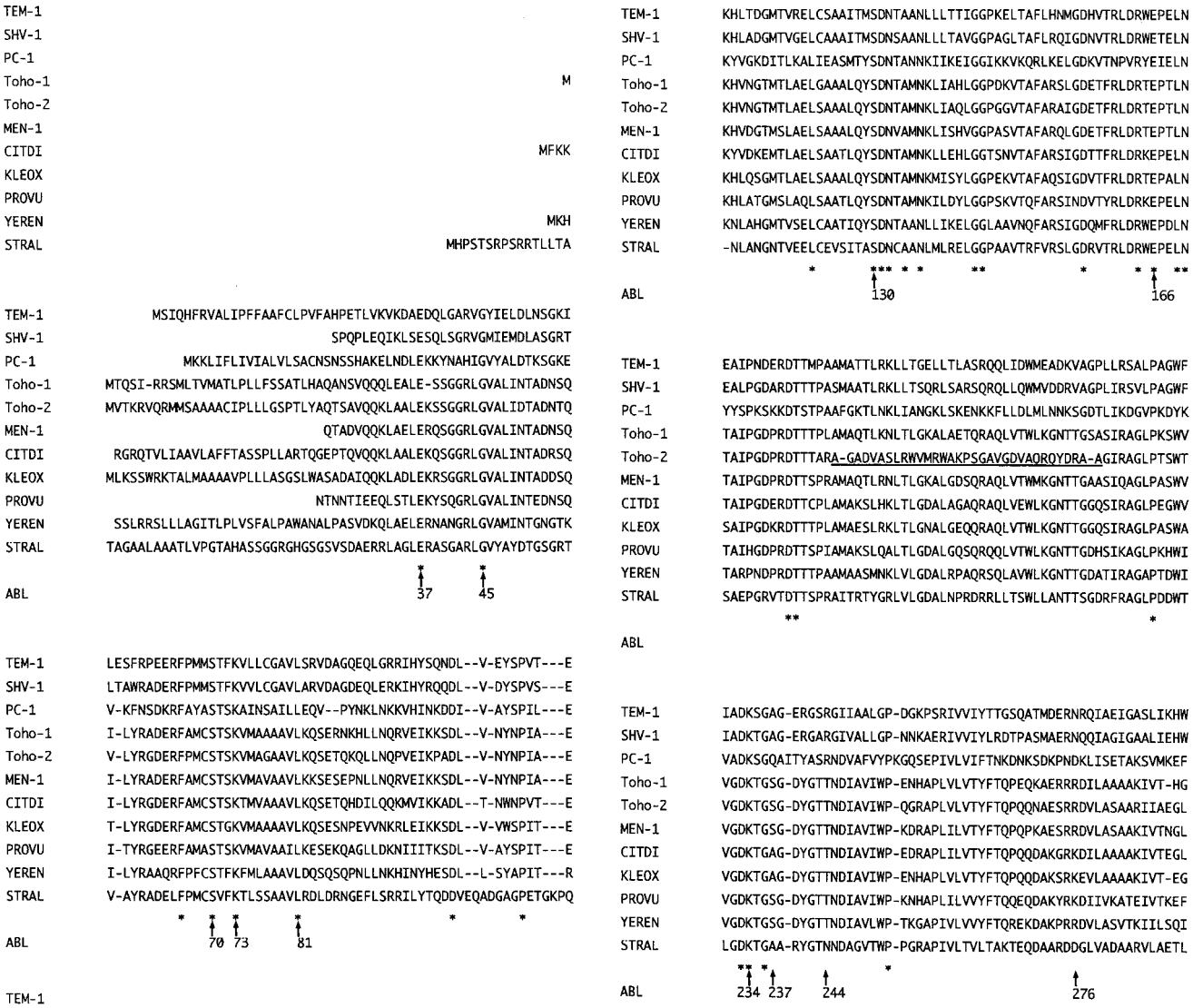

FIG. 3. Amino acid sequence alignment of 11 class A β-lactamases. Dashes indicate gaps inserted in the alignment; asterisks indicate identical residues. The unique sequence of Toho-2 is underlined (Ala-185 to Ala-219). ABL, standard numbering scheme for class A β-lactamases (1). References for the enzymes are as follows: Toho-1 (13), MEN-1 (3), KLEOX (2), CITDI (25), STRAL (20), TEM-1 (14), SHV-1 (4), PC-1 (11), PROVU (23), and YEREN (28).

Methods. The purified enzyme gave a single band on isoelectric focusing (Coomassie blue staining) and on sodium dodecyl sulfate-polyacrylamide gel electrophoresis, with an estimated molecular mass of 28,000 Da. As shown in Table 3, purified Toho-2 had high catalytic activity toward cephalothin, cephaloridine, cefotaxime, and piperacillin. the catalytic efficiency (k_{cat}/K_m) for those drugs was also much higher than for the other substrates tested. However, Toho-2 did not have high k_{cat} values toward ampicillin, oxacillin, cefoxitin, ceftazidime, and imipenem. Relatively high K_m values of these substrates reduced the catalytic efficiency (relative k_{cat}/K_m). The β-lactamase inhibitors, tazobactam and clavulanic acid, appeared to have the highest affinities of all the agents.

DNA sequencing. The nucleotide sequence of 981-bp bla_{toho} was determined by the strategy shown in Fig. 1. An 870-nucleotide open reading frame with a GC content of 70.2% was present in this sequence (Fig. 2). The sequence initiation codon (ATG) was preceded by a possible −10 region (TGGAAT) and a −35 region (TTGAAG) of a putative promoter. The termination codon was TAA. From the putative open reading frame, the precursor form of Toho-2 seemed to consist of 289 amino acid residues with a molecular mass of 30,725 Da. The processing site was determined by the comparison of the hypothetical amino acid sequence predicted by the DNA sequence with the last 10 amino acids from the N-terminal sequence determined by a peptide sequencer. The mature form consisted of 261 amino acid residues with a molecular mass of 27,752 Da. The consensus sequences such as STSK, SDN, and KTG in class A β-lactamases were found in

the amino acid sequence of Toho-2 β-lactamase. Thus, Toho-2 is a class A β-lactamase.

Homology with other β-lactamases. The DNA sequence of the gene for Toho-2 showed high homology (76% or higher) with those for plasmid-mediated β-lactamases of CTX-M-2 (5) and Toho-1. Amino acid sequence identities of Toho-2 with *E. coli* MEN-1 (3), *K. oxytoca* β-lactamase (2), and Toho-1 are 72, 72, and 70%, respectively, while Toho-2 showed less than 30% amino acid sequence identity with TEM and SHV. The multiple sequence alignment of Toho-2 and other β-lactamases is shown in Fig. 3. The consensus sequences S70XXK73, S130DN132, and K234TG236 (Ambler numbering) and the highly conserved E166 residue, which are essential for the catalysis of class A β-lactamases, were found in Toho-2.

DISCUSSION

Generally, most of the class A β-lactamases are strongly inhibited by β-lactamase inhibitors (6). Clavulanic acid is often the most potent inhibitor for these enzymes (6). For Toho-1, K_i values for sulbactam and tazobactam were 5.8 and 5.3 μM, respectively; on the other hand, the apparent K_i value of Toho-1 with clavulanic acid was 0.6 μM (13). However, the extended-spectrum β-lactamase Toho-2 was more strongly inhibited by tazobactam than by clavulanic acid or sulbactam. It is very different from the β-lactamase reported heretofore.

Toho-2 has three major sequences distinct from those of other class A β-lactamases. Two of those are found in the N- and C-terminal sequences, though Toho-1 has a sequence similar to that of Toho-2. The other distinct sequence is located at Ala-185 to Ala-219 (Fig. 3). This sequence has almost no sequence homology with the other class A β-lactamases. The sequence may constitute a loop structure at the substrate-binding site and has a deletion of 2 amino acid residues at the binding site. This deletion may well correlate with the observation that tazobactam, a β-lactamase inhibitor, showed a higher affinity for Toho-2 than for Toho-1, whereas sulbactam binds to both enzymes with similar affinities. Tazobactam has the 1,2,3-triazolylmethyl group at C-2 instead of methyl groups for sulbactam. Examination of the location of the triazolylmethyl moiety at the binding site through three-dimensional structure modeling of the acyl enzyme suggests that the triazole moiety interacts with the loop where the deletion of 2 amino acid residues occurred. Thus, the triazole moiety would find suitable room to reside in a larger substrate-binding pocket.

Ishii et al. (13) have pointed out that in Toho-1 the replacement of Asn-276 by Arg with the concomitant substitution of Thr for Arg-244 is important for the extension of the substrate specificity and that Arg-276 may function as Arg-244. The Arg-274 of Toho-1 is also a characteristic residue since this residue could also be located at a site similar to Arg-276. Toho-2 had the same substitutions as Toho-1 at positions 244 and 276. However, the Ser-274 residue was found in Toho-2, instead of the Arg-274 residue found in Toho-1. Since the Ser-274 residue is found also in the β-lactamases of *E. coli* MEN-1 (3) and *K. oxytoca* E23004 (2), the basic residue at position 274 in Toho-1 may not play an important role for the cefotaxime hydrolysis, while it would contribute to the higher affinity of aztreonam, which has the carboxylate in the oxyimino side chain.

The substitution of serine for Ala-237 is also a common mutation observed in substrate-extended class A β-lactamases. We assume that this mutation brings about an enlarged binding site for the bulky oxyimino moiety of the expanded-spectrum cephalosporins such as cefotaxime, and thus the mutation should be very important for the substrate extension.

In conclusion, Toho-2 has two characteristic mutation sites for the substrate extension. The mutations at 237, 244, and 276, which are aligned at a peripheral site of the substrate-binding site, may contribute to the specificity for oxyiminocephalosporins such as cefotaxime and ceftizoxime. The other mutation-deletion at 185 to 219, particularly at the triazole-binding loop, may be responsible for the higher affinity for tazobactam.

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