Table 3: Comparison of Lys73 Configuration for Toho-1 and Other Class A β-Lactamases

enzyme	identity[a] (%)	PDB[b]	rmsd (Å)[c]	distance to K73 Nζ (Å)[d] S70 Oγ	S130 Oγ	N132 Oδ1	E166 Oε1	Wat185 O
Toho-1 (C1)[e]				2.71[h]	3.99	2.92[h]	2.75[h]	3.06[h]
Toho-1 (C2)[f]				2.93[h]	2.70[h]	3.06[h]	4.36	4.72
E166A		1BZA	0.274	2.92[h]	3.14[h]	2.76[h]		
K1[g]	71.5	1HZO (a)	0.706	2.77[h]	3.97	2.95[h]	2.91[h]	2.84[h]
MYC	44.3	1MFO	2.015	2.89[h]	3.27	2.98[h]	3.26	
PER-1	25.4	1E25	2.667	2.75[h]	3.62	2.68[h]	3.55	
PC1	35.1	3BLM	1.950	2.54[h]	3.71	2.69[h]	2.83[h]	3.86[h]
TEM-1	38.3	1BTL	2.101	2.90[h]	4.17	2.97	3.42	
SHV-1	39.3	1SHV	2.344	2.84[h]	3.82	3.28	3.28	
BLICH[g]	41.2	4BLM (a)	1.213	2.83[h]	3.13[h]	2.56[h]	3.37	
		4BLM (b)	1.218	2.52[h]	3.24[h]	2.58	3.08[h]	3.62
ALBS	42.0	1BSG	1.224	2.77[h]	3.28	2.85[h]	3.27	
BS3[g]	40.9	1I2S (a)	0.948	2.69[h]	3.08[h]	2.86[h]	3.42	
NMC-A	49.0	1BUE	1.470	2.88[h]	3.13[h]	2.79[h]	3.44	
SME-1[g]	46.9	1DY6 (a)	1.424	2.81[h]	3.04[h]	2.63[h]	3.37	

[a] To amino acid sequence of Toho-1 β-lactamase. [b] PDB entry code; letters in parentheses indicate the molecule coordinate used in distance measurement. [c] rmsd from Toho-1 for all Cα atoms. [d] Distance between Lys73 Nζ and S70 Oγ, S130 Oγ, N132 Oδ, E166 Oε1, and a water molecule corresponding to Wat185 O in Toho-1. [e] C1 denotes conformation 1 of the Lys73 side chain. [f] C2 denotes conformation 2 of the Lys73 side chain. [g] Enzymes with two molecules in an asymmetric unit according to X-ray structural analysis, where (a) denotes the structure of the A molecule and (b) denotes the structure of the B molecule in each coordinate file. Enzymes: E166A, Toho-1 E166A mutant; K1, ESBL from *P. vulgaris* K1; MYC, *Mycobacterium fortuitum*; PER-1, *Pseudomonas aeruginosa*; PC1, β-lactamase from *S. aureus* PC1; TEM-1, *E. coli*; SHV-1, *Klebsiella pneumoniae*; ALBS, *S. albus* G; BS3, *B. licheniformis* BS3; BLICH, *B. licheniformis* 749/C; NMC-A, carbenicillinase from *Enterobacter cloacae*; SME-1, *Serratia marcescens*. [h] Denotes the hydrogen-bonding distance between Lys73 Nζ and each atom.

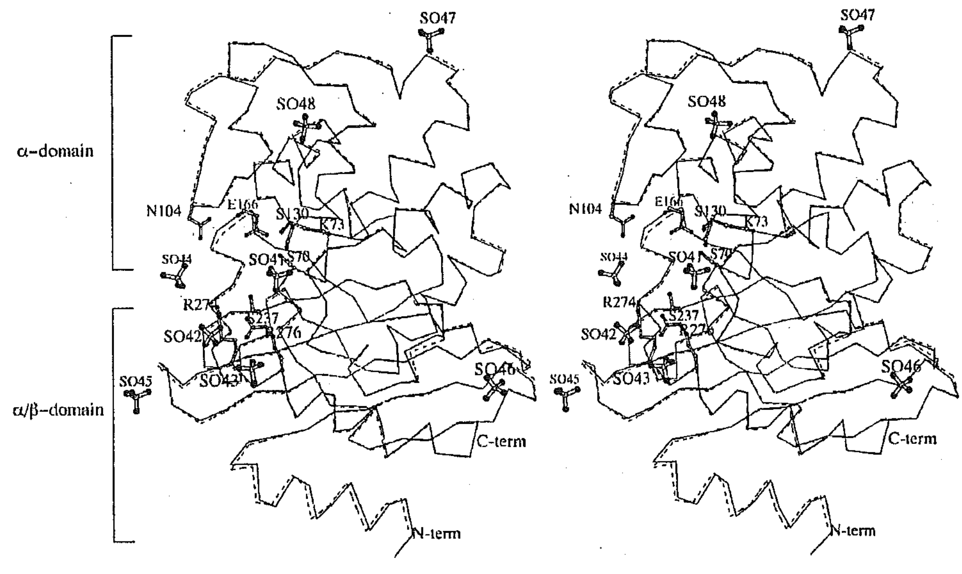

FIGURE 1: Stereoview of superimposed structures of wild-type Toho-1 and the E166A mutant. Bonds between Cα atoms in the wild-type enzyme are shown as solid lines, and those of the E166A mutant are shown as broken lines. Several residues mentioned in this study and the eight sulfate ions (SO41−SO48) observed in the wild-type enzyme are shown. The figure was generated using Molscript (*54*).

A (Figure 4) (*27*). The conformation of Lys73 is not necessarily correlated with any particular crystallization condition: Toho-1, the β-lactamase from *S. aureus* PC1, and the β-lactamase from *B. licheniformis* 749/C were all crystallized using ammonium sulfate as a precipitant, while the crystals of the β-lactamase from *P. vulgaris* K1 were obtained using PEG 6000. Similarly, no clear correlation was observed between the pH conditions for crystallization and the conformation of Lys73: Toho-1, *S. aureus* PC1 β-lactamase, *P. vulgaris* K1 β-lactamase, and *B. licheniformis* 749/C β-lactamase were crystallized at around pH 6.0, 8.5, 6.25, and 5.5, respectively. The β-lactamases listed in Table 3 were crystallized in a wide range of pH from 4.5 to 8.5.

The electron density map suggests that the side chains of Ser237 may also have alternative conformations. In the model, the hydroxyl group of Ser237 points into the active site cavity, hydrogen-bonding to the sulfate ion SO41 (Figure 3). Although the density is not clear enough to build a model for the second conformation, the map intimates that the side chain of Ser237 might rotate toward the N-terminal side of the B3 strand (Figure 2b).

Water Molecules in the Vicinity of Glu166. Two water molecules, Wat41 and Wat185, are located in positions close to the carboxyl group of Glu166, at distances from Glu166 Oε1 of 2.69 and 2.66 Å, respectively (Figure 3), though no hydrogen bonds are formed between Glu166 and these water

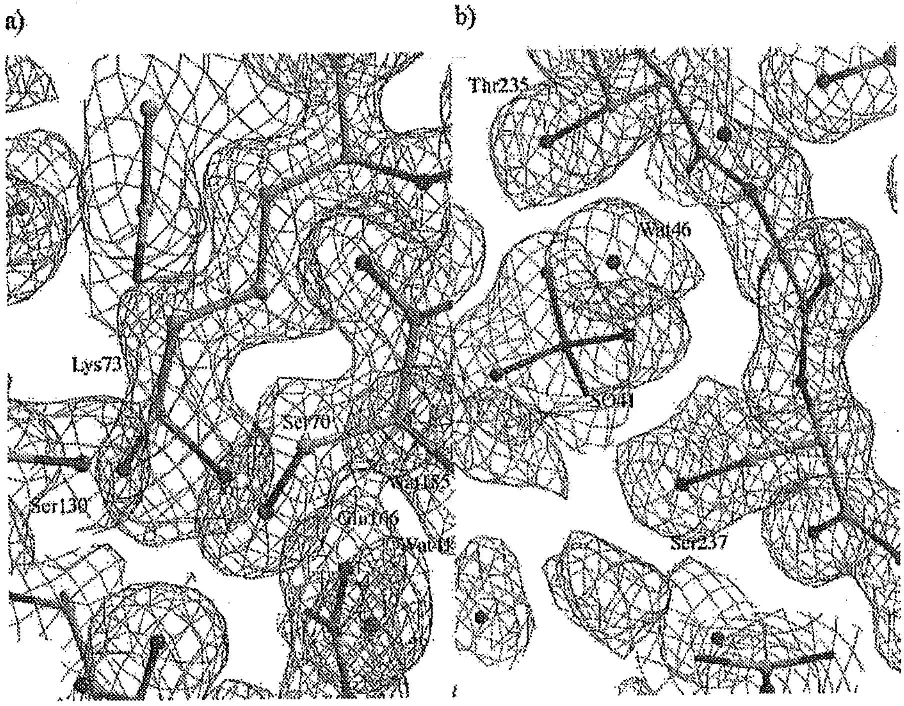

FIGURE 2: $2F_o - F_c$ electron density map around (a) Lys73 and (b) Ser237 at a contour level of 2σ. The map was calculated using the program CNS (*20*), omitting the side chain of Lys73 and Ser237. The figures were generated using Bobscript (*55*) and Raster3D (*56, 57*).

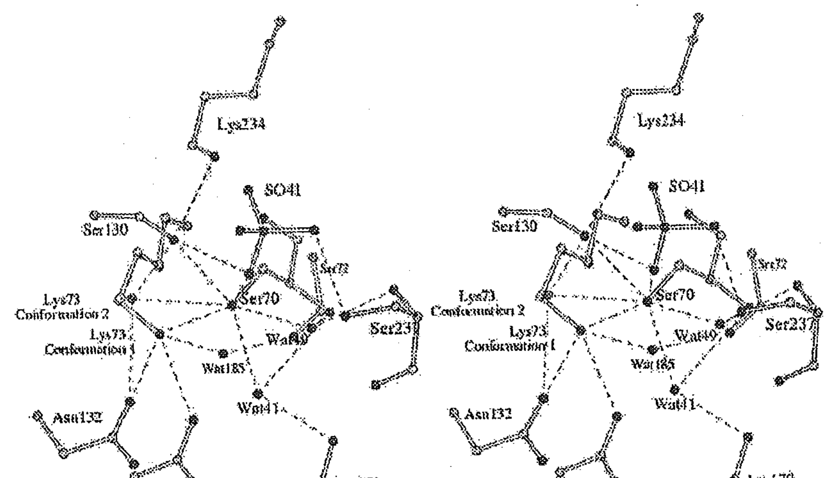

FIGURE 3: Stereoview of active site residues in the vicinity of Lys73. Hydrogen bonds are highlighted by dashed lines. Hydrogen bonds formed between Lys73 and other atoms in conformation 2 are indicated in red. The figure was generated using Molscript.

molecules. Wat41 is hydrogen-bonded to Ser70 Oγ, the backbone N of Ser70, and Asn170 Oδ1, and Wat185 is hydrogen-bonded to Ser72 Oγ and Nζ of Lys73 in conformation 1. No corresponding water molecules were observed in the structure of the E166A mutant, indicating the necessity of the Glu166 side chain to retain these water molecules. A water molecule corresponding to Wat41 exists in all the class A β-lactamase structures with the sole exception of Sme-1 and is regarded as a hydrolytic water molecule that is essential in the deacylation reaction (*25−34*). Among the class A enzymes, water molecules equivalent to Wat185 are only found in *S. aureus* PC1 β-lactamase, *P. vulgaris* K1 β-lactamase, and molecule B of *B. licheniformis* 749/C β-lactamase (*27*). In these enzymes, the conformation of the Lys73 side chain is identical or similar to conformation 1 (Figure 4). Thus, the second water molecule appears to require the Lys73 side chain to take conformation 1, in which the side chain is hydrogen-bonded to this water molecule.

Disposition of Sulfate Ions. Eight sulfate ions are modeled in the structure of Toho-1 (Figure 1). This model includes a much larger number of sulfate ions than the E166A mutant structure, in which only two sulfate ions are positioned. This difference is expected to follow simply from the difference in resolution or possibly from the difference in the quality

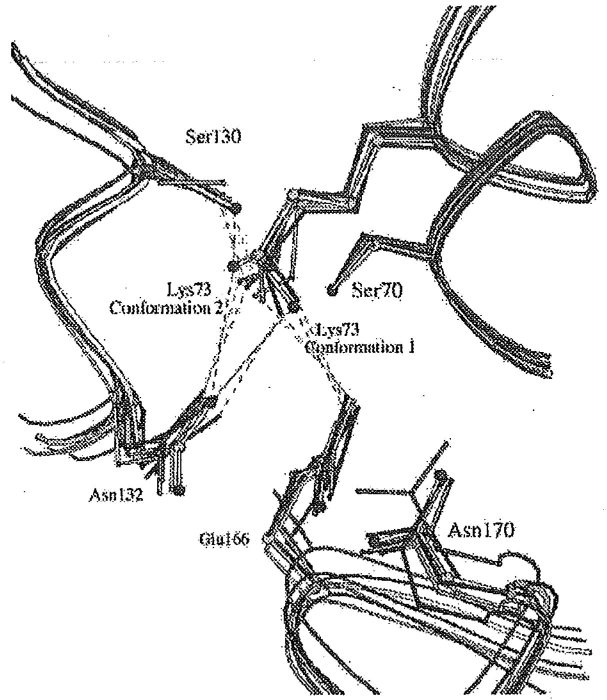

FIGURE 4: Comparison of Lys73 conformations. Structures of several enzymes listed in Table 3 are superimposed. The model of wild-type Toho-1 enzyme is depicted with the same color code as in Figure 3, with the E166A mutant rendered in cyan, β-lactamase from *P. vulgaris* K1 in blue, β-lactamase from *S. aureus* PC1 in green, TEM-1 in yellow, molecule A of *B. licheniformis* 749/C in red, and molecule B in pink. Hydrogen bonds formed between Lys73 Nζ and other residues are indicated by black broken lines for the wild-type Toho-1 enzyme and with broken lines of the corresponding color for other β-lactamases. The hydrogen bond between Lys73 Nζ and Ser70 Oγ was omitted in all structures for convenience.

of the crystals used for analysis. Of the eight sulfate ions, three sulfate ions, SO41, SO42, and SO43, are positioned in or near the active site cavity (Figures 1 and 5). SO41 is bound at the center of the active site, hydrogen-bonded to Ser70 Oγ, Ser130 Oγ, and Ser237 Oγ. In most structures of class A β-lactamases crystallized with ammonium sulfate as a precipitant, including the E166A mutant of Toho-1, a sulfate ion has been observed in the position equivalent to SO41 (*19, 26, 28, 31, 33*). Superimposing the Toho-1 structure on the structures of the acyl-enzyme complexes reveals that SO41 is in a position corresponding to the C3 (penicillins) or C4 (cephalosporins) carboxyl group of the substrates in the acyl-enzyme (*35–37*).

SO42 and SO43 are bound to the positively charged region formed by two arginine residues at positions 274 and 276 (Figure 5). Arg274 is unique to Toho-1, whereas the Arg/Lys residue at position 276 is highly conserved in the CTX-M-type enzymes (*28, 38*).

Kinetic Study. The steady-state kinetic parameters k_{cat} and K_m were determined for a set of good β-lactam substrates (Table 4). The data indicated that the Toho-1 β-lactamase exhibited a broad-spectrum activity profile. The enzyme was active against both penicillins and cephalosporins. The best substrates of the Toho-1 β-lactamase were the first-generation cephalosporins such as cephalothin and cephaloridine. Cefotaxime was also a good substrate of Toho-1, but ceftazidime was poorly hydrolyzed by this enzyme. This can be attributed mainly to the large K_m value (K_m = 7.9 mM). Comparison of the kinetic constants of cephaloridine and ceftazidime indicates that the presence of a bulky carboxypropioxyimino group on the 7β lateral chain affects the efficiency of the β-lactamase. Interestingly, new β-lactam compounds such as cefdinir, cefcapene, cefepime, and S1090 (fourth-generation cephalosporins) behaved as good substrates of Toho-1. Among the mechanism-based inactivators, sulbactam was also a good substrate of Toho-1. Cefoxitin (a cephamycin) and moxalactam (an oxacephem) were poor substrates, and interaction with Toho-1 led to the formation of a rather stable acyl enzyme, characterized by a deacylation-limiting step and by low acylation efficiency (k_2/K < 800 M^{-1} s^{-1}) (Table 5). All tested carbapenems behaved as poor substrates of Toho-1, with meropenem exhibiting the highest acylation efficiency ($k_2/K = 10^5$ M^{-1} s^{-1}). Interestingly, all deacylation rate constants and K_m values were very low. Finally, tazobactam behaved as a poor inactivator of Toho-1, with an acylation efficiency of 450 M^{-1} s^{-1}.

Table 4: Kinetic Parameters of Toho-1 against Various Antibiotics

class (generation)[a]	antibiotic	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (μM^{-1} s^{-1})
penicillin	benzylpenicillin	68	23	3.0
	piperacillin	13	8.0	1.7
cephalosporin (1)	nitrocefin[b]	160	59	2.7
	cephaloridine	200	280	0.7
	cephalothin	480	39	12
cephalosporin (3)	cefotaxime	250	120	2.1
	ceftazidime	21	7900	0.0013
	ceftizoxime	ND	ND[c]	0.12
	cefpodoxime	ND	ND[c]	0.11
cephalosporin (4)	cefepime	ND	ND[c]	0.068
	cefdinir	2.1	1.4	1.5
	cefcapene	100	46	2.2
	S1090	21	3.8	5.5
inhibitor	sulbactam	0.18	0.31	0.58

[a] Generation of cephalosporins is indicated in parentheses. [b] Classified as first generation according to structure. [c] Too high to determine.

Table 5: Kinetic Parameters of Toho-1 against Poor Substrates and Inhibitors

class[a]	antibiotics	K_{cal}[b] (μM)	k_{+2} (s^{-1})	k_{+3} (s^{-1})	k_{+2}/K (M^{-1} s^{-1})
cephamycin	cefoxitin	8	ND	6.1×10^{-3}	760
oxacephem	moxalactam	20	ND	5.0×10^{-3}	250
carbapenem	imipenem	2.4[c]	ND	5.6×10^{-3}	2250
	meropenem	0.09[c]	ND	8.9×10^{-3}	1.0×10^5
	faropenem	0.01	ND	9.6×10^{-3}	1900
	S4661	0.36	ND	4×10^{-3}	1.1×10^4
inhibitor	tazobactam	ND	ND	ND	450

[a] Generation for cephems. [b] K calculated from measured values. [c] Measured as K_i value in competition experiments.

DISCUSSION

Alternative Conformations of Lys73. As a whole, the active site structure of Toho-1 is quite similar to that of other class A β-lactamases. The most significant exception is the conformation of Lys73. The present structural analysis revealed the existence of alternative conformations of Lys73 for the first time among class A β-lactamases. Although a number of previous structural and mutagenesis studies have indicated that Lys73 is expected to play a critical role in

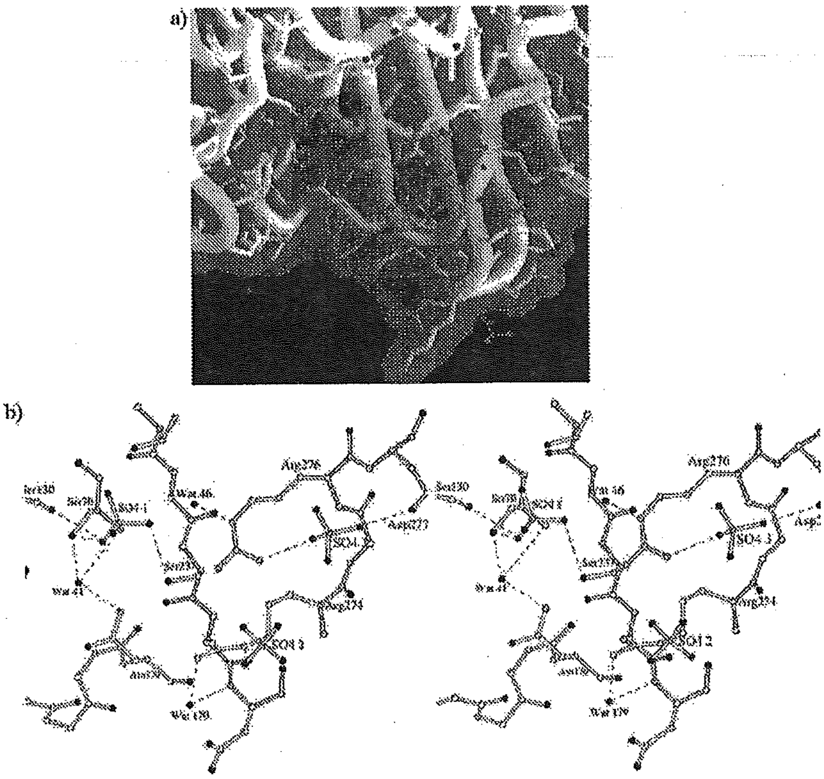

FIGURE 5: Sulfate ions binding near the active site. (a) Surface representation of the active site cleft. The transparent surface is colored according to the electrostatic potential from −30 kT (red) to +30 kT (blue). Backbones of the protein are shown as coils, and side chains within 1.5 Å of the protein surface are indicated. Water molecules are depicted in red, and sulfate ions are illustrated with green S atoms and magenta O atoms. (b) Stereoview of sulfate ion binding site near the catalytic cleft. Hydrogen bonds are indicated by dashed lines. The surface potential was calculated by GRASP (*58*).

catalysis, the role of Lys73 remains unclear (*3*, *39*). Kinetic analysis of the K73R mutant of *Bacillus cereus* 569/H β-lactamase I and the K73A mutant of *B. licheniformis* β-lactamase showed that Lys73 was involved both in acylation and in deacylation (*40*, *41*), and it was concluded that Lys73 would work to maintain an optimum electrostatic environment for fully efficient catalytic reaction, not as a general base. On the other hand, structural analysis of the acyl-enzyme suggested the role of Lys73 as a candidate of the general base to accept a proton from Ser70 prior to acylation (*35*). To function as a general base, Lys73 should be in an unprotonated state; however, the protonation state of this residue remains controversial (*42*, *43*).

The role of Glu166 in acylation is also uncertain, while it is known to act as a general base in deacylation (*35*, *44*). Precise mutagenesis studies showed that the replacement of Glu166 caused a drastic decrease in both the acylation and deacylation rates (*39*, *40*, *45*). The high-resolution structure of TEM-1 β-lactamase complexed with an acylation transition-state analogue revealed that the Glu166 side chain was protonated, which proposed that Glu166 activated Ser70 via a catalytic water molecule in acylation (*46*). Through structural analysis of the E166A mutant of *B. licheniformis* β-lactamase, it was found that removal of the Glu166 side chain did not change the conformation of Lys73. It suggested the existence of no strong salt bridge between Glu166 and Lys73, precluding the possibility of proton transfer between the two in the acylation process (*47*). In Toho-1, removal of the Glu166 side chain changes the position of Lys73 significantly. Conformation 1 of Lys73 indicates possible proton transfer between Lys73 and Glu166; conformation 2, between Lys73 and Ser130. Considering the present structural analysis and the results of previous studies, we speculate that there are several proton-relay pathways in acylation and that Lys73 would be involved in proton transfer in at least some of these pathways. Two possible speculated proton-transfer pathways in acylation are as follows: (1) after proton transfer from the Lys73 ammonium group to the Glu166 carboxylate, the unprotonated Lys73 would act as a general base to activate Ser70, or (2) the substrate carboxylate oxygen would accept a proton from the hydroxyl group of Ser130, which, then unprotonated, would accept a proton from Lys73, and finally the neutralized Lys73 would activate Ser70, as proposed by Ishiguro et al. (*48*). Proton relay involving Ser130 might represent a secondary pathway, or a water molecule might substitute for Ser130. This follows from the observation that mutants of the *Streptomyces albus* G enzyme with Ser130 replaced with alanine or glycine retained significant activity (*49*). Although this structural analysis has indicated the possibility of various proton-transfer pathways in catalysis, further research that directly shows the protonation state of these residues in each step of catalysis will be necessary before any concrete conclusions can be drawn.

The difference in Lys73 conformation observed among the structure-solved β-lactamases might reflect some subtle but significant differences in the active site properties (Table 3). The conformation of Lys73, together with the existence of the second water molecule in the active site, implies that Toho-1, *P. vulgaris* K1 β-lactamase, *S. aureus* PC1 β-lac-

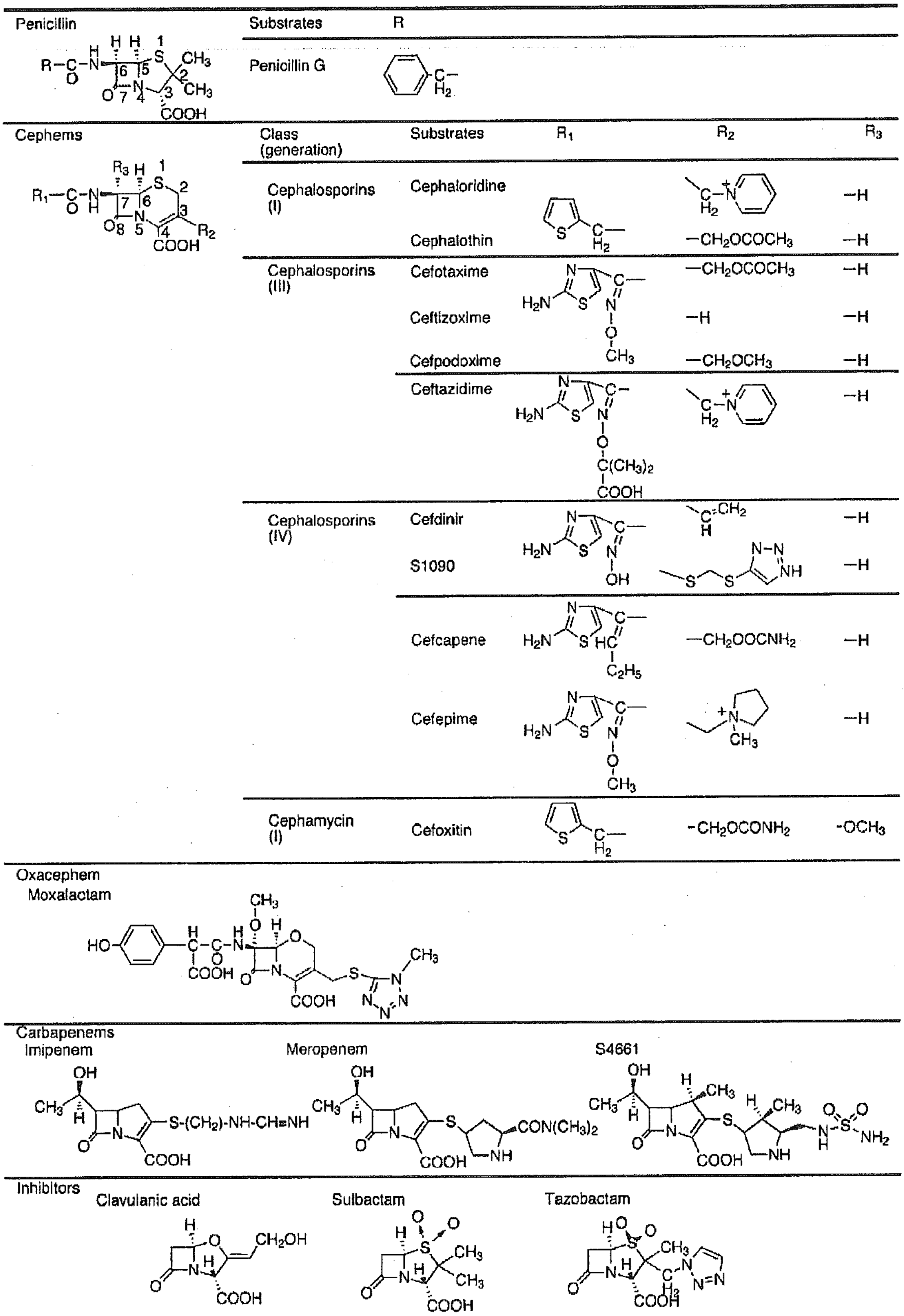

FIGURE 6: Structures of selected β-lactam antibiotics used in this study.

tamase, and possibly *B. licheniformis* β-lactamase share a common active site environment. As the β-lactamase from *P. vulgaris* K1 is an ESBL highly homologous to Toho-1, one of the necessary conditions for extending the substrate specificity of CTX-M-type ESBLs might be to maintain conformation 1 of Lys73, although this could not be the sole condition.

The Role of Water Molecules in the Vicinity of Glu166. From the comparison of the structures between wild-type Toho-1 and the E166A mutant, we speculate that Glu166 is necessary to retain Wat41 and Wat185, though there is no hydrogen bond formation between Glu166 and these water molecules. We presume that the existence of glutamate at position 166 might affect the active site environment to retain these water molecules. Since the distance between Glu166 and these water molecules is sufficiently short, a slight movement of Glu166 and/or water molecules might be sufficient to form the hydrogen bonds, thus allowing Glu166 and these water molecules to participate in the proton transfer.

The Role of Ser237. In the Toho-1 structure, the hydroxyl group of Ser237 points into the active site cavity. It is hydrogen-bonded to sulfate ion SO41, which is suggested to correspond to the C3 (penicillins) or C4 (cephalosporins) carboxylate group of β-lactam antibiotics. This interaction is suggestive of the direct participation of Ser237 in substrate binding. Position 237 is occupied by alanine in many class A β-lactamases but is populated with serine or threonine in the CTX-M-type β-lactamases, carbenicillinases, and several other β-lactamases (*28, 38*). The crystal structures of several enzymes with Ser/Thr at position 237 have been solved, and in all of these structures except Toho-1, it is found the hydroxyl group of Ser237 points toward the direction of the N-terminus of the B3 strand, not toward the active site. For example, the Ser237 side chain of *P. vulgaris* K1 β-lactamase is hydrogen-bonded to water molecule Wat547 over β-sheet B3. In the carbapenemases Sme-1 and NMC-A, the hydroxyl group of Ser237 is hydrogen-bonded to the side chain of Arg220, which is a site often occupied by a serine residue in CTX-M-type enzymes (*29, 30*). The unique conformation of Ser237 observed in Toho-1 appears to be caused by the existence of sulfate ion SO41 bound in the center of the active site, which is absent in the other structure-solved enzymes with Ser/Thr at position 237. From comparison of the crystal structures and the corresponding crystallization conditions, the side chain of Ser237 is considered to rotate as the sulfate ion and possibly substrate bind to the catalytic site. The S237A mutant of *P. vulgaris* K1 β-lactamase has been shown to retain its penicillinase activity, whereas the cephalosporinase activity, particularly for oxyimino cephalosporins, decreased dramatically, mainly due to the decrease in k_{cat} (*50*). Taken together, the hydroxyl group of Ser237 is hypothesized to be hydrogen-bonded to the C3 (penicillins) or C4 (cephalosporins) carboxyl group of substrate in the formation of the Michaelis complex and to change conformation as the carboxylate of the substrate changes position. The hydroxyl group might be involved in the exact positioning of the substrates in the hydrolytic reaction, particularly oxyimino cephalosporins. In the hydrolysis of carboxypropylimino cephalosporins, the carboxyproxylimino group might inhibit the proper functioning of Ser237, resulting in the apparently small k_{cat} and large K_m obtained in kinetic analysis.

Disposition of Sulfate Ions. The structural analysis of Toho-1 reveals that SO42 and SO43 are bound to the positively charged region formed by two arginine residues, Arg274 and Arg276 (Figure 5). Toho-1 mutants with Arg274 and/or Arg276 replaced with nonpositively charged residues exhibited an approximately 50% decrease in k_{cat}/K_m for the third-generation cephems, yet without obvious change for penicillins and first-generation cephems (unpublished data). The substitution of asparagine for arginine at position 276 of the ESBL CTX-M-4 resulted in lower resistance to oxyimino cephalosporins, whereas the level of resistance to penicillins remained unchanged (*51*). Considering these results and the fact that the arginine residue at position 276 is highly conserved in CTX-M-type enzymes, this positively charged region is speculated to function as a pseudo-substrate-binding site that would interact with the methoxyimino group of cefotaxime and other third-generation cepharosporins prior to binding in the active site. This region may also help to lead the substrate into the final binding position or facilitate binding to the active site.

Substrate Profile of Toho-1. It is the characteristic of Toho-1 and other CTX-M-type ESBLs to hydrolyze third-generation cephalosporins effectively (*9–15*). Toho-1 hydrolyzes ceftazidime far less efficiently than third-generation cephalosporins with a methoxyimino group in the 7β side chain. The size and constitution of the 7β side chain therefore appear to be critical in the processes of substrate binding and hydrolysis. The bulky carboxypropoxyimino group in the 7β side chain may cause steric conflict or unfavorable electrostatic interaction and/or repulsion with the enzyme.

Toho-1 exhibited very low activity for cefoxitin, a second-generation cephamycin, probably due to the 7α-methoxy group. As shown in the structural analysis of the β-lactamase from *B. licheniformis* BS3, the 7α-methoxy group would cause a conformational change of the 7β side chain, eliminating the hydrolytic water molecule and changing the conformation of the Ω loop, both essential in hydrolysis (*52*). Moxalactam, an oxacephem with a 7α-methoxy group, might also react in a similar fashion, resulting in the accumulation of acyl intermediates and thus inactivation of the enzyme.

Toho-1 exhibited high affinity and detectable activity against carbapenems, with small K_i and k_{cat} values. This suggests the possibility that Toho-1-like enzymes acquire higher carbapenemase activity with evolution. In the structures of carbapenemases NMC-A and Sme-1, several unique features differing from Toho-1 and other class A β-lactamases are observed. First, a disulfide bridge exists between Cys69 and Cys238, resulting in reorientation of the main-chain carbonyl O atom at position 238 toward the active site cavity. This disulfide bridge has been shown through analysis of the Cys69Ala mutant to be critical in catalysis and/or structural stability, as indicated by the full susceptibility of the strain producing the mutant to imipenem and all other antibiotics (*29*). Second, possibly as a consequence of the disulfide bond, the main-chain conformation from residue 238 to residue 240 in the β-strand B3 differs markedly from that of other class A enzymes, resulting in an increase in the space available between the Ω loop and the B3 strand. Third, the hydroxyl group of Ser237 points toward the ammonium group of Arg220, which is well conserved in carbapenemases. The Ser237 Ala mutant of Sme-1 exhibited significantly lower activity against imipenem (*53*), indicating that this conformation of Ser237 might be critical in carbapenemase activity. Mutation would occur in these regions if Toho-1-type enzymes obtained higher carbapenemhydrolyzing activity, although it is still not clear how these structural features are correlated with carbapenemase activity.

β-Lactamase inhibitors such as clavulanic acid, sulbactam, and tazobactam are known mechanism-based inactivators of class A enzymes. The k_{cat} value for sulbactam could be calculated, and the MIC value for sulbactam/cefoperazone was not small (data not shown). Toho-1 therefore appears to have acquired resistance against sulbactam. Toho-1 mutants with stronger resistance against sulbactam and other inhibitors might appear in the future.

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DNA-based diagnosis method for typhoid fever and paratyphoid fever, and the screening method for *Salmonella enterica* serovar Typhi and serovar Paratyphi A with decreased susceptibility to fluoroquinolones by PCR-restriction fragment length polymorphism (RFLP).

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Abstract

DNA-based diagnosis method for typhoid fever and paratyphoid fever were designed by using multiplex PCR, which used five pair of primers for detecting Vi antigen gene (*viaB*), H antigen gene (*fliC-d*, *fliC-a*) and O antigen synthesis gene (*tyv*, *prt*). Clinical isolates of *Salmonella enterica* serovar Typhi and Paratyphi A were correctly identified by this method. We also designed PCR- restriction fragment length polymorphism (RFLP) method for screening of the *gyrA* mutations of *S. enterica* serovar Typhi and serovar Paratyphi A with decreased susceptibility to fluoroquinolones. These two methods were useful for earlier diagnosis of typhoid fever and paratyphoid fever and earlier screening for *S. enterica* serovar Typhi and serovar Paratyphi A with decreased susceptibility to fluoroquinolones than ordinary culture methods.

Introduction

Enteric fever remains an important public health problem in many countries of the world. Typhoid fever is a sometimes fatal infection of adults and children that causes bacteremia and inflammatory destruction of the intestine and other organs. Typhoid fever is endemic in developing countries, especially in southeast Asia and Africa. Chloramphenicol has been a choice of treatment for typhoid fever for about 40 years, but alternative drugs were required for the treatment by the emergence of multidrug-resistant (MDR) *Salmonella enterica* serovar Typhi, that is resistant to ampicillin, chloramphenicol, and trimethoprim-sulfamethoxazole. Fluoroquinolones have proven to be effective for the treatment of typhoid fever caused by MDR strains in early 1990's, and have become the first line drugs of treatment for typhoid fever at moment (1, 5). But, *S. enterica* serovar Typhi strains resistant to

Table 1. Primers for the multiplex PCR amplification of *Salmonella enterica* serovar Typhi and Paratyphi A.

Oligonucleotide sequence	Length (bp)	Amplified fragment size (bp)	Primer designed from (Accession number).[b]
tyv(rfbE)			
tyv-s, 5'-gag gaa ggg aaa tga agc ttt t-3'	22	615bp	M29682
tyv-as, 5'- tag caa act gtc tcc cac cat ac-3'	23		M29682
prt(rfbS)			
parat-s, 5'-ctt gct atg gaa gac ata acg aac c-3'.	25	258bp	M29682
parat-as, 5'-cgt ctc cat caa aag ctc cat aga-3'.	24.		M29682
viaB			
vi-s, 5'-gtt att tca gca taa gga g-3'.	19	439bp	D14156
vi-as, 5'-ctt cca tac cac ttt ccg-3'.	18		D14156,
fliC			
fliCcom-s, 5'-aat caa caa caa cct gca gcg-3'.	21		L21912
fliCd-as, 5'- gca tag cca cca tca ata acc-3'.	21		L21912
fliCa-as, 5'-t[illegible] tgc tta atg tag ccg aag g-3'.	22		X03393
fliCcom/fliCd-as		750bp(489bp)[a]	
fliCcom/fliCa-as		329bp	

a: Number in parenthses represents size of PCR product of H:j gene.
b: Primers were designed using sequences corresponding to indicated GenBank/EMBL/DDBJ nucleotide sequence database accession number.

primers detect only *S. enterica* serovar Typhi *tyv* gene. We designed the primers for the *viaB* gene which specifically detect the *Salmonella* Vi antigen gene, because *viaB* primers previously reported by other researchers detected both *S. enterica* serovar Paratyphi C, *S. enterica* serovar Dublin and the *Citrobacter freundii* Vi antigen genes.

The multiplex PCR using five sets of primer pairs, which were targeted for the *viaB*, *prt*, *tyv*, *fliC-d*, and *fliC-a* genes, correctly identified *S. enterica* serovar Typhi and serovar Paratyphi A and differentiated the two serovars by the combinations of the different-size bands produced: four positive bands, which consist of *viaB*, *prt*, *tyv* and *fliC-d* PCR products, in *S. enterica* serovar Typhi and two positive bands, which consist of *prt* and *fliC-a* PCR products, in *S. enterica* serovar Paratyphi A (Fig. 1).

The primers for *tyv* specifically detected the *tyv* gene of *S. enterica* serovar Typhi. The *prt* primers also detected strains belonging to the O2 and O9 groups of *Salmonella*, and the *tyv* primers detected isolates of the *Salmonella* O9 group (Table 2). The primer pairs for *fliC-d* and *fliC-a* specifically detected the *fliC-d* and *fliC-a* genes, respectively, for the *Salmonella* serovars, and were able to distinguish *fliC-d* and *fliC-a* genes from other *Salmonella* serovar *fliC* genes. The primers for *fliC-d* also detected the *fliC-j* gene, which is an alternate phase of *S. enterica* serovar Typhi H-1 antigen genes (6, 14). Since *fliC-j* is a 261-bp deletion derivative of the *fliC-d* gene (ref), the PCR product was smaller than fliC-d

Table 2. Bacterial strains used to evaluate the specificity of multiplex PCR and the multiplex PCR results.

Strain No.		Serotype	Antigen structure				PCR results [1]				
			Phage Type	O antigen	H-1	H-2 [2]	*tyv*	*fliC-d*	*viaB*	*fliC-a*	*prt*
990116	*Salmonella*	Typhi	D1	9,12,[Vi]	d	-	+	+	+	-	+
990120	*Salmonella*	Typhi	E1	9,12,[Vi]	d	-	+	+	+	-	+
990005	*Salmonella*	Typhi	UVS1	9,12,[Vi]	d	-	+	+	+	-	+
990006	*Salmonella*	Typhi	A	9,12,[Vi]	d	-	+	+	+	-	+
990007	*Salmonella*	Typhi	E1	9,12,[Vi]	d	-	+	+	+	-	+
990008	*Salmonella*	Typhi	E1	9,12,[Vi]	d	-	+	+	+	-	+
990009	*Salmonella*	Typhi	E1	9,12,[Vi]	d	-	+	+	+	-	+
990012	*Salmonella*	Typhi	E1	9,12,[Vi]	d	-	+	+	+	-	+
990014	*Salmonella*	Typhi	E1	9,12,[Vi]	d	-	+	+	+	-	+
990037	*Salmonella*	Typhi	D1	9,12,[Vi]	d	-	+	+	+	-	+
980096	*Salmonella*	Typhi	46	9,12,[Vi]	d	-	+	+	+	-	+
980111	*Salmonella*	Typhi	DVS	9,12,[Vi]	d	-	+	+	+	-	+
980077	*Salmonella*	Typhi	UVS1	9,12,[Vi]	d	-	+	+	+	-	+
980014	*Salmonella*	Typhi	UVS1	9,12,[Vi]	j	-	+	+[3]	+	-	+
GIFU9954	*Salmonella*	Typhi		Rough	d	-	+	+	+	-	+
000055	*Salmonella*	Paratyphi A	1	1,2,12	a	[1,5]	-	-	-	+	+
000056	*Salmonella*	Paratyphi A	1	1,2,12	a	[1,5]	-	-	-	+	+
990110	*Salmonella*	Paratyphi A	2	1,2,12	a	[1,5]	-	-	-	+	+
970083	*Salmonella*	Paratyphi A	2	1,2,12	a	[1,5]	-	-	-	+	+
960007	*Salmonella*	Paratyphi A	3	1,2,12	a	[1,5]	-	-	-	+	+
000001	*Salmonella*	Paratyphi A	4	1,2,12	a	[1,5]	-	-	-	+	+
000041	*Salmonella*	Paratyphi A	4	1,2,12	a	[1,5]	-	-	-	+	+
990081	*Salmonella*	Paratyphi A	5	1,2,12	a	[1,5]	-	-	-	+	+
970032	*Salmonella*	Paratyphi A	5	1,2,12	a	[1,5]	-	-	-	+	+
990046	*Salmonella*	Paratyphi A	6	1,2,12	a	[1,5]	-	-	-	+	+
990103	*Salmonella*	Paratyphi A	6	1,2,12	a	[1,5]	-	-	-	+	+
99023	*Salmonella*	Chester		1,4,[5],12	e,h	e,n,x	-	-	-	-	-
99076	*Salmonella*	Agona		1,4,[5],12	f,g,s	[1,2]	-	-	-	-	-
99026	*Salmonella*	Oranienburg		6,7,14	m,t	[z_{57}]	-	-	-	-	-
99063	*Salmonella*	Infantis		6,7,14	r	1,5	-	-	-	-	-
99087	*Salmonella*	Litchfield		6,8	l,v	1,2	-	-	-	-	-
99114	*Salmonella*	Hadar		6,8	z_{10}	e,n,x	-	-	-	-	-
99109	*Salmonella*	Enteritidis		1,9,12	f],g,m,[p	[1,7]	+	-	-	-	+
99112	*Salmonella*	Javiana		1,9,12	l,z_{28}	1,5	+	-	-	-	+
99017	*Salmonella*	Senftenberg		1,3,19	g,[s],t	-	-	-	-	-	-
99089	*Salmonella*	Grumpensis		13,23	d	1,7	-	+	-	-	-
99108	*Salmonella*	Poona		1,13,22	z	1,6	-	-	-	-	-
1363	*Salmonella*	Typhimurium		1,4,[5],12	i	1,2	-	-	-	-	-
1364	*Salmonella*	Enteritidis		1,9,12	f],g,m,[p	[1,7]	+	-	-	-	+
1365	*Salmonella*	Weltevreden		3,10[15]	r	z_6	-	-	-	-	-
S-222	*Salmonella*	Durban		9,12	a	e,n,z_{15}	+	-	-	+	+
S-214	*Salmonella*	Strasbourg		9,46	d	1,7	+	+	-	-	+
S-154	*Salmonella*	Ndolo		1,9,12	d	1,5	+	+	-	-	+
GIFU12823	*Salmonella*	Paratyphi C		6,7,[Vi]	c	1,5	-	-	+	-	-
GIFU13011	*Salmonella*	Dublin		1,9,12[Vi]	g,p	-	+	-	+	-	+
	Citrobacter	*freundii*		Vi+			-	-	-[4]	-	-
	Yersinia	*pseudotuberculosis*		1b			-	-	-	-	-
	Yersinia	*pseudotuberculosis*		2a			-	-	-	-	-
	Yersinia	*pseudotuberculosis*		2b			-	-	-	-	-
	Yersinia	*pseudotuberculosis*		4a			-	-	-	-	-
	Yersinia	*pseudotuberculosis*		4b			-	-	-	-	-
	Yersinia	*pseudotuberculosis*		5b			-	-	-	-	-
	Yersinia	*enterocolitica*		O3			-	-	-	-	-
	Yersinia	*enterocolitica*		O5			-	-	-	-	-

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(10). In most strains, the acquired fluoroquinolone resistance was attributed to mutations in the genes encoding DNA gyrase (GyrA, GyrB) (18, 30-32)or DNA topoisomerase IV (ParC, ParE) (12, 13). We analyzed the association of quinolone resistance with mutations in the genes coding for gyrase and topoisomerase IV of *S. enterica* serovar Typhi and serovar Paratyphi A, which are especially clinically important serotypes of *Salmonella* spp.

The point mutations in *gyrA* and *parC* of *S. enterica* serovar Typhi and serovar Paratyphi A with decreased susceptibility to fluoroquinolones.

We determined *gyrA* and *parC* mutation of several strains with -resistant to fluoroquinolones or decreased susceptibility to fluoroquinolones (8). The strains resistant to fluoroquinolones were obtained from experimentally selection in vitro. The data are shown in Table 3. The *S. enterica* serovar Typhi and serovar Paratyphi A clinical isolates with decreased susceptibility to fluoroquinolone had only a single mutation in the *gyrA* gene, at either position 83 or 87 of GyrA. The strains with high-level resistance to fluoroquinolones induced by in vitro selection with ciprofloxacin had double mutations in the *gyrA* gene at both position 83 and position 87 of GyrA (Table 3). Only one *S. enterica* serovar Paratyphi A strain (strain NIHP3-1 in Table 3) had a mutation in the *parC* gene, at Glu-84 of ParC, in addition to double mutations in the *gyrA* gene. For the *parC* gene, the mutation was a change of GAA (Glu) to AAA (Lys) at codon 84. Alterations in the quinolone resistance determining regions (QRDRs) of the *gyrB* and *parE* genes were not found in any of the strains tested. These findings indicate that *gyrA* mutations are of principal importance for the fluoroquinolone resistance of *S. enterica* serovar Typhi and serovar Paratyphi A. Alterations at position 83 or 87 of the GyrA amino acid sequence have been described previously for *Salmonella* strains. Double mutations at positions 83 and 87 of the GyrA amino acid sequence were also reported in clinical isolates of *S. enterica* serovar Schwarzengrund, which caused nosocomial infections in the United States and which exhibited ciprofloxacin resistance (19). Although strains with high-level fluoroquinolone resistance due to double mutations at codons 83 and 87 in the GyrA amino acid sequence have not been found in clinical isolates of *S. enterica* serovar Typhi and serovar Paratyphi A, several cases of the failure of treatment for typhoid fever due to strains with decreased susceptibilities to fluoroquinolones have been reported. Since we obtained isolates with double mutations in the *gyrA* gene by in vitro selection and a mutation in *parC* caused by a novel substitution in Lys-84, such mutations in clinical isolates of *S. enterica* serovar Typhi and serovar Paratyphi A may appear in the future. Establishment of a surveillance system for the detection of *gyrA* mutations will be important for the detection of fluoroquinolone resistance in *S. enterica* serovar Typhi and serovar Paratyphi A.

PCR-RFLP for screening *S. enterica* serovar Typhi and serovar Paratyphi A with decreased susceptibility to fluoroquinolones.

The alterations at the codon Ser-83 and Asp-87 of GyrA are the most frequently found in the clinical isolates with reduced susceptibility to fluoroquinolones in *S. enterica* serovar Typhi and serovar Paratyphi A (2, 8, 28). We previously reported that only *gyrA* mutations contribute the fluoroquinolone resistance in *S.*

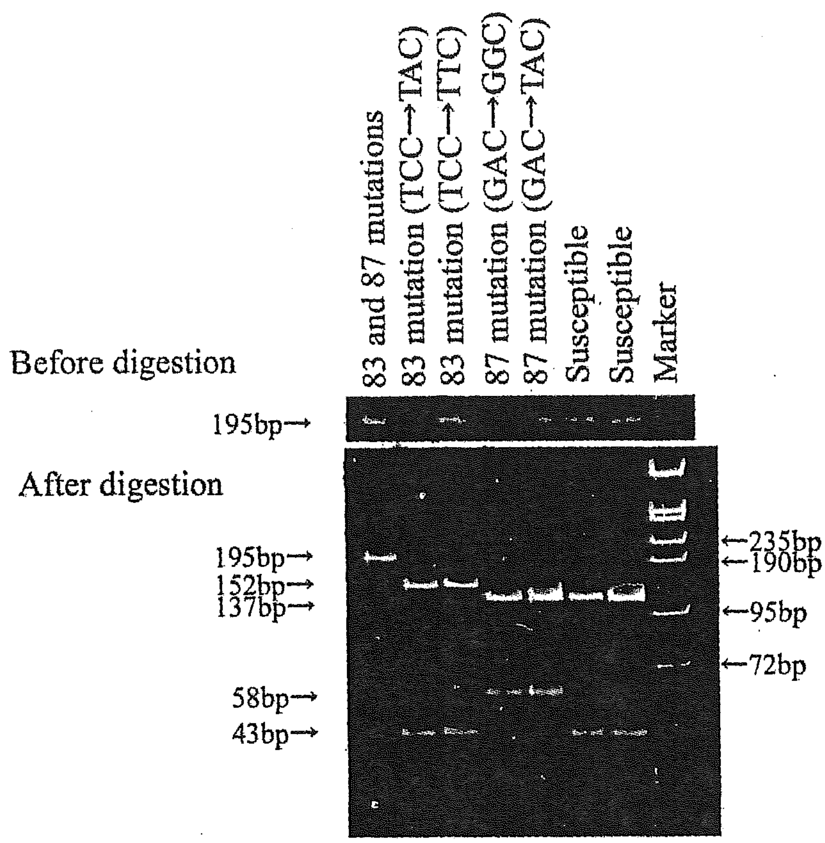

Fig. 3 PCR-RFLP patterns of the representative strains after *Hinf*I digestion. The digested PCR products were separated by 15% polyacrylamide gel electrophoresis.

Table 4. Primers used for PCR-RFLP.

Primers	Sequence		primer position [1]
gyrA-F	5'- TGT CCG AGA TGG CCT GAA GC	-3'	108-127
gyrA-HinfI-as	5'- ATG TAA CGC AGC GAG AAT GGC TGC GCC ATA CGA ACG CTG GA*[2]G	-3'	302-261

1) The primer positions were indicated by the number of nucleotide sequences from the start codon of *gyrA* gene.
2) The mismatch sequence to produce a *Hinf*I site into the amplified fragment is indicated by asterisc on the primer of gyrA-HinfI-as

susceptibility to fluoroquinolones. The purpose of this study is to develop more rapid screening method for the detection of fluoroquinolone resistant strains and reduced susceptibility strains than the ordinary culture method.

We designed the PCR-RFLP to detect common mutations related to fluoroquinolone resistance at codon 83 and 87 of GyrA. The PCR was performed with the primers, gyrA-F and gyrA-HinfI-as, which are expected to produce a 195-bp amplified fragment with a *Hinf*I restriction site at the codon corresponding to reduced susceptibility to fluoroquinolone, and fluoroquinolone susceptible strains, and we successfully screened the strains with reduced susceptibility to fluoroquinolones by this method (Table 3). Establishment of surveillance system for the detection of *gyrA* mutations will be the most important to find out the fluoroquinolone resistance of *S. enterica* serovar Typhi and serovar Paratyphi A. PCR-RFLP method described here may be one of methods for the rapid detection of such mutations.

Conclusions

The surveillance for antimicrobial resistance of *S. enterica* serovar Typhi and serovar Paratyphi A should be continued. Particularly monitoring the emergence of strains with double mutations in the *gyrA* genes, that are fully resistant to fluoroquinolones, is important for the antimicrobial resistance surveillance of clinically important *S. enterica* serovar Typhi and serovar Paratyphi A.

In conclusion, PCR diagnosis method for typhoid fever and paratyphoid fever and PCR-RFLP for screening for *gyrA* mutations, that are described here, may make it possible to get earlier diagnosis and earlier screening for fluoroquinolone resistance.

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