Roles of Residues Cys69, Asn104, Phe160, Gly232, Ser237, and Asp240 in Extended-Spectrum β -Lactamase Toho-1[∇]

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Toho-1, which is also designated CTX-M-44, is an extended-spectrum class A β -lactamase that has high activity toward cefotaxime. In this study, we investigated the roles of residues suggested to be critical for the substrate specificity expansion of Toho-1 in previous structural analyses. Six amino acid residues were replaced one by one with amino acids that are often observed in the corresponding position of non-extended-spectrum β -lactamases. The mutants produced in *Escherichia coli* strains were analyzed both for their kinetic properties and their effect on drug susceptibilities. The results indicate that the substitutions of Asn104 and Ser237 have certain effects on expansion of substrate specificity, while those of Cys69 and Phe160 have less effect, and that of Asp240 has no effect on the hydrolysis of any substrates tested. Gly232, which had been assumed to increase the flexibility of the substrate binding site, was revealed not to be critical for the expansion of substrate specificity of this enzyme, although this substitution resulted in deleterious effects on expression and stability of the enzyme.

β -Lactam antibiotics inhibit transpeptidases that synthesize the bacterial cell wall and have been the most widely utilized antibiotics for decades. Bacteria have acquired several resistance mechanisms, of which the most common is the production of enzymes called β -lactamases that hydrolyze the amide bond of the β -lactam ring of β -lactam antibiotics to inactivate them.

β -Lactamases have been classified into four classes (A, B, C, and D) according to sequence similarity (1). Although class A enzymes had been labeled originally as penicillinases, many enzymes with hydrolysis activity toward cephalosporins emerged with clinical use of oxymino-cephalosporins (18). Such class A enzymes are called “extended-spectrum β -lactamases (ESBLs),” and they have been classified into the 2be group in a functional classification proposed by Bush et al. (9). This group consists of a large number of ESBLs that evolved from TEM-1 and SHV-1 through a limited number of point mutations together with other ESBLs only distantly related to such classical penicillinases.

Among non-TEM/SHV class A ESBLs, CTX-M β -lactamases with powerful hydrolysis activity against cefotaxime are the most widespread. CTX-M enzymes exhibit less than 40% identity with TEM and SHV β -lactamases in their primary structures. Typically, they hydrolyze cefotaxime more efficiently than ceftazidime and are inhibited by clavulanate and tazobactam. Early reports of CTX-M enzymes appeared around 1990 (3, 24). The first amino acid sequence was reported for

MEN-1, later found to be identical to that of CTX-M-1, in 1992 (2). It has already been proposed that the Ser237 in MEN-1 might be responsible for the strong activity toward oxymino-cephalosporins. Since then, the CTX-M enzymes have formed a growing family of ESBLs (5, 37).

Many researchers have investigated the mechanism of substrate specificity extension in class A ESBLs, and several residues have been proposed to be responsible. Ser237 is regarded as a key residue, since its importance in substrate specificity has been reported in TEM- and SHV-type ESBLs, in an ESBL from *Proteus vulgaris*, and also in carbapenem-hydrolyzing Sme-1 (22, 34, 36). In CTX-M-4, a Ser237Ala substitution resulted in an increased $V_{\max}$ for penicillins and also in a decreased $V_{\max}/K_m$ ratio for cefotaxime and cefuroxime (17). The residue at position 240 is also regarded as having a role in substrate specificity. In TEM- and SHV-type enzymes, the replacement of Glu240 with lysine introduces the ability to hydrolyze ceftazidime and aztreonam, possibly by the formation of an electrostatic bond between the lysine residue and these substrates (22). In a kinetic study of CTX-M-9 and its derivative CTX-M-16 (which differ only by the substitution Asp240Gly), it was shown that the Asp240Gly substitution increased catalytic efficiency toward ceftazidime and aztreonam (6). A structural study indicated that ceftazidime could be inserted more deeply into the binding site of CTX-M-16 than in CTX-M-9, because of higher flexibility resulting from the Asp240Gly substitution (13). This was recently confirmed by anisotropic vibration analysis of their crystal structures at ultra-high resolution (12). All of these results suggest that the accommodation of substrates seems to occur in a different manner in CTX-M enzymes from that in TEM/SHV-type ESBLs. Substitution at position 167, which resides in the omega loop, was also proposed to explain the hydrolysis of ceftazidime by

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FIG. 1. Partial alignment of class A β -lactamases. Shown is the primary sequence of Toho-1 compared with those of other class A β -lactamases. The eight β -lactamases below Toho-1 are the ESBLs: CTX-M-1 (MEN-1) (2), CTX-M-2 (4), CTX-M-8 (7), CTX-M-9 (28), GES-1 (15), NMC-A (26), SME-1 (8), and KPC-2 (33). The next four are non-ESBLs: Blich, the β -lactamase from *B. licheniformis* (27); SHV-1 (25); TEM-1 from *E. coli* (35); and PC1 from *Staphylococcus aureus* PC1 (11). Three conserved regions critical for activity are boxed. Amino acids identical to those of Toho-1 are indicated in gray. The positions mutated in this study are shown with white letters in black, marked with asterisks. On the top of the alignment, secondary structure elements for Toho-1 are indicated. β -Strands are shown as black arrows, α -helices as black coils, and the 3_{10} helix as gray coils. These elements are labeled according to the notation used in the structural analysis of Toho-1 (19).

CTX-M-19 (30), but not in all CTX-M enzymes (38). Arg276 conserved in the CTX-M enzymes had been thought to be equivalent to Arg244 of IRT-TEM derivative enzymes, the residue which is critical for inhibition by mechanism-based inhibitors such as clavulanic acid and sulbactam (22). However, substitution of Arg276 in the CTX-M enzymes revealed that this residue is not equivalent to Arg244 of IRT-TEM enzymes, but is important for cefotaxime hydrolysis (16, 29). Mutation at position 69 in TEM-type enzymes is also known to result in resistance to the mechanism-based inhibitors (10, 22), but analysis of this residue has not been performed in CTX-M enzymes.

Toho-1, lately designated as CTX-M-44, is a CTX-M-type enzyme encoded by a plasmid in *Escherichia coli* TUH12191, which was isolated from the urine of a patient treated with β -lactam antibiotics (21). Toho-1 is the first CTX-M enzyme whose tertiary structure was solved by X-ray analysis (19). The crystal structure showed that there is no apparent difference between Toho-1 and non-ESBLs in overall tertiary structures. However, the structure suggested that Toho-1 may have higher flexibility in the B3 β -strand and Ω loop, two critical components of the active-site structure. Several residues, including Gly232, Asp240, and Phe160, were proposed to cause the flex-

ibility in Toho-1, contributing to the expansion of substrate specificity of this enzyme (19). Furthermore, the analysis of the acyl intermediate structures of Toho-1, in which the enzyme was complexed with cefotaxime, cephalothin, and benzylpenicillin, indicated that the side chain of Asn104 interacts with all of the substrates while the side chain of Asp240 interacts only with cefotaxime (32).

In this study, we prepared eight Toho-1 mutants in which the amino acid residues at positions 69, 104, 160, 232, 237, and 240 were replaced with those often observed in non-extended-spectrum β -lactamases. Figure 1 shows these residues in the partial sequence alignment with other class A enzymes. Cys69 is conserved in all the CTX-M enzymes and also in other ESBLs. Position 104 is occupied by an asparagine residue in CTX-M enzymes, but it is not conserved in the other class A β -lactamases (6, 21). The phenylalanine at position 160 is also well conserved in CTX-M β -lactamases and many other ESBLs (6), but this position is often occupied by threonine in non-ESBLs (21). Glycine occupies position 232 in many ESBLs, while an alanine residue is more common at this position in non-ESBLs (21). The serine residue at position 237 is conserved in the CTX-M group and also in other ESBLs (6, 23). Asp240 is often observed in CTX-M enzymes (6), but less so in other enzymes

FIG. 2. Stereo views of the substrate binding site in Toho-1. The mutated residues are indicated by a stick model in the C α trace of the Toho-1 structure.

(21, 23). In the tertiary structure of Toho-1, the residues Asn104, Ser237, and Asp240 are positioned in the substrate-binding cavity, while Cys69, Phe160, and Gly232 are in positions where they cannot form direct interactions with substrates (Fig. 2). Cys69 is next to the catalytic serine residue Ser70, but its side chain is not directed toward the substrate binding site. Phe160 is behind the α -helix, which contains the catalytic residue Ser70. Gln232 is in the middle of the B3 β -strand, on which are the catalytically important KTG sequence residues (19). All of the Toho-1 mutants were expressed in *E. coli* and were analyzed kinetically and for their effects on drug susceptibility.

MATERIALS AND METHODS

Bacterial strains and plasmids. *E. coli* strains CJ236 [*dut1 ung1 thi-1 relA1/pCJ105*(F' Cam^r)], MV1184 [*ara Δ(lac-proAB) rpsL thi (Δ80 lacZ ΔM15) Δ(srl-recA)306::Tn10*(Tet^r)/F'(*traD36 proAB⁺ lacI^q lacZ ΔM15*)], and BL21(DE3)pLysS [F⁻ *dcn ompT hsdS_B (r_B⁻ m_B⁻) gall(DE3) pLysS*(Cam^r)] were used for site-directed mutagenesis, subsequent genetic constructions, and overexpression of proteins, respectively. *E. coli* strain AS226 (F⁻ *thr-1 lenB6 thi-1 hsaS1 lacY1 tonA21 supE44 ampCΔ λ⁻*) was used for drug susceptibility tests. The Toho-1 gene was cloned and sequenced as described previously. A previously constructed plasmid, pET-bla, was used for the overexpression of wild-type Toho-1 (20).

Site-directed mutagenesis. All mutants other than S237A protein were constructed by the Kunkel method with the Muta-Gene *in vitro* mutagenesis kit (Bio-Rad). The plasmid pre119PA, which has the gene encoding the wild-type Toho-1 enzyme inserted into the multiple cloning region of pUC119 in the reverse direction, was utilized for the mutagenesis. The synthesized oligonucleotide primers for the mutagenesis were as follows (with the mutated residues underlined): 5'-AGA GCG ACC TGG TGG CCT ACA ATC CCA T-3' for N104A mutation, 5'-AGA GCG ACC TGG TCG ATT ACA ATC CCA TT-3' for N104D, 5'-ACC GGC AGC GGA GCA TAT GGC ACC ACC-3' for D240A, 5'-ACC GGC AGC GGA TCC TAT GGC ACC ACC-3' for D240S, 5'-CGT TTT GCG ATG GCC AGT ACC AGT AAG-3' for C69A, 5'-TTG GGT GAT GAG ACC ACG CGT CTG GAC AGA ACC-3' for F160T, and 5'-TCA TGG GTA GTG GCC GAT AAA ACC GGC-3' for G232A. The S237A mutant was constructed with the GeneTailor site-directed mutagenesis kit (Invitrogen). For the S237A mutation, a forward primer with a mutation site, 5'-TGG GCG ATA AAA CCG GCG CCG GAG ATT AT-3', and a reverse primer, 5'-GCC GGT TTT ATC GCC CAC TAC CCA TGA T-3', were synthesized. All mutagenesis was performed according to the protocol provided by

each manufacturer. The nucleotide sequence of the mutants was confirmed by DNA sequencing using standard procedures. The mutated gene was isolated by digestion with two appropriate restriction enzymes and inserted into the plasmid vector pET-bla, which was digested with the same enzymes, to yield the plasmids that express each mutant.

Expression and purification of the protein. The expression and purification of the wild-type and mutant enzymes were performed according to procedures described previously (20). The purity of the enzyme was assessed to be more than 95% by Coomassie blue staining after SDS-polyacrylamide gel electrophoresis. All mutations were confirmed by matrix-assisted laser desorption ionization-tandem time of flight (MALDI-TOF/TOF) mass spectrometry with a Bruker Ultraflex III mass spectrometer (Billerica, MA).

Antibacterial agents. The following antibiotics and chemicals were used for kinetic assays and/or drug susceptibility testing. Ampicillin and cefotaxime were purchased from Wako Chemicals (Tokyo, Japan). Benzylpenicillin, cephalothin, cephaloridine, ceftazidime, cefoxitin, imipenem, and aztreonam were purchased from Sigma Chemical Co. (St. Louis, MO). Cefuzoname was from Lederle Japan, Ltd. (Tokyo, Japan).

Kinetic assays. The activity of the purified proteins was measured by spectrophotometric assay either with a JASCO V-530 spectrophotometer or with a Shimadzu UV-2400PC spectrophotometer. The peak wavelength of each antibiotic for the measurement was set according to the previous reports (20, 21). Complete time courses of progress curves were measured at least three times for each substrate. A precise description of the analysis had been detailed previously (14, 20).

Drug susceptibility tests. MICs were determined by the broth microdilution method with Mueller-Hinton broth (Difco, Detroit, MI). The organisms were inoculated at about 5×10^5 cells per well. The MIC was defined as the lowest concentration preventing visible growth after incubation for 18 h at 37°C. The Toho-1 genes were cloned into plasmid pUC-kan-118a, a pUC118-type plasmid with the kanamycin resistance gene, to yield pBSD-H2, pBSD-C69A, pBSD-N104A, pBSD-N104D, pBSD-F160T, pBSD-G232A, pBSD-S237A, pBSD-D240A, and pBSD-D240S for expression of the wild-type, C69A, N104A, N104D, F160T, G232A, S237A, D240A, and D240S Toho-1 enzymes, respectively. These plasmids were transformed into *E. coli* AS226 and used for MIC measurement.

Expression level and stability of the enzymes. The strains used for drug susceptibility tests were grown in 4 ml of 2 $\times$ tryptone-yeast extract (TY) medium for 14 h at 37°C. The cells were collected by centrifugation, suspended in 1 ml 20 mM MES (morpholineethanesulfonic acid)-NaOH buffer (pH 6.5), and then sonicated. Lysate was centrifuged, and the supernatant was used as crude extract. The enzyme activity of crude extract was measured with 0.2 mM ampicillin as the substrate. From the initial rates and the k_{cat} values determined by kinetic analysis, the amount of enzyme was calculated. Stability of the enzymes was estimated by measuring the activity of the crude extract incubated for 5 h at 37°C.

RESULTS AND DISCUSSION

Construction, expression, and purification of the Toho-1 mutants. By site-directed mutagenesis, the residues located at positions 69, 104, 160, 232, 237, and 240 in Toho-1 were replaced by amino acids found at the same positions in non-ESBLs resulting in the C69A, N104A, N104D, F160T, G232A, S237A, D240S, and D240A mutant proteins. All of the purified enzymes appeared as a single band on SDS-polyacrylamide gels and were subjected to mass spectrometry to confirm the mutations.

Kinetic study of the N104A, N104D, S237A, D240S, and D240A mutants. The steady-state kinetic parameters of wild-type Toho-1 and its mutants are summarized in Table 1. The residues Asn104, Ser237, and Asp240 are in positions where the side chains of these residues can make direct interaction with substrates. Compared to the kinetic values of the wild-type enzyme, mutation at position 104 tended to cause higher K_m values and lower k_{cat} , resulting in lower k_{cat}/K_m values for all of the substrates. This tendency was more obvious in the N104A enzyme than in the N104D enzyme. In the N104A protein, the decreases in the k_{cat}/K_m ratio were most drastic for

TABLE 1. Kinetic parameters for hydrolysis of β -lactam antibiotics by Toho-1 and its mutants

Antibiotic	Toho-1 type	K_m (μ M)	k_{cat} (s^{-1})	k_{cat}/K_m ratio ($s^{-1} \cdot M^{-1}$)
Ampicillin	Wild type	24 ± 3	42 ± 2	1.8×10^6
	N104A	38 ± 1	27 ± 0	7.2×10^5
	N104D	25 ± 4	28 ± 2	1.1×10^6
	S237A	120 ± 10	310 ± 20	2.5×10^6
	D240S	25 ± 5	33 ± 3	1.3×10^6
	D240A	21 ± 3	27 ± 3	1.2×10^6
	C69A	20 ± 3	38 ± 1	1.9×10^6
	F160T	26 ± 2	39 ± 0	1.5×10^6
	G232A	35 ± 3	37 ± 1	1.1×10^6
Benzylpenicillin	Wild type	18 ± 2	65 ± 1	3.6×10^6
	N104A	29 ± 6	31 ± 4	1.1×10^6
	N104D	51 ± 5	38 ± 2	7.4×10^5
	S237A	46 ± 1	280 ± 10	6.1×10^6
	D240S	17 ± 1	37 ± 1	2.2×10^6
	D240A	16 ± 3	30 ± 3	1.8×10^6
	C69A	16 ± 0	54 ± 1	3.5×10^6
	F160T	22 ± 0	64 ± 3	2.9×10^6
	G232A	25 ± 1	56 ± 0	2.3×10^6
Cephalothin	Wild type	190 ± 10	790 ± 30	4.3×10^6
	N104A	140 ± 10	180 ± 1	1.2×10^6
	N104D	70 ± 2	200 ± 10	2.8×10^6
	S237A	77 ± 7	370 ± 30	4.8×10^6
	D240S	120 ± 4	410 ± 20	3.3×10^6
	D240A	98 ± 4	320 ± 10	3.3×10^6
	C69A	200 ± 10	780 ± 10	4.0×10^6
	F160T	170 ± 10	690 ± 30	4.1×10^6
	G232A	150 ± 0	620 ± 20	4.1×10^6
Cephaloridine	Wild type	280 ± 10	420 ± 10	1.5×10^6
	N104A	450 ± 2	99 ± 4	2.2×10^5
	N104D	400 ± 10	120 ± 4	3.0×10^5
	S237A	270 ± 30	320 ± 30	1.2×10^6
	D240S	250 ± 30	190 ± 20	7.6×10^5
	D240A	200 ± 20	140 ± 10	7.1×10^5
	C69A	300 ± 3	300 ± 10	1.0×10^6
	F160T	430 ± 30	470 ± 10	1.1×10^6
	G232A	290 ± 10	440 ± 10	1.5×10^6
Cefotaxime	Wild type	210 ± 5	390 ± 10	1.9×10^6
	N104A	ND ^a	ND	1.2×10^5
	N104D	ND	ND	2.4×10^5
	S237A	230 ± 40	94 ± 9	4.1×10^5
	D240S	320 ± 20	370 ± 20	1.2×10^6
	D240A	360 ± 40	440 ± 10	1.3×10^6
	C69A	350 ± 20	370 ± 20	1.0×10^6
	F160T	360 ± 10	410 ± 10	1.2×10^6
	G232A	259 ± 10	440 ± 30	1.7×10^6
Cefuzoname	Wild type	150 ± 1	530 ± 1	3.5×10^6
	N104A	ND	ND	2.8×10^5
	N104D	ND	ND	5.7×10^5
	S237A	140 ± 10	130 ± 3	9.4×10^5
	D240S	220 ± 10	430 ± 40	2.0×10^6
	D240A	260 ± 0	550 ± 20	2.1×10^6
	C69A	240 ± 10	560 ± 30	2.3×10^6
	F160T	220 ± 4	470 ± 30	2.1×10^6
	G232A	190 ± 3	630 ± 20	3.3×10^6
Aztreonam	Wild type	45 ± 2	8.3 ± 0.2	1.9×10^5
	N104A	240 ± 4	8.6 ± 0.4	3.5×10^4
	N104D	ND	ND	1.9×10^4
	S237A	31 ± 1	9.1 ± 0.4	2.9×10^5
	D240S	93 ± 11	12 ± 1	1.3×10^5
	D240A	100 ± 0	15 ± 0	1.5×10^5
	C69A	58 ± 0	4.5 ± 0.3	7.7×10^4
	F160T	69 ± 1	6.6 ± 0.4	9.6×10^4
	G232A	64 ± 7	6.7 ± 0.3	1.1×10^5

^a ND, not determined.

oxymino-cephalosporins, being 16-fold for cefotaxime and 12-fold for cefuzoname, while a 3-fold decrease was observed for penicillins. In the TEM family, position 104 is generally occupied by glutamic acid, and it is suggested that a hydrogen bond between the side chains of Glu104 and Asn132 stabilizes the catalytically essential Ser130 (22). In the acyl intermediate structure of Toho-1, an additional second hydrogen bond was observed between the side chain of Asn104 and the carbonyl oxygen of the acylamide side chain of all the substrates tested, including cefotaxime, cephalothin, and benzylpenicillin (32). The result of our kinetic analysis seems to be consistent with the results of these structural analyses. The change in the kinetic parameters was much more apparent for oxymino-cephalosporins than for penicillins especially in the N104A mutant. This suggests that the existence of an appropriate polar residue at this position is more significant for the binding of oxymino-cephalosporins.

Mutation at position 237 resulted in an increased K_m and k_{cat} for penicillins, with the larger increase of k_{cat} resulting in the increase of k_{cat}/K_m values. In contrast, there was a decrease in k_{cat} values for oxymino-cephalosporins, resulting in a 4- to 5-fold reduction in the value of k_{cat}/K_m . The K_m values for oxymino-cephalosporins were not affected by this mutation, and little change was observed in kinetic parameters for narrow-spectrum cephalosporins. For aztreonam, the k_{cat}/K_m value for aztreonam increased 1.6-fold due to a decrease in the K_m value. In the structural analysis of the cefotaxime-Toho-1 intermediate, a hydrogen-bonding interaction possibly favorable for the catalytic reaction was formed between Ser237 and cefotaxime (32). Our kinetic result is consistent with such structural features, suggesting that Ser237 plays a particular role in oxymino-cephalosporin hydrolysis by Toho-1, while the serine residue at this position has rather negative effects on the hydrolysis of classical penicillins. The decrease of k_{cat} and k_{cat}/K_m values for cefotaxime in this mutant was about 4-fold, and the k_{cat}/K_m value was still higher than that of TEM-19, which is a TEM-1 derivative with a single mutation (G238S) which has a 150-fold-increased k_{cat}/K_m value for cefotaxime (31). This result indicates that Ser237 is not the only residue which causes the expansion of substrate specificity in Toho-1.

Replacement of the aspartic acid residue at position 240 with serine or alanine resulted in a moderate decrease in the k_{cat}/K_m values for all substrates. All of the changes in k_{cat}/K_m values were 2-fold or less. For penicillins and narrow-spectrum cephalosporins, the decrease of the k_{cat}/K_m ratio was due to decrease of k_{cat} . For oxymino-cephalosporins, the decrease was due to the increase of K_m . There was only a minor difference between the D240A and D240S mutants in terms of their kinetic parameters. Asp240 resides at the end of the B3 β -strand, almost on the edge of the substrate-binding cavity and exposed to the solvent (Fig. 2). In the crystal structure of β -lactamase from *Bacillus licheniformis*, the side chain of Ser240 is hydrogen bonded to the main-chain oxygen atom of Asn170 in the omega loop, but no such interaction is observed in Toho-1, implying that the lack of interaction may cause the flexibility of strand B3 and the omega loop in Toho-1 (19). Subsequent structural analysis of the cefotaxime intermediate of Toho-1 showed that there was an interaction between the Asp240 side chain and the amino group in the aminothiazole ring of the acylamide side chain, which wasn't observed in the

benzylpenicillin intermediate structure, suggesting that such interaction could promote oxymino-cephalosporinase activity of CTX-M enzymes (32). The result of our kinetic analysis has revealed that the substitution causes little change to the flexibility of the active-site components and that there is no specific interaction between this residue and oxymino-cephalosporins.

Kinetic study of C69A, F160T, and G232A mutants. The residues Cys69, Phe160, and Gly232 are not reported to have direct interactions with substrates. The mutations at positions 69 and 160 had little effect on the activity toward penicillins and cephalothin. A decrease in k_{cat}/K_m values for cephaloridine and oxymino-cephalosporins was observed, but it was less than 2-fold in these mutants. In the C69A mutant, such a decrease was caused both by the increase of K_m and the decrease of k_{cat} . In the F160T mutant, the decrease was mainly due to the increase in K_m value. In these mutants, the hydrolytic activity toward aztreonam also decreased 2-fold as a result of both the increase of K_m and decrease of k_{cat} . Previous studies of TEM-type enzymes with a mutation at position 69 have led to the idea that the hydrophobicity and the steric constraints at this position are critical in fixing other residues, such as Ser70 and the residues at positions 237 and 238 (on the β -strand B3) that are essential for the binding and hydrolysis of substrates (10, 22). In CTX-M enzymes and several other ESBLs, position 69 is occupied by cysteine, which is not observed in non-ESBLs. Our result suggests that the role of this cysteine residue in expansion of substrate specificity is limited in Toho-1.

Structural comparison between Toho-1 and non-ESBLs has suggested that in non-ESBLs a hydrogen bond between the side chain of Thr160 and that of Ser/Thr181, which connects the N and C termini of the omega loop, stabilizes this loop conformation and lowers the flexibility of the substrate binding site. The loss of such a hydrogen bond could increase the flexibility of the omega loop, resulting in the ability of Toho-1 and some other ESBLs to hydrolyze expanded-spectrum cephalosporins (19). Since Ser/Thr181 is also well conserved in CTX-M enzymes, we replaced phenylalanine at position 160 in Toho-1 with threonine, expecting this mutation to introduce the hydrogen bond observed in non-ESBLs. The mutation of F160T in Toho-1 did cause a decrease in k_{cat}/K_m for cephaloridine, oxymino-cephalosporins, and aztreonam, but the change was not drastic. This indicates that Phe160 is not critical for the substrate specificity expansion of this enzyme.

The replacement of the glycine residue at position 232 by alanine also resulted in little change in the substrate specificity. The k_{cat}/K_m values for penicillins and aztreonam decreased about 1.7-fold as a result of both the increase in K_m and the decrease in k_{cat} . Glycine occupies position 232 in many ESBLs, including the CTX-M enzymes (Fig. 1). Since there are glycine residues at positions 232, 236, and 238 on the B3 β -strand of many ESBLs, including Toho-1, it was proposed that the presence of these glycine residues increased the flexibility of the β -strand, making it possible for the enzyme to bind to bulky substrates like expanded-spectrum cepheims (19). The result of our kinetic analysis indicates that the glycine residue at position 232 probably does not play a specific role in the hydrolysis of oxymino-cephalosporins.

Susceptibility toward antibiotics. Table 2 shows MICs of β -lactam antibiotics against *E. coli* AS226 expressing Toho-1

TABLE 2. β -Lactams MICs for *E. coli* AS226 producing Toho-1 enzymes

Antibiotic	MIC (μ g/ml) for <i>E. coli</i> AS226 with plasmid ^a :									No plasmid
	pBSD-H2 (wild type)	pBSD-N104A (N104A)	pBSD-N104D (N104D)	pBSD-S237A (S237A)	pBSD-D240S (D240S)	pBSD-D240A (D240A)	pBSD-C69A (C69A)	pBSD-F160T (F160T)	pBSD6 (G232A)	
Ampicillin	256	256	1,024	4,096	512	512	1,024	512	128	1
Cephalothin	2,048	512	2,048	2,048	2,048	2,048	2,048	2,048	128	2
Cephaloridine	1,024	32	256	2,048	1,024	1,024	1,024	512	8	2
Cefotaxime	256	16	256	32	1,024	512	1,024	256	2	<0.125
Cefuzoname	2,048	16	64	1,024	256	1,024	2,048	512	2	<0.125
Ceftazidime	0.25	<0.125	<0.125	0.5	4	8	4	1	0.25	<0.125
Aztreonam	64	8	1	128	64	128	64	16	2	<0.125
Cefoxitin	1	1	2	2	1	1	2	2	2	1
Imipenem	<0.125	0.125	0.125	<0.125	<0.125	0.125	<0.125	<0.125	<0.125	<0.125

^a The phenotype (wild type or mutant) is shown in parentheses.

and its mutants. Compared to the strain producing the wild-type Toho-1, the strains producing the N104A and N104D proteins exhibited a similar or higher level of resistance to ampicillin, but were more sensitive to most of the other β -lactams, especially to cephaloridine (32- to 128-fold) and aztreonam (8- to 64-fold). The decrease in resistance was more drastic in the N104A protein-producing strain than in the N104D protein-producing strain, while the resistance to aztreonam was lower in the N104D protein-producing strain. In the S237A protein-producing strain, an increase in resistance to ampicillin and a decrease in resistance to cefotaxime were observed. The D240S- and D240A protein-producing strains showed a level of resistance similar to that of the wild-type-producing strain for most of the antibiotics tested, with the exception of increased resistance to ceftazidime in both strains and decreased cefuzoname resistance in the D240S protein-producing strain. The C69A protein-producing strain showed higher resistance to ceftazidime. The F160T protein producer had slightly higher resistance to ceftazidime and was a little more sensitive to cefuzoname and aztreonam. All of these changes were 4-fold or less. The G232A protein-producing strain was revealed to be more sensitive to most of the cephalosporins and aztreonam, showing 128-fold, 256-fold, and 1,000-fold decreased levels of resistance to cephaloridine, cefotaxime, and cefuzoname, respectively. On the whole, with the exception of the G232A protein-producing mutant, the results of the susceptibility tests were consistent with those of the kinetic analyses. Subsequently, we examined the expression level and stability of the mutants, since MIC values can be affected to a large degree by changes in these parameters.

Expression level and stability of the enzymes. The expression level and stability of the enzymes in the strains used for the susceptibility test were estimated approximately by measuring β -lactamase activity in crude extract. The expression level of the C69A mutant was 1.5 times higher than that of the wild-type enzyme. The N104D, D240S, and D240A mutants were even more highly expressed, in a range from 2- to 4-fold that of the wild-type enzyme. The expression levels of the D104A, F160T, and S237A mutants were decreased, being in a range between 50% and 80% that of the wild-type enzyme. The most drastic decrease in expression level was observed for G232A protein, which was only 20% of the wild-type enzyme. For stability estimation, the remaining activity of each enzyme

after 5 h of incubation at 37°C was measured. For the wild-type enzyme, the remaining activity was 90%. A severe decrease of activity was observed in the C69A, F160T, and G232A mutants, with remaining activities of 29%, 17%, and 16%, respectively. A large decrease in expression and stability of the G232A mutant is likely to be the cause of the large decrease in MIC for *E. coli* expressing this enzyme. The residues Cys69, Phe160, and Gly232, whose mutation had little effect on substrate specificity of Toho-1, seem to be important in maintaining stability of the enzyme.

Our main purpose in this study was to investigate the hypotheses based on the structural analysis of Toho-1, through systematic mutagenesis followed by kinetic analyses of this enzyme and by susceptibility testing. On the whole, the kinetic parameters for "classical" substrates were not much affected by the mutations, while in some cases those for oxyimino-cephalosporins and aztreonam were more influenced. The ratio of k_{cat} to K_m for cefotaxime relative to ampicillin (k_{cat}/K_m value for cefotaxime divided by that of ampicillin) is 1.1 for the wild-type enzyme, while the ratios are 0.16 for N104A enzyme, 0.21 for N104D enzyme, 0.16 for S237A enzyme, 0.91 for D240S enzyme, 1.0 for D240A enzyme, 0.53 for C69A enzyme, 0.80 for F160T enzyme, and 1.5 for G232A enzyme. These values indicate that the substitutions of Ser237 and Asn104 have a clear effect on the expansion of substrate specificity. Substitution of Cys69 and Phe160 has much less effect, while Gly232 and Asp240 are not involved at all. The fact that the N104A/D and S237A mutants still retain activity for cefotaxime suggests that the cefotaxime-hydrolyzing activity of Toho-1 is not due to just one residue, but that it relies on the more general architecture of the active site. Since the hydrolytic activity of Asn104 mutants decreased for most of the substrates, this residue seems to participate not only in the substrate specificity expansion, but also in the general substrate recognition and hydrolysis reaction in Toho-1. As a consequence, our study shows that Ser237 does play the role that was proposed by previous structural analysis and that Asn104 can have multiple functions both for general substrate recognition/hydrolysis and for substrate specificity expansion, while the residues at positions 69, 160, 232, and 240 were clearly defined as not being critical to substrate specificity expansion.

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