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Cloning and Sequence of the Gene Encoding a Cefotaxime-Hydrolyzing Class A β-Lactamase Isolated from *Escherichia coli*

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***Escherichia coli* TUH12191, which is resistant to piperacillin, cefazolin, cefotiam, ceftizoxime, cefuzonam, and aztreonam but is susceptible to cefoxitin, latamoxef, flomoxef, and imipenem, was isolated from the urine of a patient treated with β-lactam antibiotics. The β-lactamase (Toho-1) purified from the bacteria had a pI of 7.8, had a molecular weight of about 29,000, and hydrolyzed β-lactam antibiotics such as penicillin G, ampicillin, oxacillin, carbenicillin, piperacillin, cephalothin, cephaloridine, cefoxitin, cefotaxime, ceftazidime, and aztreonam. Toho-1 was markedly inhibited by β-lactamase inhibitors such as clavulanic acid and tazobactam. Resistance to β-lactams, streptomycin, spectinomycin, sulfamethoxazole, and trimethoprim was transferred by conjugational transfer from *E. coli* TUH12191 to *E. coli* ML4903, and the transferred plasmid was about 58 kbp, belonging to incompatibility group M. The cefotaxime resistance gene for Toho-1 was subcloned from the 58-kbp plasmid by transformation of *E. coli* MV1184. The sequence of the gene for Toho-1 was determined, and the open reading frame of the gene consisted of 873 or 876 bases (initial sequence, ATGATG). The nucleotide sequence of the gene (DDBJ accession number D37830) was found to be about 73% homologous to the sequence of the gene encoding a class A β-lactamase produced by *Klebsiella oxytoca* E23004. According to the amino acid sequence deduced from the DNA sequence, the precursor consisted of 290 or 291 amino acid residues, which contained amino acid motifs common to class A β-lactamases (^{70}SXXK, ^{130}SDN, and ^{234}KTG). Toho-1 was about 83% homologous to the β-lactamase mediated by the chromosome of *K. oxytoca* D488 and the β-lactamase mediated by the plasmid of *E. coli* MEN-1. Therefore, the newly isolated β-lactamase Toho-1 produced by *E. coli* TUH12191 is similar to β-lactamases produced by *K. oxytoca* D488, *K. oxytoca* E23004, and *E. coli* MEN-1 rather than to mutants of TEM or SHV enzymes. Toho-1 has shown the highest degree of similarity to *K. oxytoca* class A β-lactamase. Detailed comparison of Toho-1 with other β-lactamases implied that replacement of Asn-276 by Arg with the concomitant substitution of Thr for Arg-244 is an important mutation in the extension of the substrate specificity.**

Expanded-spectrum cephalosporins have chemical structures which confer stability to many β-lactamases from gram-negative bacteria. However, many members of the family *Enterobacteriaceae* other than *Escherichia coli* developed resistance to the expanded-spectrum cephems (40). The primary mechanism of this resistance was demonstrated to be excessive production of a chromosomal β-lactamase (AmpC) (23). However, bacteria that show resistance mediated by other β-lactamases appeared in 1984 (8). Species of the *Enterobacteriaceae* such as *Klebsiella pneumoniae*, *Klebsiella oxytoca*, and *E. coli* acquired resistance against expanded-spectrum cephem antibiotics by producing extended-spectrum β-lactamase. The extended spectrum of the β-lactamase was often acquired by the variation of β-lactamase genes on transmissible plasmids (43, 44). Under the influence of antimicrobial agents, bacteria producing primarily TEM-type or SHV-type β-lactamases developed point mutations in structural genes which served to extend the substrate specificity of the enzymes (44). These TEM-type and SHV-type β-lactamases show about 65% amino acid sequence homology, with isoelectric points of 5.5 to 6.3 and 7.0 to 8.2, respectively (8, 9). The plasmid containing the gene encoding TEM-type extended-spectrum β-lactamase usually belongs to incompatibility group 7 or M, and simultaneous increases in the resistance to aminoglycoside antibiotics such as gentamicin and tobramycin have been reported by a number of investigators (43). Thus, it would be quite interesting to determine which mutation is important in the extension of the substrate specificity of β-lactamase.

In this study, we analyzed biochemically and genetically the β-lactamase produced by *E. coli* TUH12191, a cefotaxime-resistant strain isolated from the urine of a patient at Toho University School of Medicine Omori Hospital in November 1993 and compared the amino acid sequence with those of the β-lactamases from *Staphylococcus aureus* PC-1 (15), *E. coli* TEM-1 (26), and *Streptomyces albus* G (32), whose crystal structures are known, and with those of other β-lactamases by a multiple sequence alignment to search for new mutant sites in the extended-spectrum β-lactamase.

MATERIALS AND METHODS

Bacterial strains and plasmids. Table 1 shows the bacterial strains and plasmids used in this study. *E. coli* TUH12191 was isolated in November 1993 from the urine of a 1-year-old female who developed cystitis. This patient had received β-lactam antibiotics, primarily cefoperazone-sulbactam or cefpodoxime proxetil. This strain did not ferment lactose but was identified as *E. coli* by tests using API 20E (Asuka, Tokyo, Japan) and the Vitek system (bioMérieux, Hazelwood, Mo.). *E. coli* ML4903, used for plasmid conjugation, was provided by Matsuhisa Inoue of Kitasato University. *E. coli* AS226, used for β-lactamase purification, was provided

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TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Characteristic(s)	Reference or source
Strains		
E. coli TUH12191	Clinical isolate, cefotaxime-resistant strain	This study
E. coli ML4903	*F*$^-$ *galK2 galT22 hsdR lacY1 metB1 relA supE44* Rif^r	M. Inoue
E. coli C600	*F*$^-$ *thi-1 thr-1 leuB6 lacY1 tonA21 supE44* λ$^-$ Rif^r	39
E. coli MV1184	*ara* Δ(*lac-proAB*) Δ(*stl-racA*)*306*::Tn*10* φ80 d*lacZ* ΔM15 *rpsL thi* [*F′ lacI*^{q} *lacZ*ΔM15 *proAB traD36*]	48
E. coli AS226	*F*$^-$ *thr-1 leuB6 thi-1 hsdS1 lacY1 tonA21 supE44 ampC*Δ λ$^-$	K. Sugimoto
Plasmids		
pHSG397	*lac cat*	45
pMTY001	Cefotaxime resistant	This study
pMTY010	4.3-kb *Sau*3AI fragment from pMTY001 cloned into pHSG397	This study
R388	IncW	50
R386	IncFI	41
R100-1	IncFII	41
R124	IncFIV	41
R64-11	IncIα	41
R621a	IncIγ	13
R27	IncHI	41
N3	IncN	41
R751	IncP	27
R446B	IncM	41
R401	IncT	41
R6K	IncX	41

by Kazunori Sugimoto of Hokkaido University. *E. coli* MV1184 (48) was used for transformation.

Transconjugation. Transconjugation was done by the broth method (10). *E. coli* TUH12191 and *E. coli* ML4903 (recipient) were incubated for 30 min at 35°C before selection of transconjugants.

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., Osaka, Japan), piperacillin (Toyama Chemical Co., Ltd., Tokyo, Japan), cefuzonam (Lederle Japan Ltd., Tokyo, Japan), cephalothin, cephaloridine, and latamoxef (Shionogi & Co., Ltd., Osaka, Japan), ceftazidime (Nippon Glaxo Ltd., Toyko, Japan), cefotaxime (Hoechst Japan Ltd., Tokyo, Japan), aztreonam (Sankyo Co., Ltd., Tokyo, Japan), and tazobactam and YP-14 (Taiho Pharmaceutical Co., Ltd., Tokyo, Japan), all with known potencies, were used. YP-14 is a combination of tazobactam and piperacillin in a ratio of 1 to 4.

Drug susceptibility 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 per well by using 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 as described in a previous report (10). The plasmids and strains used in this study were provided by Somay Y. Murayama of Teikyo University.

The β-lactamase was purified from *E. coli* AS226 in which pMTY010 was transformed. The organisms were incubated overnight in 2 liters of Mueller-Hinton broth containing cefotaxime at 10 μg/ml and centrifuged at 6,000 × *g* for 15 min at 4°C. A periplasmic fraction was separated from the sediment according to the osmotic shock method (33). This periplasmic fraction was dialyzed for 24 h against 0.005 M phosphate buffer (pH 6.5) and was then applied to CM-Bio gel A (Bio-Rad, Richmond, Calif.) and washed overnight with 0.005 M phosphate buffer (pH 6.5). Elution was performed with 0.05 M phosphate buffer (pH 6.5). The activities of the eluted fractions were checked with nitrocefin (Oxoid, Basingstoke, England), and the active fractions were pooled and concentrated by using a Centriprep-10 (Amicon, Beverly, Mass.), as an apparatus for dialyzation, dialyzed overnight against 0.005 M phosphate buffer (pH 6.5), and repeatedly purified with the same ion-exchange resin. This partially purified β-lactamase was dialyzed against 0.05 M phosphate buffer (pH 7.0) and purified by using TSK gel Toyopearl HW-50 (Tosoh, Tokyo, Japan) with 0.05 M phosphate buffer (pH 7.0) containing 0.1 M NaCl as the mobile phase. The purified β-lactamase was concentrated with a Centriprep-10 (Amicon). This enzyme was used in the β-lactamase assay, which was done as follows. Isoelectric focusing was carried out with a Multiphor II electrophoresis system (Pharmacia Biotech, Uppsala, Sweden) and a gel plate containing 5% Ampholine (pH 3.5 to 9.5). 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 (SDS)-polyacrylamide gel electrophoresis (28). The β-lactamase activity was measured by spectrophotometric assay (49) using a double-beam spectrophotometer with a thermoregulator (model 3200; Hitachi, Tokyo, Japan). The peak wavelength for each antibiotic used for the measurement was set according to previous reports (21). K_m and V_{max} values were derived by the linear regression analysis of Lineweaver-Burk plots (31) of initial velocity data that were obtained at different substrate concentrations ranging from 1 to 100 μM. V_{max} and V_{max}/K_m were expressed relative to those of penicillin G (taken as 100%). K_i was determined with cephalothin at concentrations from 1 to 100 μM as the substrate, after preincubation with β-lactamase inhibitor at concentrations from 1 to 25 μM for 1 min, and data were analyzed by a Dixon plot (11).

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 (39) at 70,000 rpm for 16 h with a TLA centrifuge (Beckman, Fullerton, Calif.). Restriction enzymes and T4 DNA ligase used were purchased from Takara Shuzo Co., Ltd. (Kyoto, Japan). The plasmid size was calculated from the size of the fragments obtained by cleaving the plasmid with restriction enzymes using 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 (45). *E. coli* MV1184 was transformed with the ligated DNA, and cefotaxime-resistant colonies were selected on an L agar plate (39) supplemented with 10 μg of cefotaxime per ml.

DNA sequencing analysis. After pMTY010 was double digested with *Kpn*I and *Ava*I, deletion mutants were prepared by using a Takara deletion kit (Takara Shuzo). From these deletion mutants, four subclones were sequenced by using the universal M13 pUC sequencing primer (Takara Shuzo Co., Ltd.), the *Taq* DyeDeoxy TM Terminator cycle sequencing kit (Perkin Elmer Co., Norwalk, Conn.), and a model 373A DNA sequencer (Perkin Elmer Co.). Then, we prepared a 17-mer oligonucleotide reversed primer on the basis of the results obtained with the universal primer and determined the sequence (see the scheme in Fig. 1).

Computer analysis. The DNA sequence data were analyzed primarily by using a UNIX computer and software of 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 (34), and the multiple sequence alignment was examined by using ODEN and karashi (20). The sequences extracted from database and used for examination of the multiple alignment were those of *E. coli* MEN-1 (4), *K. oxytoca* E23004 (3), *K. oxytoca* D488 (37), *Citrobacter diversus* ULA 27 (38), *S. albus* G (32), *E. coli* TEM-3 (44), *E. coli* TEM-1 (25), *E. coli* SHV-2 (16), *E. coli* SHV-1 (5), *S. aureus* PC1 (15), *Proteus vulgaris* RO104 (35), *Yersinia enterocolitica* (42), and *Mycobacterium fortuitum* D316 (46).

Nucleotide sequence accession numbers. The nucleotide sequence data for the Toho-1 gene appear in the EMBL/GenBank/DDBJ data libraries under accession number D37830. The amino acid sequence data for Toho-1 appear in the JIPID database under accession number JP0074.

RESULTS

Plasmid profile. Transconjugants which acquired cefotaxime resistance by conjugation appeared at a frequency of 10^{-4}. A plasmid profile of 30 transconjugants revealed the presence in each of a single 58-kbp plasmid. pMTY001 (pMTY is registered with the Plasmid Reference Center [29]) was cleaved into seven segments by *Eco*RI but was not cleaved by *Hin*dIII, *Bam*HI, or *Sal*I. From the size of the fragments obtained by the cleavage with *Eco*RI, *Ava*I, and *Sph*I, the size of pMTY001 was estimated to be about 58 kbp. Then, the incompatibility of pMTY001 was examined by conjugation with *E. coli* C600 strains containing the various Inc plasmids listed in Table 1. In this way, pMTY001 was shown to belong to incompatibility group M.

Cloning of the β-lactamase gene. The fragments of pMTY 001 generated by the partial digestion with *Sau*3AI were inserted into pHSG397 and transformed into *E. coli* MV1184. Two clones of about 5.5 kbp (pMTY010) and about 7.2 kbp (pMTY011) were isolated from the transformants selected on L agar plates containing 10 μg of cefotaxime per ml. Of these fragments, pMTY010 was used in this study. Its plasmid map

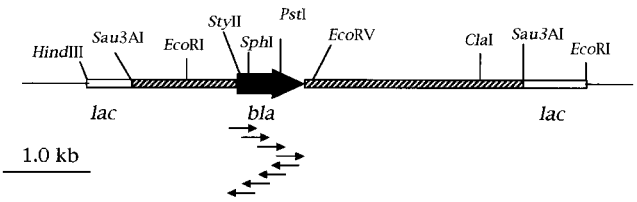

FIG. 1. Sequencing strategies for the *bla* gene from pMTY010. Sequence strategy is indicated by arrows. Small arrows represent overlapping deletion mutants for sequencing of the *bla* gene.

and the position of the β-lactamase structural gene in this fragment are shown in Fig. 1.

Susceptibility to antibiotics. Table 2 shows MICs of β-lactam antibiotics against *E. coli* TUH12191 and *E. coli* ML4903 containing and not containing pMTY001. We tried susceptibility tests using 25 clones of transconjugants. MICs of β-lactam antibiotics except imipenem, latamoxef, and flomoxef against *E. coli* ML4903 containing plasmid pMTY001 were more than four times higher than those against *E. coli* ML4903 alone. MICs of piperacillin for *E. coli* TUH12191 and *E. coli* ML4903(pMTY001) were 256 μg/ml. The MICs of the inhibitors alone against *E. coli* TUH12191 and *E. coli* ML4903 (pMTY001) were 512 μg/ml or more. However, MICs of piperacillin for these strains were markedly decreased to 4 and 2 μg/ml in the presence of the β-lactamase inhibitors tazobactam and clavulanic acid, respectively. MICs of aminoglycoside antibiotics such as streptomycin and spectinomycin against transformants were increased 32 times or more, while no increase was observed in the susceptibility of *E. coli* ML4903 (pMTY001) to other aminoglycoside antibiotics. Thus, the gene encoding aminoglycoside 3″-adenyltransferase, an aminoglycoside-modifying enzyme, is likely to be present also on this plasmid. Furthermore, MICs of sulfamethoxazole and trimethoprim against *E. coli* ML4903(pMTY001) were more than 4,000 times higher than those against the parent strains. This suggests that pMTY001 simultaneously encodes sulfamethoxazole and trimethoprim resistance genes.

TABLE 2. MICs of various drugs for strains producing Toho-1 β-lactamase

Drug	MIC (μg/ml) for:		
	E. coli TUH12191	*E. coli* ML4903 (pMTY001)	*E. coli* ML4903
Ampicillin	512	512	≤0.25
Carbenicillin	>512	>512	≤0.25
Piperacillin	256	256	≤0.25
Cephalothin	>512	>512	0.5
Cefoxitin	1	1	≤0.25
Ceftizoxime	32	32	≤0.25
Cefotaxime	>512	>512	≤0.25
Ceftazidime	8	4	≤0.25
Cefuzonam	512	>512	≤0.25
Latamoxef	≤0.25	≤0.25	≤0.25
Flomoxef	≤0.25	≤0.25	≤0.25
Aztreonam	32	32	0.125
Imipenem	0.0625	0.0315	0.015
YP14	4	2	≤0.25
Streptomycin	128	64	2
Kanamycin	0.5	0.25	≤0.25
Gentamicin	8	≤0.25	≤0.25
Tobramycin	16	≤0.25	≤0.25
Spectinomycin	>512	>512	8
Nalidixic acid	128	≤0.25	≤0.25
Ciprofloxacin	2	≤0.25	≤0.25
Sulfamethoxazole	>512	>512	≤0.25
Trimethoprim	>512	>512	≤0.25

TABLE 3. Kinetic parameters for Toho-1 β-lactamase

Drug	Relative V_{max} (%)[a]	K_m or K_i (μM)	Relative V_{max}/K_m (%)[b]
Penicillin G[c]	100	15	100
Ampicillin	37	11	51
Oxacillin	11	32	5.1
Carbenicillin	15	2.9	78
Piperacillin	21	23	14
Cephalothin	480	1,300	5.3
Cephaloridine	1,000	1,100	13
Cefoxitin	4	200	0.3
Ceftizoxime	140	480	4.3
Ceftazidime	17	68	3.8
Cefotaxime	1,600	1,400	17
Aztreonam	4	75	0.9
Imipenem	<0.1		
Clavulanic acid		0.6	
Sulbactam		5.8	
Tazobactam		5.3	

[a] Values are percentages of the V_{max} for penicillin G.
[b] Values are percentages of the V_{max}/K_m ratio for penicillin G.
[c] k_{cat} = 170 s^{-1}.

Purification of β-lactamase. The purified enzyme yielded a single band in SDS-polyacrylamide gel electrophoresis and isoelectric focusing, and no other bands were detected by Coomassie blue staining. Hence, the purity of the enzyme was above 90%. We estimated an apparent molecular weight of 29,000 and an isoelectric point of 7.8 for the enzyme, which was named Toho-1.

Kinetic parameters. As seen in Table 3, purified Toho-1 had the greatest activity (relative V_{max}) against cefotaxime (1,600% that for penicillin G) and cephaloridine (1,000%). However, the extremely high K_m values with these substrates reduced the catalytic efficiency (relative V_{max}/K_m) to below those with penicillin G (100%), ampicillin (51%), piperacillin (14%), or carbenicillin (78%). Nonetheless, the catalytic efficiency with cefotaxime and cephaloridine was still at least severalfold higher than with the other substrates that were tested. Imipenem hydrolysis was not detectable (the relative V_{max} was <0.1%). The low K_i values for β-lactamase inhibitors such as clavulanic acid, tazobactam, and sulbactam reflected a good affinity of the enzyme for these compounds (Table 3).

DNA sequencing. The nucleotide sequence of 1,037 bp was determined by the strategy shown in Fig. 2. A 876- or 873-nucleotide open reading frame with a GC content of 56.7% was present in this sequence (Fig. 2). The two possible sequence initiation codons (ATGATG) were preceded by a possible −10 region (TGGAAT) and −35 region (TGAAGG) of a putative promotor. The termination codon was TGA. From the putative open reading frame, the precursor form of Toho-1 should consist of 291 (or 290) amino acid residues and have a molecular weight of 31,481, and the mature form should consist of 262 amino acid residues and have a molecular weight of 28,463. The consensus sequences STSK, SDN, and KTG found in class A β-lactamases were found in the amino acid sequence of Toho-1. Thus, Toho-1 is a class A β-lactamase.

Homology with other β-lactamases. The DNA sequence of Toho-1 showed extensive homology (70% or more) with β-lac-

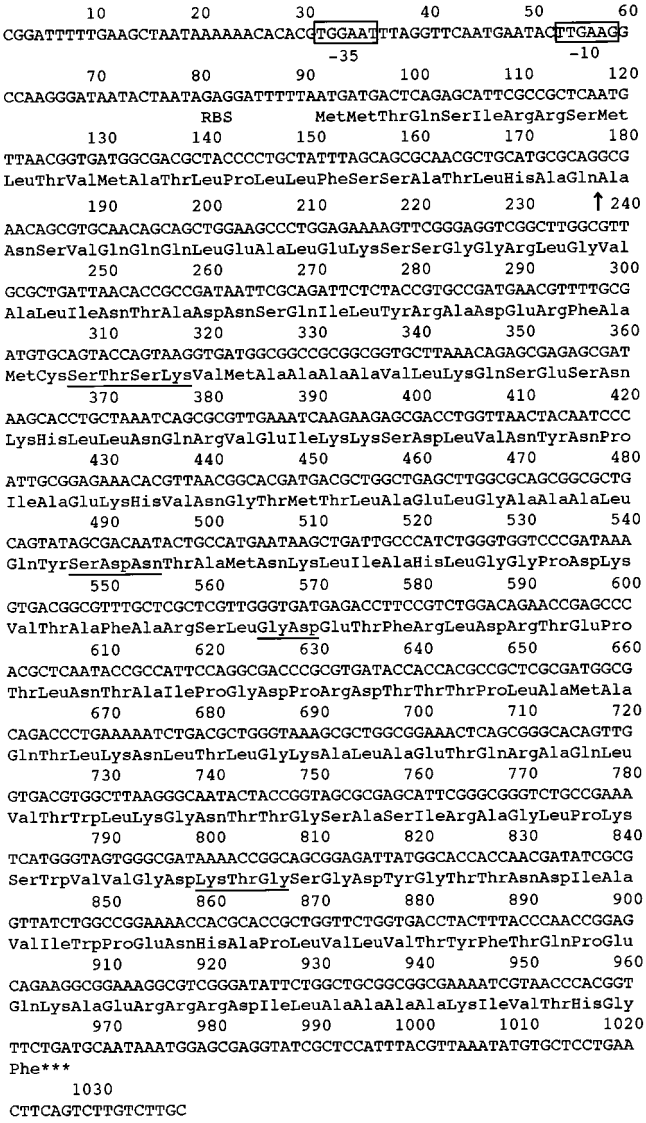

FIG. 2. Nucleotide sequence of the pMTY010 *bla* gene. The conserved amino acid residues found in the class A β-lactamase serine active site are underlined. The deduced leader peptide is shown by an arrow.

tamases of *K. oxytoca* (3) and *C. diversus* (38). The degree of amino acid sequence homology of Toho-1 with TEM and SHV enzymes was rather low (about 60%), while the enzyme showed more than 80% sequence homology with *K. oxytoca* D488, *K. oxytoca* E23004, and *E. coli* MEN-1. The multiple alignment of Toho-1 and other β-lactamases is shown in Fig. 3. The consensus sequences ^{70}SXXK^{73}, ^{130}SDN^{132}, and ^{234}KTG^{236} (numbering system of Ambler et al. [2]) and the highly conserved E-166 residue, which are essential for the catalysis by class A β-lactamases, were found in Toho-1. However, the degree of homology of the precursor form of Toho-1 with that of *K. oxytoca* E23004 was lower (76%).

DISCUSSION

Differences in amino acid residues of the signal peptides and C-terminal regions of β-lactamases are unlikely to affect the active-site structure. Since the β-lactamase reported here has highly conserved amino acid residues such as ^{70}SXXK^{73}, ^{130}SDN^{132}, ^{234}KT(S)G^{236}, and E-166, which are important residues for the catalysis by class A β-lactamases (1), we can categorize the enzyme as a member of class A. The similarity of the substrate specificity to Toho-1 should be due to a close resemblance of the three-dimensional structure, particularly the active site, to those of *K. oxytoca* D488, *K. oxytoca* E23004, and *E. coli* MEN-1, which are class A enzymes.

Recent studies with TEM and SHV β-lactamase variants have identified residues at positions 238 and 240 (14, 17, 35, 46) which contribute to their extended substrate specificity. In OHIO-1 and TEM enzymes, mutations at 69, 104, 164, 240, and 242 are important for an extension of substrate specificity (6, 24). However, Toho-1 has no substitution at those positions. On the other hand, the enzyme has a serine residue at 237. This mutation could be one of the factors affecting the substrate specificity. Barthelemy et al. (4) have suggested that Ser-237 is important for the extension of the substrate specificity of the MEN-1 β-lactamase, similar to the Ser-238 mutation in TEM enzymes (17, 36, 47). However, it is hard to rationalize this since the Ser-237 residue would protrude to a surface of the enzyme while the Ser-238 residue would be directed toward the inside of the active site, hydrogen bonding to the methoxyimino group.

It has been pointed out that the Arg residue at 220 in the *S. albus* G β-lactamase or at 244 in the class A enzymes plays an important role in the enzyme catalysis (22, 51). The crystal structures of class A enzymes indicate that the Arg-244 residue forms a hydrogen bond with the Asn-276 residue (30). A mutation of this residue at 276 resulted in a change in substrate specificity in the OHIO-1 β-lactamase (7). Because of the lack of a hydrogen bond, the Arg-244 residue became more flexible, changing its location at the enzyme surface. The Toho-1 enzyme has no basic residue at 220 (Ser) or at 244 (Thr). Instead, the enzyme has a basic residue at 276. The three-dimensional-structure model indicates that the arginine at 276 can overlap with those at 220 or 244 (30). However, the Arg-276 residue would not have an alignment identical to that of the Arg-244 residue. Thus, such a slight but significant change of the position of the basic residue should contribute to the substrate specificity. We note that a cephalosporinase activity has been reported for the *S. albus* G enzyme. We found seven class A enzymes, *K. oxytoca* E23004 (3), *K. oxytoca* D488 (37), *E. coli* MEN-1 (4), *C. diversus* ULA27 (12, 38), *P. vulgaris* RO104 (35), *Y. enterocolitica* (42), and *M. fortuitum* D316 (46), which have a basic residue, Arg or Lys, at 276 but no basic residues at 220 and 244. All of those enzymes show cephalosporinase activity and constitute a group with highly homologous amino acid sequences (except *M. fortuitum* D316). Although it is hard to account for the extended specificity for cefotaxime, the substitution of the basic residue at 276 may be a major factor for the extension of the specificity.

A substitution of serine for the Arg-244 residue altered the substrate specificity, particularly breakdown of carbapenems and the β-lactamase inhibitor clavulanic acid (19, 52). The proton transfer from water, anchored by the side chain of Arg-244 and the main-chain carbonyl of Val-216, to the clavulanate was proposed for the mechanism of inhibition of TEM-1 by clavulanic acid. Since the lack of an Arg guanidinium moiety by the mutation of Arg-244 to Ser would affect the appropriate position of the water molecule, the mutant enzyme had the ability to hydrolyze the acylenzyme formed with clavulanic acid. The basic residue at 276 in Toho-1 could have the same function as the Arg-244 residue in the TEM-1 enzyme in affecting the change of the clavulanate structure. Thus, Toho-1 is also inactivated by clavulanic acid (Table 3). Clavulanic acid had a K_i value approximately

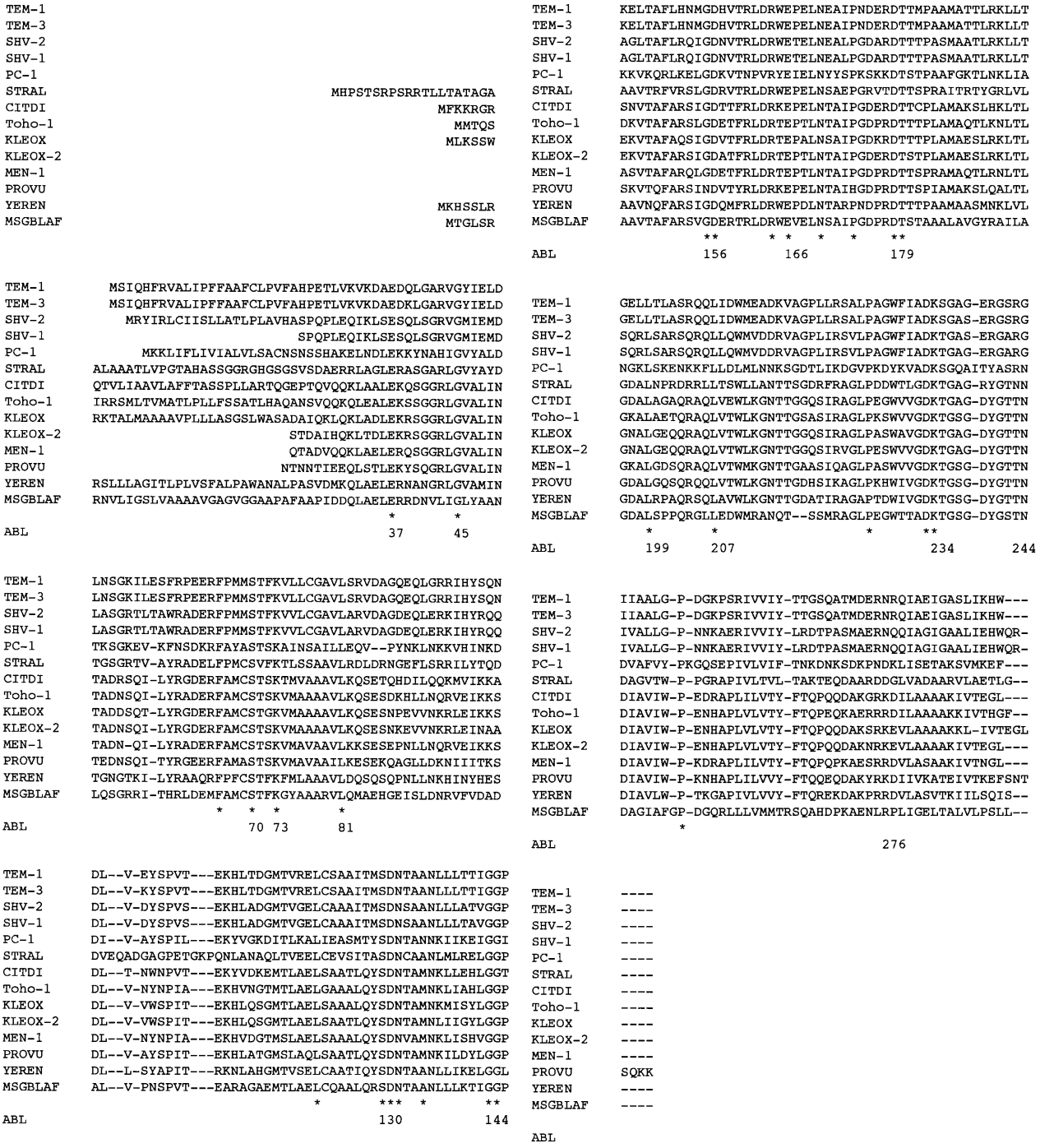

FIG. 3. Alignment of 14 amino acid sequences for class A β-lactamases. Dashes indicate gaps inserted in the alignment; asterisks indicate identical residues. ABL, standard numbering scheme for class A β-lactamases (2). References for the enzymes are as follows: Toho-1, this study; MEN-1, 4; KLEOX, 3; KLEOX-2, 37; CITDI, 38; STRAL, 32; TEM-3, 44; TEM-1, 25; SHV-2, 16; SHV-1, 5; PC-1, 15; PROVU, 35; YEREN, 42; and MSGBLAF, 46.

10-fold lower than those of the penicillin-1-sulfone derivatives sulbactam and tazobactam. This result is reasonable, since the formation of the stable acyl complex with clavulanic acid is facilitated by the cationic guanidinium moiety of the arginine residue at 220, 244, or 276 (18) whereas the residue will not have such an effect on the sulfone derivatives.

In this report, we describe the amino acid sequence deduced from the DNA sequence of the Toho-1 β-lactamase. Toho-1 contained amino acid motifs common to class A β-lactamases and was about 83% homologous to β-lactamase of *K. oxytoca* D488 and *E. coli* MEN-1. Therefore, the newly isolated β-lactamase Toho-1 is categorized as being similar to β-lactamases produced by *K. oxytoca* D488, *K. oxytoca* E23004 and *E. coli* MEN-1. The multiple alignment of Toho-1 and other β-lactamases suggested that replacement of Asn-276 by Arg with the concomitant substitution of Thr for Arg-244 is one of important

mutations responsible for the extension of the substrate specificity.

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