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Nosocomial Spread of Cephem-Resistant *Escherichia coli* Strains Carrying Multiple Toho-1-Like β-Lactamase Genes

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Escherichia coli* HKY56, which demonstrated resistance to various β-lactams except carbapenems, was isolated from the throat swab of an inpatient in 1994. Conjugal transfer of cephem resistance from HKY56 to *E. coli* CSH2 was not successful. Three cefotaxime-resistant *E. coli* clones harboring plasmid pMRE001, pMRE002, or pMRE003, each of which carried a 3.4-, 5.8-, or 6.2-kb *Eco*RI fragment insert, respectively, were obtained from HKY56. Although restriction analysis suggested their different origins, these clones showed similar profiles of resistance to various β-lactams. The sequence of 10 amino acid residues at the N terminus of β-lactamase purified from *E. coli* HB101(pMRE001) was identical to that of Toho-1. This Toho-1-like β-lactamase-1 (TLB-1) was able to hydrolyze cefoperazone and cefotaxime efficiently, but it failed to hydrolyze cephamycins. A Toho-1-specific DNA probe was hybridized with three distinct *Eco*RI fragments derived from the chromosomal DNA of strain HKY56, and these fragments corresponded to DNA inserts carried by pMRE001, pMRE002, and pMRE003, respectively. PCR and Southern hybridization analysis suggested that all six cephem-resistant *E. coli* strains, strains HKY273, HKY285, HKY288, HKY305, HKY316, and HKY335, which were isolated in 1996 at the same hospital where strain HKY56 had been isolated, also possessed multiple Toho-1-like β-lactamase (TLB) genes, and the hybridization patterns obtained with the Toho-1-specific probe were quite similar among these six isolates. The DNA fingerprinting patterns observed by pulsed-field gel electrophoresis revealed that among the *E. coli* isolates tested, all isolates except HKY56 possessed a similar genetic background. These findings suggested that *E. coli* strains that carry chromosomally multiplied TLB genes may have been proliferating and transmitted among patients in the same hospital.

Escherichia coli is one of the most common nosocomial pathogens that cause urinary tract infections (1, 27) and enterocolitis (18). It also causes a variety of opportunistic infections including pneumonia (7), bacteremia (9), and meningitis (28), especially in neonates and immunocompromised hosts, whose numbers have been increasing with the recent development of medical technology. Therefore, the emergence of antibiotic-resistant *E. coli* poses a substantial threat to antimicrobial chemotherapy (15, 24). *E. coli* is inherently susceptible to cephems, although it usually produces a low level of an uninducible chromosomal AmpC-type cephalosporinase. Recently, however, with the increasing use of the broad-spectrum cephalosporins, resistant *E. coli* isolates are more commonly encountered in various clinical settings. In some *E. coli* isolates, moderate to high-level cephem resistance depends on hyperproduction of the chromosomal AmpC enzyme (19). In other strains, the acquisition of plasmid-mediated secondary β-lactamases, such as TEM- or SHV-derived extended-spectrum β-lactamases (14), class A enzymes not related to TEM- or SHV-derived β-lactamases (2, 3, 12), and AmpC enzymes derived from other bacteria (8, 20), was also responsible for resistance to broad-spectrum cephalosporins. Some TEM- or SHV-derived extended-spectrum β-lactamases are widespread in Europe and the United States, while the other types of β-lactamases have been detected infrequently, and neither their prevalence nor their clinical relevance is known (2, 3, 8, 12, 20). So far as we know, most of the previous reports of nosocomial outbreaks of resistant bacteria have focused on *Klebsiella pneumoniae*, and few have described outbreaks of drug-resistant *E. coli*. In this paper, we describe the background of multiple cephem resistance in clinical *E. coli* isolates which may have been proliferating in nosocomial settings.

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MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The strains and plasmids used in this study are listed in Table 1. For plasmid preparations, bacteria were grown in Luria-Bertani (LB) broth supplemented with the appropriate antibiotics.

E. coli CSH2 was used as the recipient of a transferable large plasmid in the transconjugation analysis. Both the host strain and strain CSH2 were grown to a mid-logarithmic phase in 1 ml of LB broth. A mixture of 500 μl of each bacterial culture was centrifuged to yield a bacterial pellet, and the pellet was incubated for 6 h at 37°C. Transconjugants were selected on LB agar plates supplemented with 50 μg of ampicillin per ml, 100 μg of rifampin per ml, and 100 μg of nalidixic acid per ml.

E. coli HKY56, HKY273, HKY285, HKY288, HKY305, HKY316, and HKY335 are all clinical isolates from the same hospital in Aichi Prefecture in Japan. *E. coli* HKY56 was isolated in September 1994, while strains HKY273, HKY285, HKY288, HKY305, HKY316, and HKY335 were isolated from January through April 1996. Four strains, strains HKY273, HKY285, HKY305, and HKY335, were isolated from urine; strains HKY288 and HKY316 were isolated from sputum; and strain HKY56 was isolated from a throat swab. Strains HKY273 and HKY285 were isolated from the same patient at different times.

E. coli TUH12191, from which Toho-1 was originally isolated, and clone pMTY011, which carried the gene fragment encoding Toho-1, were kindly provided by Y. Ishii, Toho University (12).

Antibiotics and susceptibility testing. The antibiotics used in the study were obtained from the indicated sources: ampicillin and kanamycin, Meiji-seika Kai-

TABLE 1. Bacterial strains and plasmids used in this study

Bacterial strain or plasmid	Characteristics	Source or reference
Strains		
E. coli HKY56	Multidrug-resistant clinical isolate recovered in 1994 at one hospital in Japan	This study
E. coli HKY273, HKY285, HKY288, HKY305, HKY316, and HKY335	Multidrug-resistant clinical isolates recovered at the same hospital as HKY56 in 1996	This study
E. coli TUH12191	Original strain harboring the Toho-1 gene	Y. Ishii, Toho University (12)
E. coli HB101	F^- *hsdS20* (r^-, m^-) *recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1 supE44* λ^-	23
E. coli CSH2	*metB* F^- NA^r Rif^r	T. Sawai, Chiba University
Plasmids and clones		
pMK16	Cloning vector; Km^r Tc^r	A. Ohta
pMTY011	Toho-1 gene from TUH12191 cloned into pHSG397	Y. Ishii, Toho University (12)
pMTY100	4.0-kb *Eco*RI fragment encoding Toho-1 of pMTY011 subcloned into pMK16	This study
pMRE001, pMRE002, and pMRE003	Recombinant plasmids encoding Toho-1-like β-lactamase of *E. coli* HKY56	This study

sha, Ltd., Tokyo, Japan; aztreonam, Eizai Co., Ltd., Tokyo, Japan; cefmetazole, Sankyo Co., Ltd., Tokyo, Japan; cefotaxime, Fabwerke Hoechst AG, Frankfurt, Germany; cefoperazone and cefoperazone-sulbactam, Pfizer Pharmaceutical Inc., Tokyo, Japan; ceftazidime, Japan Glaxo Co., Ltd., Osaka, Japan; cephaloridine, moxalactam, and tobramycin, Shionogi and Co., Ltd., Osaka, Japan; imipenem, Banyu Pharmaceutical Co., Ltd., Tokyo, Japan; meropenem, Sumitomo Pharmaceutical Co., Ltd., Osaka, Japan; piperacillin, Toyama Chemical Co., Ltd., Toyama, Japan; amikacin, Bristol-Meyers Squibb K. K., Tokyo, Japan; nalidixic acid, Daiichi Pharmaceutical Co., Ltd., Tokyo, Japan; ciprofloxacin, Bayer Yakuhin, Ltd., Osaka, Japan; chloramphenicol and tetracycline, Sigma Chemical Co., St. Louis, Mo.; and rifampin, Nippon Ciba-Geigy Co., Ltd., Hyogo, Japan.

Antimicrobial susceptibility testing was performed by the agar dilution method according to National Committee for Clinical Laboratory Standards document M7-A3 (21). Mueller-Hinton II agar and Mueller-Hinton broth (BBL Microbiology Systems, Cockeysville, Md.) were used for susceptibility testing.

DNA techniques and cloning of β-lactamase genes. Manipulation of DNA was carried out by the method outlined by Sambrook et al. (23). Restriction endonucleases and T4 DNA ligase were purchased from Nippon Gene Co. Ltd. (Toyama, Japan).

Total DNA from *E. coli* HKY56 was digested completely with a restriction endonuclease and was ligated into the same restriction site of pMK16 by using T4 DNA ligase. Recombinant plasmids were introduced into *E. coli* HB101, and resistant colonies were selected on LB agar plates supplemented with 50 μg each of ampicillin and kanamycin per ml.

Purification of β-lactamase. For purification of Toho-1-like β-lactamase-1 (TLB-1), *E. coli* HB101 harboring plasmid pMRE001 was cultured overnight in 1 liter of LB broth supplemented with 30 μg of cefotaxime per ml with shaking at 37°C. The cells were harvested by centrifugation and were suspended in 10 ml of 100 mM phosphate buffer (pH 7.0). For preparation of the cell extract, the suspension was frozen at −80°C and was thawed at 30°C. This step was repeated three times, and the cell suspension was then ultracentrifuged at 25,000 × *g* for 1 h at 4°C. The supernatant was applied to a DEAE-Sephacel column preequilibrated with 100 mM phosphate buffer (pH 7.0). The fractions with β-lactamase activity were pooled and concentrated to 2 ml by ultrafiltration with a Centricut 10 filter (Kurabo Co., Osaka, Japan). For further purification, the enzyme solution was diluted with 15 ml of 50 mM MOPS (3-morpholinopropanesulfonic acid) buffer (pH 6.0) and was then chromatographed through a HiTrap SP column by using a high-pressure liquid chromatography system (Pharmacia Biotech, Uppsala, Sweden). Elution was carried out with 50 mM MOPS buffer (pH 6.0) containing 120 mM NaCl. The purified enzyme was used for β-lactamase assays and determination of the N-terminal amino acid sequence. Toho-1 was also purified from clone pMTY011 as described above, except that elution was with 50 mM MOPS buffer (pH 6.0) containing 110 mM NaCl.

Enzyme assays. The activities of TLB-1 from *E. coli* HB101(pMRE001) and Toho-1 were assayed with a spectrophotometer (model 557; Hitachi, Tokyo, Japan) at 30°C in 50 mM phosphate buffer. The absorption maxima of the substrates used were as follows: ampicillin, 235 nm; aztreonam, 315 nm; ceftazidime, 272 nm; cephaloridine, 295 nm; cefoperazone, 276 nm; cefotaxime, 264 nm; imipenem, 297 nm; moxalactam, 274 nm. The molar extinction coefficients were calculated by the method of Seeberg et al. (25). K_m and V_{max} values were obtained by least-squares fit to plots of the initial steady-state velocities at different substrate concentrations. Relative V_{max} and relative V_{max}/K_m values were calculated for comparison of the enzyme activities, as recommended by Bush and Sykes (6).

Isoelectric focusing was carried out with a Multiphor II electrophoresis system (Pharmacia Biotech) 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, and β-lactamase activity was confirmed with nitrocefin.

N-terminal amino acid sequencing of β-lactamases. Ten amino acid residues in the N terminus of the purified TLB-1 were sequenced with a peptide sequencer (model 473A; Applied Biosystems Inc., Foster City, Calif.).

PCR. *Taq* polymerase was purchased from Takara Shuzo Co., Ltd. (Shiga, Japan). Primers A, 5′-d(ACG CTA CCC CTG CTA TTT)-3′, and B, 5′-d(CCT TTC CGC CTT CTG CTC)-3′, which were derived from the coding region of the Toho-1 β-lactamase gene (12), were synthesized for PCR analysis. Plasmid pMTY011, supplied by Y. Ishii, Toho University, was used as a positive control template for the Toho-1 gene. We tested the reagent with distilled water instead of template DNA as a negative control. PCR was performed with 30 cycles of denaturation at 94°C for 1 min, annealing at 57°C for 1 min, and extension at 72°C for 1.5 min by using a GeneAmp PCR System 2400 thermal cycler (Perkin-Elmer).

Southern hybridization analysis. The DNA probe used for hybridization was amplified by PCR. The 780-bp PCR product from pMTY011 template DNA was labelled with fluorescein-11-dUTP by using a Fluorescein Gene Images random prime module (Amersham International plc, Little Chalfont, England). Large plasmids extracted from seven clinical *E. coli* isolates by the method of Kado and Liu (16) and *Eco*RI or *Bam*HI digests of total DNA from these isolates were blotted onto nylon membranes (Hybond-N; Amersham International plc) after agarose gel electrophoresis. DNA hybridization was performed with a Fluorescein Gene Images dioxetane detection module (Amersham International plc) as follows. The membrane was soaked for 30 min at 60°C in hybridization buffer (5× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate; pH 7.0]–0.5% liquid blocking agent [Amersham International plc]–0.1% sodium dodecyl sulfate [SDS]–5% dextran sulfate) and was hybridized overnight at 60°C with the denatured probe (1 ng/ml). The blot was washed once for 15 min at 60°C with 1× SSC–0.1% SDS, washed again for 15 min at 60°C with 0.5× SSC–0.1% SDS, rinsed in diluent buffer (100 mM Tris-HCl, 300 mM NaCl [pH 7.5]), and incubated for 1 h in 10% liquid blocking agent in diluent buffer at room temperature. After rinsing briefly in diluent buffer, the blot was incubated for 1 h at room temperature with a 1:5,000 dilution of anti-fluorescein-alkaline phosphatase conjugate in a solution of 0.5% bovine serum albumin in diluent buffer. To remove unbound conjugate the blot was washed three times for 10 min each time in 0.3% Tween 20 in diluent buffer at room temperature. After a brief rinse in diluent buffer, dioxetane was sprayed on the blot, and the blot was exposed to X-ray film (Hyperfilm MP; Amersham International plc) for 30 to 40 min.

PFGE. Total DNA was prepared from seven clinically isolated *E. coli* strains and *E. coli* TUH12191 for analysis by pulsed-field gel electrophoresis (PFGE) by the method of Smith and Cantor (26) and the modified method of Ichiyama et al. (11). *Xba*I-digested DNA was electrophoresed with a contour-clamped homogeneous electric field system (Pulsaphor Plus; Pharmacia Biotech, Uppsala, Sweden) as described previously (11). The condition for electrophoresis was 170 V for 24 h, with pulse times ranging from 10 to 30 s.

RESULTS

Transfer of β-lactam resistance and cloning of the β-lactamase gene. After the confirmation that *E. coli* HKY56 had a large plasmid, transfer of ampicillin resistance from *E. coli* HKY56 to *E. coli* CSH2 by conjugation and electroporation

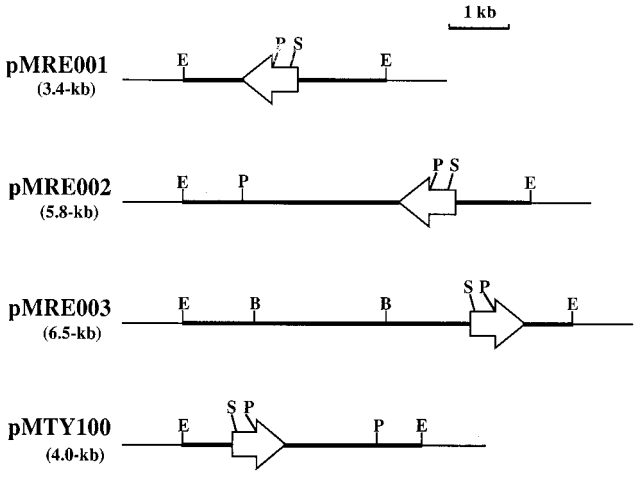

FIG. 1. Restriction map of four clones: pMRE001, pMRE002, pMRE003, and pMTY100. *Eco*RI inserts of the clones are represented by heavy lines. Open arrows indicate the coding regions of the β-lactamase genes, and the direction of the arrows represents the transcriptional orientation. The DNA molecules are drawn to scale with the indicated restriction endonuclease sites: B, *Bgl*II; E, *Eco*RI; P, *Pst*I; S, *Sph*I.

with the plasmids harbored by HKY56 to *E. coli* HB101 were performed, but the results were unsuccessful, despite several attempts. Subsequently, total DNA from *E. coli* HKY56 was digested with *Eco*RI and was ligated with *Eco*RI-cleaved pMK16. Transformation of *E. coli* HB101 with these recombinants yielded three distinct clones, each of which harbored a plasmid containing an insert of approximately 3.4, 5.8, or 6.5 kb of DNA, respectively. Three representative plasmids, plasmids pMRE001, pMRE002, and pMRE003, were mapped with several restriction endonucleases (Fig. 1). The 4.0-kb fragment of pMTY011 digested with *Eco*RI was also subcloned into pMK16, and the resultant plasmid, plasmid pMTY100, was introduced into *E. coli* HB101. A restriction map of pMTY100 is also shown in Fig. 1, along with those of pMRE001, pMRE002, and pMRE003. The *Pst*I and *Sph*I sites found in the coding region of the β-lactamase genes appeared to be conserved. However, in the flanking region, there are some differences in the *Pst*I or *Bgl*II sites among these four clones.

Antibiotic susceptibility. The seven clinical *E. coli* isolates were resistant to all β-lactam antibiotics tested except the carbapenems, and the MICs of ceftazidime and moxalactam ranged from 32 to 128 μg/ml (Table 2). These isolates were also resistant to quinolones, including ciprofloxacin, although they remained susceptible to amikacin. The MICs of tobramycin, tetracycline, and chloramphenicol for these strains varied widely. The *E. coli* HB101 clones harboring plasmid pMRE001, pMRE002, or pMRE003 were resistant to all β-lactams except cephamycins and carbapenems. Their levels of resistance to ceftazidime were lower than that of the parent strain HKY56. The profile of *E. coli* HB101(pMTY011) resistance to β-lactams was similar to those of the HB101 strains harboring pMRE001, pMRE002, or pMRE003. Interestingly, these three clones from HKY56 also demonstrated high-level resistance to the combination of cefoperazone and sulbactam, as has been observed in *E. coli* HB101(pSB23), which carries the *Klebsiella oxytoca* β-lactamase gene bla_{RBI} (17).

Purification of β-lactamase and N-terminal amino acid sequence of the purified β-lactamase. The enzyme isolated from the clone *E. coli* HB101(pMRE001) was purified to give a single band on SDS-polyacrylamide gel electrophoresis (data not shown). The amino acid sequence at the N terminus of the purified β-lactamase was identified as 1-QANSVQQQLE-10 which is identical to that of Toho-1 (12). Therefore, this β-lactamase was designated TLB-1.

Kinetic parameters and isoelectric focusing. Toho-1 had the greatest catalytic efficiency (relative V_{max}/K_m) against cefotaxime and cefoperazone, while the TLB-1 produced by HKY56 hydrolyzed cefoperazone and cephaloridine most efficiently (Table 3). The latter enzyme efficiently hydrolyzed cefotaxime as well. Both enzymes had poor hydrolytic activities (relative V_{max}) against moxalactam and imipenem. The catalytic efficiency with ceftazidime was substantially lower than those with the other β-lactams tested.

We estimated a pI of 8.2 for TLB-1, which is different from that for Toho-1 (pI 7.8) (12). Isoelectric focusing analysis of the crude enzyme extract from HKY56 revealed that HKY56 produced at least two different β-lactamases with pIs of 8.2 and about 9.0.

Genetic backgrounds of the determinants for TLBs. Similar 780-bp products were obtained by PCR from all seven clinical *E. coli* isolates tested (Fig. 2). The large plasmid profiles for the seven isolates were similar, and the DNA probe specific for the Toho-1 gene hybridized to chromosomal positions on the Southern blot (Fig. 3). These results combined with the failure to transfer resistance by both transconjugation and electropo-

TABLE 2. Antibiotic susceptibilities of clinical *E. coli* isolates, *E. coli* HB101, and transformants

E. coli strain	MIC (μg/ml)[a]																	
	ABPC	PIPC	CER	CMZ	CTX	CAZ	CPZ	CPZ-SBT	LMOX	AZT	IPM	MEPM	AMK	TOB	NA	CPFX	TC	CP
HKY56	>128	>128	>128	>128	>128	128	>128	>128	64	>128	1	<0.5	4	16	>128	128	>128	8
HKY273	>128	>128	>128	>128	>128	128	>128	>128	128	>128	1	1	2	16	>128	>128	>128	128
HKY285	>128	>128	>128	>128	>128	128	>128	>128	64	128	<0.5	<0.5	4	16	>128	>128	>128	128
HKY288	>128	>128	>128	>128	>128	32	>128	>128	32	128	1	<0.5	4	8	>128	64	2	32
HKY305	>128	>128	>128	>128	>128	64	>128	128	64	128	2	<0.5	8	16	>128	128	8	32
HKY316	>128	>128	>128	>128	>128	64	>128	>128	64	128	2	1	8	2	>128	64	4	32
HKY335	>128	>128	>128	>128	>128	64	>128	64	128	>128	<0.5	<0.5	4	16	>128	128	4	32
HB101	2	<0.5	2	1	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	4	1	2	<0.5	1	2
HB101(pMTY011)	>128	>128	>128	1	>128	16	>128	128	<0.5	>128	<0.5	<0.5	<0.5	<0.5	2	<0.5	1	>128
HB101(pMRE001)	>128	>128	>128	1	>128	16	>128	>128	<0.5	128	<0.5	<0.5	<0.5	2	4	<0.5	>128	4
HB101(pMRE002)	>128	>128	>128	1	>128	16	>128	>128	<0.5	>128	<0.5	<0.5	2	2	4	<0.5	>128	4
HB101(pMRE003)	>128	>128	>128	2	>128	32	>128	>128	1	>128	<0.5	<0.5	<0.5	1	4	<0.5	64	4

[a] Abbreviations: ABPC, ampicillin; PIPC, piperacillin; CER, cephaloridine; CMZ, cefmetazole; CTX, cefotaxime; CAZ, ceftazidime; CPZ, cefoperazone; CPZ-SBT, cefoperazone-sulbactam; LMOX, moxalactam; AZT, aztreonam; IPM, imipenem; MEPM, meropenem; AMK, amikacin; TOB, tobramycin; NA, nalidixic acid; CPFX, ciprofloxacin; TC, tetracycline; CP, chloramphenicol.

TABLE 3. Comparison of kinetic parameters for various β-lactams between Toho-1 and TLB-1 purified from *E. coli* HB101(pMRE001)

Drug	Toho-1			TLB-1 from strain with pMRE001		
	Relative V_{max} (%)	K_m (μM)	Relative V_{max}/K_m (%)	Relative V_{max} (%)	K_m (μM)	Relative V_{max}/K_m (%)
Ampicillin	100	85.7	100	100	284	100
Cephaloridine	950	654	124	810	925	249
Cefotaxime	824	327	216	654	1,310	142
Cefoperazone	17	4.7	310	10.5	8.9	335
Ceftazidime	0.9	121	0.6	0.3	99.6	0.9
Aztreonam	58.3	63.8	78.3	16.3	373	12.4
Moxalactam	<0.1			<0.1		
Imipenem	<0.1			<0.1		

ration of the plasmids from HKY56 suggest that the determinants for Toho-1-like β-lactamases (TLBs) are located on the chromosome. The Toho-1 gene-specific DNA probe hybridized to at least two different positions on the blot of *Eco*RI digests of total DNA prepared from seven *E. coli* isolates (Fig. 4). In HKY56, the same DNA probe hybridized to at least three different-sized *Eco*RI fragments corresponding to the sizes of the inserts of clones pMRE001, pMRE002, and pMRE003. This observation suggests that the seven clinical isolates possessed multiple TLB genes. The hybridization signals detected in five clinical isolates, isolates HKY285, HKY288, HKY305, HKY316, and HKY335, gave quite similar patterns. Two hybridization signals of about 3.4 and 6.5 kb were detected in these five isolates and were similar to those detected in HKY56. This observation suggests that the nucleotide sequences of TLB genes and their flanking regions are highly conserved among these five isolates. Although strains HKY273 and HKY285 were both isolated from the same patient, their hybridization patterns appeared to be different. Hybridization signals in HKY273 were detected at two additional positions (about 4.0 kb). Genetic rearrangements occurring around the TLB genes in HKY273 may have resulted in the emergence of the genetically different strain HKY285.

The Toho-1 gene-specific DNA probe hybridized to 22-kb *Bam*HI fragments in all seven strains, while in HKY273 an additional smaller signal was noted (Fig. 4). This result indicates that three TLB genes in HKY56 are located on the 22-kb *Bam*HI fragments.

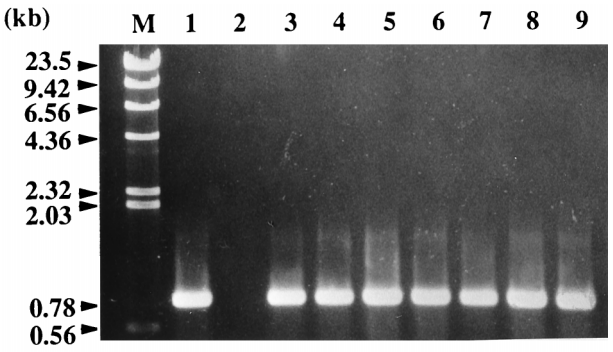

FIG. 2. PCR detection of the TLB gene in seven clinical *E. coli* isolates. PCR was performed as described in Materials and Methods. The same apparent 780-bp products were identified by PCR from all seven clinical *E. coli* isolates. Lanes: M, *Hin*dIII-digested DNA marker; 1, positive control; 2, negative control; 3, *E. coli* HKY56; 4, HKY273; 5, HKY285; 6, HKY288; 7, HKY305; 8, HKY316; 9, HKY335.

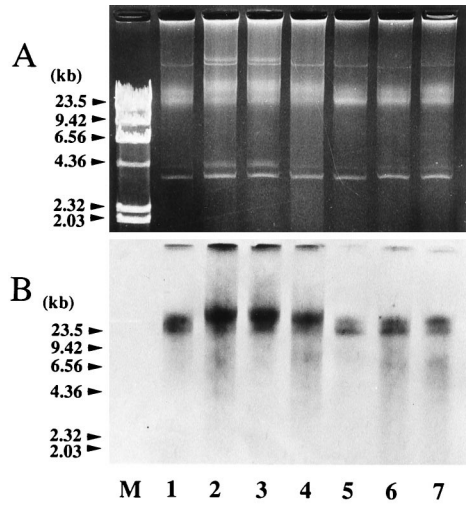

FIG. 3. Large plasmid profiles for and Southern hybridization analysis of seven *E. coli* isolates. Plasmids were prepared from the seven multidrug-resistant clinical *E. coli* isolates by the method of Kado and Liu (16). The 780-bp PCR product isolated by using pMTY011 as template DNA was used as a probe. (A) Plasmid profile of each strain. (B) Hybridization to the chromosomal positions were detected in all strains tested. Lanes: M, *Hin*dIII-digested DNA marker; 1, *E. coli* HKY56; 2, HKY273; 3, HKY285; 4, HKY288; 5, HKY305; 6, HKY316; 7, HKY335.

PFGE. Six distinctive restriction patterns were observed in the seven *E. coli* isolates tested in this study and *E. coli* TUH12191 (Fig. 5). Strain TUH12191 was unrelated to the seven *E. coli* isolates tested in this study. The restriction pattern of strain HKY56 was very different from those of the other six isolates. Strains HKY288, HKY305, and HKY335 showed indistinguishable restriction patterns (pattern A), while the restriction pattern of HKY316 differed from pattern A by two bands. The fingerprinting patterns of strains HKY273 and HKY285, isolated from the same patient at different times,

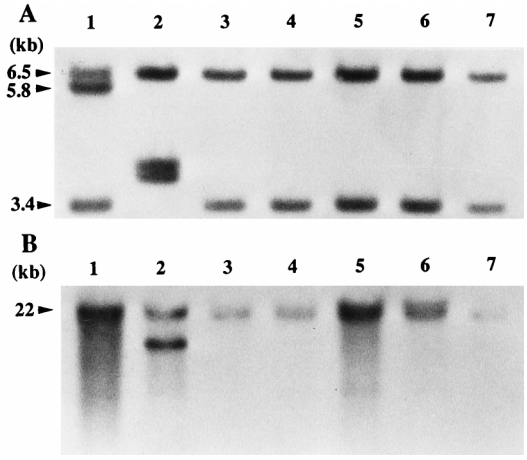

FIG. 4. Hybridization of *Eco*RI or *Bam*HI digests of total DNA from seven *E. coli* isolates with the Toho-1-type probe. Total DNA preparations from the seven clinical *E. coli* isolates were subjected to agarose gel electrophoresis following digestion with *Eco*RI (A) or *Bam*HI (B) and were blotted onto a nylon membrane. The 780-bp PCR product obtained by using pMTY011 as template DNA was used as a probe. Lanes: 1, *E. coli* HKY56; 2, HKY273; 3, HKY285; 4, HKY288; 5, HKY305; 6, HKY316; 7, HKY335.

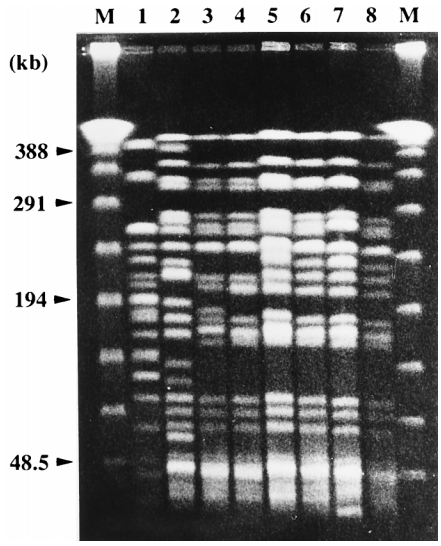

FIG. 5. DNA fingerprinting patterns for seven clinical *E. coli* isolates and *E. coli* TUH12191 obtained by PFGE. Total DNA containing both genomic and plasmid DNAs was prepared from each clinical isolate, digested with *Xba*I, and then subjected to PFGE (see text). Lanes: M, DNA molecular markers; 1, *E. coli* TUH12191; 2, HKY56; 3, HKY273; 4, HKY285; 5, HKY288; 6, HKY305; 7, HKY316; 8, HKY335.

were different from each other by one band and differed by one or two bands from pattern A. Therefore, these six strains had a similar genetic background. With our limited number of isolates and limited epidemiologic information, we supposed that these six strains had probably spread as a result of nosocomial transmission.

DISCUSSION

Toho-1 was identified as a novel plasmid-mediated class A enzyme from the clinical isolate *E. coli* TUH12191 (12) in Japan. This enzyme has an efficient cefotaxime-hydrolyzing capacity and belongs to group 2be according to the functional classification (5). In this study, we determined that *E. coli* HKY56 also produced an enzyme (TLB-1) similar to Toho-1. Although the amino acid sequence at the N terminus of TLB-1 is identical to that of Toho-1, comparison of the kinetic parameters between these enzymes suggests minor differences in substrate specificities. Furthermore, the difference in pIs between Toho-1 and TLB-1 indicates several amino acid substitutions in TLB-1. Determination of the overall sequence of the TLB-1 gene, together with the enzymologic assays, including K_i analysis, is under way for further characterization of this enzyme.

Notably, the genes encoding TLBs were replicated on the chromosome in seven *E. coli* isolates tested in the present study. Multiplication of the β-lactamase gene may be advantageous to bacteria from the point of view of evolution, since it may increase the chance of incurring point mutations responsible for extension of the substrate specificity of the β-lactamase. It has recently been reported that a single *K. pneumoniae* isolate produced both TEM-12 and TEM-10, two β-lactamases encoded by plasmids that were indistinguishable by their restriction digest patterns (4). In another report, both TEM-1 and TEM-12 genes were identified on the chromosome of one *E. coli* isolate (29). Those studies suggest that the evolution of TEM-type β-lactamase proceeded sequentially in one organism, implying that multiplication and mutations in these genes lead to the extension of the spectrum of the enzyme. A similar evolutionary event might occur in the seven *E. coli* isolates tested in this study.

Although strain HKY56 was genetically distinguishable from the six isolates by PFGE, Southern hybridization analysis suggested that these strains shared one or two TLB genes located on the same *Eco*RI fragments as those in HKY56. Since the determinants for TLBs were located on the chromosome in seven strains tested in this study, it is possible that mobile elements such as transposons or integrons may have been involved in the translocation of the TLB genes. The gene encoding TEM-12, a β-lactamase prevalent in the United States, was reported to be located on a transposon (10), and the spread of the genes encoding the PSE-1, CARB-4, and OXA-type β-lactamases or the IMP-1 metallo-β-lactamase has been associated with integrons (22). It is therefore possible to speculate that a TLB gene on the chromosome of HKY56 may first have been transposed to a plasmid via a transposon or become integrated as a gene cassette into an integron on a plasmid, as was observed in *E. coli* TUH12191. Next, the plasmid was transferred to other *E. coli* strains by conjugation, and then the β-lactamase genes translocated on their chromosome. During these processes, genetic rearrangements, including deletions and multiplications, may have occurred. Finally, clonal proliferation and transmission of the *E. coli* strains which possess TLB genes may have occurred in a nosocomial setting. To clarify the mobile elements of these genes, further sequencing of the TLB genes, as well as their flanking regions, is now under way.

Three TLB-producing *E. coli* clones harboring pMRE001, pMRE002, and pMRE003, respectively, were susceptible to cephamycins, so that the production of TLBs accounted for most but not all of the cephem resistance found in strain HKY56. We, however, could not isolate a β-lactamase from HKY56 except for the presumed AmpC, despite repeated cloning experiments. A crude enzyme preparation from HKY56 contained two detectable β-lactamase activities with different pIs. One, with a pI of 8.2, corresponded to TLB-1, and the other, with a pI of 9.0, probably corresponded to AmpC. Therefore, the possibility of the presence of other β-lactamases is low. It is possible that another mechanism such as alteration in outer membrane permeability (13) might be responsible for resistance to cephamycins in HKY56.

Broad-spectrum cephalosporins have been widely used in Japan to control various infectious diseases caused by gram-negative bacteria. Under such antibiotic stress, even *E. coli*, which is inherently susceptible to cephems, has developed high-level resistance to broad-spectrum cephalosporins. In the present study, the spread of multiple cephem-resistant *E. coli* strains has been identified as a possible nosocomial transmission. This finding represents a substantial potential threat to successful antimicrobial chemotherapy for bacterial infections in the near future. Rigorous use of antibiotics and the establishment of practical infection control measures are necessary for the prevention of the further proliferation and transmission of these multidrug-resistant bacteria. Moreover, a survey of TLB-producing clinical *E. coli* isolates would be also required for rigorous infection control.

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REFERENCES

1. **Ackermann, R. J., and P. W. Monroe.** 1996. Bacteremic urinary tract infection in older people. J. Am. Geriatr. Soc. **44:**927–933.
2. **Barthelemy, M., J. Peduzzi, H. Bernard, C. Tancrede, and R. Labia.** 1992. Close amino acid sequence relationship between the new plasmid-mediated extended-spectrum β-lactamase MEN-1 and chromosomally encoded enzymes of *Klebsiella oxytoca*. Biochim. Biophys. Acta **1122:**15–22.
3. **Bauernfeind, A., I. Stemplinger, R. Jungwirth, S. Ernst, and J. M. Casellas.** 1996. Sequences of β-lactamase genes encoding CTX-M-1 (MEN-1) and CTX-M-2 and relationship of their amino acid sequences with those of other β-lactamases. Antimicrob. Agents Chemother. **40:**509–513.
4. **Bradford, P. A., C. E. Cherubin, V. Idemyor, B. A. Rasmussen, and K. Bush.** 1994. Multiply resistant *Klebsiella pneumoniae* strains from two Chicago hospitals: identification of the extended-spectrum TEM-12 and TEM-10 ceftazidime-hydrolyzing β-lactamases in a single isolate. Antimicrob. Agents Chemother. **38:**761–766.
5. **Bush, K., G. A. Jacoby, and A. A. Medeiros.** 1995. A functional classification scheme for β-lactamases and its correlation with molecular structure. Antimicrob. Agents Chemother. **39:**1211–1233.
6. **Bush, K., and R. B. Sykes.** 1986. Methodology for the study of β-lactamases. Antimicrob. Agents Chemother. **30:**6–10.
7. **Craven, D. E., K. A. Steger, and T. W. Barber.** 1991. Preventing nosocomial pneumonia: state of the art and perspectives for the 1990s. Am. J. Med. **91**(Suppl. 3B)**:**44S–53S.
8. **Fosberry, A. P., D. J. Payne, E. J. Lawlor, and J. E. Hodgson.** 1994. Cloning and sequence analysis of bla_{BIL-1}, a plasmid-mediated class C β-lactamase gene in *Escherichia coli* BS. Antimicrob. Agents Chemother. **38:**1182–1185.
9. **Gransden, W. R., S. J. Eykyn, I. Phillips, and B. Rowe.** 1990. Bacteremia due to *Escherichia coli*: a study of 861 episodes. Rev. Infect. Dis. **12:**1008–1018.
10. **Heritage, J., P. M. Hawkey, N. Todd, and I. J. Lewis.** 1992. Transposition of the gene encoding a TEM-12 extended-spectrum β-lactamase. Antimicrob. Agents Chemother. **36:**1981–1986.
11. **Ichiyama, S., M. Ohta, K. Shimokata, N. Kato, and J. Takeuchi.** 1991. Genomic DNA fingerprinting by pulsed-field gel electrophoresis as an epidemiological marker for study of nosocomial infections caused by methicillin-resistant *Staphylococcus aureus*. J. Clin. Microbiol. **29:**2690–2695.
12. **Ishii, Y., A. Ohno, H. Taguchi, S. Imajo, M. Ishiguro, and H. Matsuzawa.** 1995. Cloning and sequence of the gene encoding a cefotaxime-hydrolyzing class A β-lactamase isolated from *Escherichia coli*. Antimicrob. Agents Chemother. **39:**2269–2275.
13. **Jacoby, G. A., and I. Carreras.** 1990. Activities of β-lactam antibiotics against *Escherichia coli* strains producing extended-spectrum β-lactamases. Antimicrob. Agents Chemother. **34:**858–862.
14. **Jacoby, G. A., and A. A. Medeiros.** 1991. More extended-spectrum β-lactamases. Antimicrob. Agents Chemother. **35:**1697–1704.
15. **Jones, R. N.** 1996. Impact of changing pathogens and antimicrobial susceptibility patterns in the treatment of serious infections in hospitalized patients. Am. J. Med. **100**(Suppl. 6A)**:**3S–12S.
16. **Kado, C. I., and S. T. Liu.** 1981. Rapid procedure for detection and isolation of large and small plasmids. J. Bacteriol. **145:**1365–1373.
17. **Kimura, K., Y. Arakawa, S. Ohsuka, H. Ito, K. Suzuki, H. Kurokawa, N. Kato, and M. Ohta.** 1996. Molecular aspects of high-level resistance to sulbactam-cefoperazone in *Klebsiella oxytoca* clinical isolates. Antimicrob. Agents Chemother. **40:**1988–1994.
18. **Levine, M. M.** 1987. *Escherichia coli* that cause diarrhea: enterotoxigenic, enteropathogenic, enteroinvasive, enterohemorrhagic, and enteroadherent. J. Infect. Dis. **155:**377–389.
19. **Livermore, D. M.** 1995. β-Lactamases in laboratory and clinical resistance. Clin. Microbiol. Rev. **8:**557–584.
20. **Matsumoto, Y., F. Ikeda, T. Kamimura, Y. Yokota, and Y. Mine.** 1988. Novel plasmid-mediated β-lactamase from *Escherichia coli* that inactivates oxyimino-cephalosporins. Antimicrob. Agents Chemother. **32:**1243–1246.
21. **National Committee for Clinical Laboratory Standards.** 1993. Methods for dilution antimicrobial susceptibility testing for bacteria that grow aerobically, 2nd ed. Approved standard. NCCLS document M7-A3. National Committee for Clinical Laboratory Standards, Villanova, Pa.
22. **Recchia, G. D., and R. M. Hall.** 1995. Gene cassettes: a new class of mobile element. Microbiology **141:**3015–3027.
23. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
24. **Sanders, C. C., and W. J. Sanders.** 1992. β-Lactam resistance in gram-negative bacteria: global trends and clinical impact. Clin. Infect. Dis. **15:**824–839.
25. **Seeberg, A. H., R. M. Tolxdorff-Neutzling, and B. Wiedemann.** 1983. Chromosomal β-lactamases of *Enterobacter cloacae* are responsible for resistance to third-generation cephalosporins. Antimicrob. Agents Chemother. **23:**918–925.
26. **Smith, C. L., and C. R. Cantor.** 1987. Purification, specific fragmentation, and separation of large DNA molecules. Methods Enzymol. **155:**449–467.
27. **Stamm, W. E., and T. M. Hooton.** 1993. Management of urinary tract infections in adults. N. Engl. J. Med. **329:**1328–1334.
28. **Unhanand, M., M. M. Mustafa, G. H. McCracken, Jr., and J. D. Nelson.** 1993. Gram-negative enteric bacillary meningitis: a twenty-one-year experience. J. Pediatr. **122:**15–21.
29. **Weber, D. A., C. C. Sanders, J. S. Bakken, and J. P. Quinn.** 1990. A novel chromosomal TEM derivative and alterations in outer membrane proteins together mediate selective ceftazidime resistance in *Escherichia coli*. J. Infect. Dis. **162:**460–465.