

Nosocomial Spread of Ceftazidime-Resistant *Klebsiella pneumoniae* Strains Producing a Novel Class A β -Lactamase, GES-3, in a Neonatal Intensive Care Unit in Japan

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Klebsiella pneumoniae strain KG525, which showed high-level resistance to broad-spectrum cephalosporins, was isolated from the neonatal intensive care unit (NICU) of a Japanese hospital in March 2002. The ceftazidime resistance of strain KG525 was transferable to *Escherichia coli* CSH-2 by conjugation. Cloning and sequence analysis revealed that production of a novel extended-spectrum class A β -lactamase (pI 7.0), designated GES-3, which had two amino acid substitutions of M62T and E104K on the basis of the sequence of GES-1, was responsible for resistance in strain KG525 and its transconjugant. The *bla*_{GES-3} gene was located as the first gene cassette in a class 1 integron that also contained an *aacA1-orfG* fused gene cassette and one unique cassette that has not been described in other class 1 integrons and ended with a truncated 3' conserved segment by insertion of IS26. Another five ceftazidime-resistant *K. pneumoniae* strains, strains KG914, KG1116, KG545, KG502, and KG827, which were isolated from different neonates during a 1-year period in the same NICU where strain KG525 had been isolated, were also positive for GES-type β -lactamase genes by PCR. Pulsed-field gel electrophoresis and enterobacterial repetitive intergenic consensus-PCR analyses displayed genetic relatedness among the six *K. pneumoniae* strains. Southern hybridization analysis with a GES-type β -lactamase gene-specific probe showed that the locations of *bla*_{GES} were multiple and diverse among the six strains. These findings suggest that within the NICU setting genetically related *K. pneumoniae* strains carrying the *bla*_{GES} gene were ambushed with genetic rearrangements that caused the multiplication and translocation of the *bla*_{GES} gene.

Resistance to β -lactam antibiotics mainly depends on the production of β -lactamases. To date, a large variety of β -lactamases which were classified by their amino acid sequences and functional substrate specificity profiles in various gram-negative bacilli such as *Pseudomonas* spp. and members of the family *Enterobacteriaceae* have been documented (6). Since the late 1980s, extended-spectrum β -lactamases (ESBLs) derived from TEM- and SHV-type penicillinases capable of hydrolyzing the oxymino-cephalosporins have been spreading globally, mainly in the *Enterobacteriaceae*, including *Klebsiella pneumoniae* and *Escherichia coli* (5, 23, 29). Moreover, various non-TEM-, non-SHV-type class A β -lactamases exhibiting extended-spectrum activities, including CTX-M-type (13, 31, 38, 39, 41), SFO-type (18), VEB-type (12, 20, 25), and GES-type (10, 11, 19, 24, 28, 37) β -lactamases, have also been reported in various gram-negative bacilli. Among the GES-type β -lactamases, GES-1, which was found to be produced by *K. pneumoniae* ORI-1, identified from a child transferred from French Guiana to France in 1998, was the first report of the GES-type class A β -lactamase (24); and GES-1-producing *K. pneumoniae* strains have caused nosocomial infections in Portugal (9). IBC-1 was

identified in an *Enterobacter cloacae* clinical isolate from Greece in 1999 (11), and IBC-1-producing *E. cloacae* has also been reported to cause nosocomial infections in a neonatal intensive care unit (NICU) (17). GES-2, which displayed more extended-spectrum activity against imipenem compared with that of GES-1, was reported in *Pseudomonas aeruginosa* from South Africa (28) in 2000, and GES-2 producers also caused a nosocomial infection (27). All three genes, *bla*_{GES-1}, *bla*_{GES-2}, and *bla*_{IBC-1}, were found to be located as a gene cassette within similar class 1 integrons.

Recently, six clinical isolates of *K. pneumoniae* showing high-level resistance to various broad-spectrum cephalosporins, including ceftazidime, were identified from the NICU of a Japanese hospital, and conventional PCR analyses for TEM-derived ESBLs and CTX-M enzymes failed to specify their genetic determinants. In the present study, therefore, we characterized the molecular mechanism underlying the multiple-cephalosporin resistance among these six strains, as well as the organizations of their genetic environments.

MATERIALS AND METHODS

Bacterial strains and plasmids. The strains and plasmids used in this study are listed in Table 1. Six *K. pneumoniae* clinical strains had been isolated from neonates over 1 year, from September 2001 to August 2002, and were stored in the clinical microbiology laboratory of the hospital until this study. Biochemical phenotypic identification of these strains was carried out by the analytical profile index procedure (API 20E system; bioMérieux, Marcy l'Etoile, France). A pre-

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Characteristics	Source or Reference
<i>K. pneumoniae</i> KG914, KG1116, KG525, KG545, KG502, KG827	Clinical isolates from neonatal specimens	This study
<i>E. coli</i>		
CSH-2	<i>metB</i> F ⁻ nalidixic acid ^r rifampin ^r	T. Sawai, Chiba University
XL1-Blue	<i>supE44 recA1 endA1 gyrA96 thi hsdR17</i> (rK ⁻ mK ⁺) <i>relA1 lac</i> [F ⁻ <i>proAB</i> ⁺ <i>lacI</i> qZ Δ M15::Tn10(Tet ^r)]	Stratagene
BL21(DE3)pLysS	F ⁻ <i>ompT hsdS_B</i> (r _B ⁻ m _B ⁻) <i>gal dcm</i> (DE3) pLysS (Cam ^r)	Invitrogen
Plasmids		
pKGC525	A natural plasmid carrying <i>bla</i> _{GES-3} of <i>K. pneumoniae</i> KG525	This study
pKGB525	A recombinant plasmid carrying a 6.7-kb BamHI fragment containing <i>bla</i> _{GES-3} of <i>K. pneumoniae</i> KG525	This study
pKGM525	A recombinant plasmid carrying a 11.6-kb BamHI fragment containing <i>bla</i> _{GES-3} of <i>K. pneumoniae</i> KG525	This study
pTAGES3	A recombinant plasmid carrying a PCR fragment with the entire <i>bla</i> _{GES-3} sequence and its promoter region cloned into the pCR2.1 vector	This study
pGES3	A recombinant plasmid carrying EcoRI fragment from pTAGES3	This study
pIBC1	A recombinant plasmid carrying <i>bla</i> _{IBC-1} constructed from pGES3	This study
pBCSK+	A cloning vector; chloramphenicol ^r	Stratagene
pCR2.1	A cloning vector; ampicillin ^r kanamycin ^r	Invitrogen
pET29a(+)	An expression vector; kanamycin ^r	Novagen
pET-GES3	A recombinant plasmid carrying PCR-amplified <i>bla</i> _{GES-3} gene ligated to pET29a(+)	This study

liminary double-disk synergy test was carried out with disks containing ceftazidime and amoxicillin-clavulanate. Bacteria were grown in Luria-Bertani (LB) broth supplemented with appropriate antibiotics, unless specified otherwise.

Antibiotic susceptibility testing. The following antibiotics were obtained from the indicated sources: ampicillin, amoxicillin, and cefminox, Meiji Seika Kaisha, Ltd., Tokyo, Japan; piperacillin, Toyama Chemical Co., Ltd., Toyama, Japan; cephaloridine and moxalactam, Shionogi & Co., Ltd., Osaka, Japan; cefmetazole and chloramphenicol, Sankyo Co., Ltd., Tokyo, Japan; cefotaxime and cefiprome, Aventis Pharma, Ltd., Tokyo, Japan; ceftazidime and clavulanic acid, GlaxoSmithKline K. K., Tokyo, Japan; sulbactam, Pfizer Pharmaceutical Inc., Tokyo, Japan; tazobactam, Taiho Pharmaceutical Co., Ltd., Tokyo, Japan; cefepime, Bristol Pharmaceuticals K. K., Tokyo, Japan; aztreonam, Eisai Co., Ltd., Tokyo, Japan; imipenem, Banyu Pharmaceutical Co., Ltd., Tokyo, Japan; and rifampin, Daiichi Pharmaceutical Co., Ltd., Tokyo, Japan. The MICs of the β -lactams were determined by the agar dilution method, according to the recommendations of National Committee for Clinical Laboratory Standards document M7-A5 (21). *E. coli* ATCC 25922 and ATCC 35218 were purchased from the American Type Culture Collection (ATCC) and served as control strains in the antimicrobial susceptibility testing.

PCR amplification. To amplify the broad-spectrum β -lactamase genes from the six clinical strains, PCR analyses were performed with sets of primers specific for various β -lactamase genes found in Japan—including the TEM-derived extended-spectrum β -lactamase (39); CMY-2-, MOX-1-, and DHA-1-type β -lactamases (8, 40, 41); and CTX-M-1-, CTX-M-2-, CTX-M-9-, IMP-1-, IMP-2-, and VIM-2-type β -lactamases (13, 26, 30, 31, 33, 39)—under the conditions described elsewhere (33). Detection of the SHV-type β -lactamase gene was not performed because most clinical *K. pneumoniae* strains carry the LEN-1 and/or SHV-1 β -lactamase gene on their chromosomes (1, 7). In order to detect the GES-type β -lactamase gene, an 827-bp internal fragment of the gene was amplified with primers GES-A (5'-CTT CAT TCA CGC ACT ATT AC-3') and GES-B (5'-TAA CTT GAC CGA CAG AGG-3') under the conditions described above.

Conjugal transfer of β -lactam resistance. Conjugal transfer of the ceftazidime resistance of *K. pneumoniae* KG525 to a recipient *E. coli* strain, strain CSH-2 (F⁻ *metB*, resistant to nalidixic acid and rifampin), was performed by the filter mating method. Transconjugants were selected on LB agar plates containing ceftazidime (2 μ g/ml), rifampin (100 μ g/ml), and nalidixic acid (50 μ g/ml).

Cloning experiment and DNA sequencing. Basic recombinant DNA techniques were performed as described by Sambrook et al. (32). Total DNA of *K. pneumoniae* KG525 was extracted and digested with BamHI. The resultant fragments were ligated into cloning vector pBCSK+ (Stratagene, La Jolla, Calif.) restricted with the same enzyme. Transformants were selected on LB agar plates

containing chloramphenicol (30 μ g/ml) and ampicillin (50 μ g/ml) or ceftazidime (2 μ g/ml). The nucleotide sequence of the cloned fragment was determined with BigDye terminator cycle Sequencing Ready Reaction kits and an ABI 3100 DNA sequencer (Applied Biosystems, Foster City, Calif.) by using custom sequencing primers.

Site-directed mutagenesis for comparison of GES-3 with IBC-1. PCR-based site-directed mutagenesis of the *bla*_{GES-3} gene was performed with the LA PCR In Vitro Mutagenesis kit (TAKARA Bio Inc., Ohtsu, Japan). In brief, the entire *bla*_{GES-3} gene and its promoter region were amplified by PCR and cloned into plasmid pCR2.1 with the TA cloning kit (Invitrogen, NV, Leek, The Netherlands). One plasmid, pTAGES3, was selected after it was confirmed that it contained no amplification error and was then digested with EcoRI. The resultant fragment was recloned into pBCSK+. The resultant plasmid, pGES3, with an insert carrying the *bla*_{GES-3} gene and its promoter region was used to introduce a single nucleotide mutation (C to T) at nucleotide position 167, which leads to an amino acid substitution (T to M) at position 62 in GES-3, resulting in the conversion of the gene product from GES-3 to IBC-1 expressed under the same promoter.

Pulsed-field gel electrophoresis (PFGE) and enterobacterial repetitive intergenic consensus (ERIC)-PCR analyses. Total DNA was prepared from six *K. pneumoniae* strains (34) and digested overnight with XbaI (New England Biolabs, Beverly, Mass.). The digested DNA was electrophoresed with a CHEF-DRII Drive Module (Bio-Rad Laboratories, Hercules, Calif.) under the following conditions: pulses ranging from 10 to 40 s at 6 V/cm for 20 h at 16°C. Six *K. pneumoniae* strains were also typed with the primer ERIC-2 (5'-AAG TAA GTG ACT GGG GTG AGG G-3'). The PCR was carried out under the conditions described elsewhere (36).

Southern hybridization. Large plasmids were prepared from six *K. pneumoniae* strains by the procedure described by Kado and Liu (16). The chromosomal DNA was extracted from each isolate by the method of Stauffer et al. (35). Both plasmid and chromosomal DNA preparations were separately subjected to Southern hybridization experiments. The 827-bp DNA probes were amplified by a PCR with primers 5'-CTT CAT TCA CGC ACT ATT AC-3' and 5'-TAA CTT GAC CGA CAG AGG-3'. The PCR amplicons were labeled with digoxigenin (DIG) by a random priming labeling method with the PCR DIG detection system, as recommended by the manufacturer (Roche Diagnostics, Tokyo, Japan). Southern hybridization was performed by the protocol of the manufacturer (Roche Diagnostics).

Purification of GES-3 β -lactamase. To overproduce GES-3 β -lactamase in *E. coli*, the *bla*_{GES-3} gene was amplified by using two primers, primer GES-F (5'-CAT ATG CGC TTC ATT CAC GCA CTA TTA CTG-3'), which was designed to add an NdeI linker (underlined), and primer GES-R (5'-GTC GAC

TABLE 2. MICs for six *K. pneumoniae* clinical isolates from a NICU

<i>K. pneumoniae</i> strain	Date of isolation (mo/day/yr)	Site of isolation	MIC ($\mu\text{g/ml}$) ^a													
			AMX + CLA	PIP	PIP + TZB	CAZ	CAZ + CLA	CTX	ATM	CMZ	FEP	IPM	GEN	AMK	LVX	CIP
KG914	9/14/01	Bronchial secretion	>128	>128	128	>1,024	512	64	64	16	32	0.13	1	32	<0.06	<0.06
KG1116	11/16/01	Bronchial secretion	>128	>128	128	>1,024	128	64	64	16	32	0.13	0.5	32	<0.06	<0.06
KG525	3/4/02	Stool	>128	128	128	>1,024	256	64	64	16	16	0.13	2	64	<0.06	<0.06
KG545	3/7/02	Nasal mucosa	>128	>128	>128	>1,024	1,024	128	128	128	64	0.5	2	64	0.25	0.25
KG502	5/2/02	Pus	>128	128	64	1,024	512	16	32	>128	8	8	2	32	0.13	<0.06
KG827	8/27/02	Bronchial secretion	>128	128	32	>1,024	256	16	64	>128	32	0.5	2	32	2	1

^a Abbreviations: AMX, amoxicillin; CLA, clavulanic acid; PIP, piperacillin; TZB, tazobactam; CAZ, ceftazidime; CTX, cefotaxime; ATM, aztreonam; CMZ, cefmetazole; FEP, cefepime; IPM, imipenem; GEN, gentamicin; AMK, amikacin; LVX, levofloxacin; CIP, ciprofloxacin.

CTA TTT GTC CGT GCT CAG GAT GAG-3'), which was designed to add an Sall linker (underlined), and DNA polymerase (Expand High Fidelity PCR System; Roche Diagnostics), according to the instructions of the manufacturers. The resulting products were cloned into plasmid pCR2.1 with the TA cloning kit (Invitrogen, NV) and subjected to confirmatory sequencing. One plasmid with no amplification error was selected and was partially double digested with NdeI and Sall and then subcloned into pET-29a(+) (Novagen, Madison, Wis.), which had been digested with the same enzymes. The expression vector constructed, named pET-GES3, was introduced into *E. coli* BL21(DE3) pLysS (Novagen). *E. coli* BL21(DE3) pLysS carrying plasmid pET-GES3 was cultured in 1 liter of LB broth containing kanamycin (50 $\mu\text{g/ml}$). Isopropyl- β -D-thiogalactopyranoside (final concentration, 1 mM) was added when the culture reached an A_{600} of 0.6, and the culture was incubated for an additional 2 h. The cells were harvested by centrifugation and were suspended in 5 ml of 20 mM bis-Tris buffer (pH 6.5). The suspension was passed through a French pressure cell twice and was then centrifuged at 100,000 $\times g$ for 1 h at 4°C. The supernatant was used for subsequent chromatographic purification. Size-exclusion chromatography was performed on a HiLoad 16/60 Superdex 200 prep-grade column (Pharmacia Biotech, Uppsala, Sweden) preequilibrated with 20 mM bis-Tris buffer (pH 6.5). Fractions containing β -lactamase activity were collected and applied to an anion-exchange Hitrap Q HP column with the same buffer. β -Lactamase activity was recovered in the flowthrough and was dialyzed against 20 mM Tris-HCl buffer (pH 8.0) overnight at 4°C. This partially purified enzyme was loaded onto a Hitrap Q HP column (Pharmacia Biotech) preequilibrated with the same buffer and eluted with a linear gradient of NaCl. Fractions presenting high levels of activity were pooled and dialyzed against 50 mM phosphate buffer (pH 7.0).

Isoelectric focusing (IEF). Fifty milliliters of the bacterial culture was centrifuged, and the cell pellet was suspended in 5 ml of distilled water. A crude periplasmic preparation containing β -lactamase was obtained by freezing-thawing the bacterial suspension three times, followed by ultracentrifugation (40,000 $\times g$) for 1 h. The supernatant was condensed to 1/10 volume with an Ultrafree-15 Centrifugal Filter Device (Millipore Corporation, Bedford, Mass.). To determine the isoelectric point (pI), 5 μl of the condensed supernatant containing β -lactamase was loaded onto an Ampholine PAG plate (pH 3.5 to 9.5; Pharmacia Biotech) with a Multiphor II electrophoresis system (Pharmacia Biotech). The pI of the β -lactamase was measured by staining the gel with a 0.05% solution of nitrocefin. Purified GES-3 β -lactamase was also electrophoresed on the Ampholine PAG plate and stained with Coomassie blue.

Nucleotide sequence accession number. The nucleotide sequence data reported in this paper are available in the GenBank nucleotide database under accession number AB113580.

RESULTS

Characteristics of six *K. pneumoniae* clinical isolates. The susceptibilities of the six isolates to β -lactams are presented in Table 2. All isolates were resistant to piperacillin, ceftazidime, and aztreonam. The MICs of cefotaxime, cefmetazole, cefepime, and imipenem for the isolates were variable. Despite the addition of clavulanic acid, these isolates kept their high-level resistance to ceftazidime (MICs, ≥ 128 $\mu\text{g/ml}$). This observation was consistent with the negative results of the double-disk synergy test with two disks containing ceftazidime and

amoxicillin-clavulanate, respectively. Metallo- β -lactamase production was not detected by using a thiol compound (2). PCR analyses performed preliminarily to detect broad-spectrum β -lactamase genes including TEM derivatives, CTX-M-1, CTX-M-2, CTX-M-9, MOX-1 (CMY-9), CMY-2, DHA-1, IMP-1, IMP-2, and VIM-2, all of which had already been identified in Japan, failed to give positive results.

Transfer and cloning of β -lactamase genes. The ceftazidime resistance determinant of representative strain *K. pneumoniae* KG525 was successfully transferred to a recipient strain, *E. coli* CSH-2; and this finding indicated that the genetic determinant was located on a transferable plasmid. Two ceftazidime-resistant *E. coli* clones, each of which harbored a plasmid containing BamHI fragment inserts of approximately 6.7 and 11.6 kb, respectively, were obtained as a result of the cloning experiment. These two recombinant plasmids contained the same 864-bp open reading frame (ORF) encoding a putative β -lactamase which had conserved structural features of the active site of Ambler class A β -lactamases. The deduced amino acid sequence of the β -lactamase showed an amino acid substitution of M62T (a point mutation of T to C at nucleotide position 167) compared with the amino acid sequences of GES-1 (24), GES-2, and IBC-1, as well as an additional E104K substitution in comparison with the amino acid sequences of GES-1 and GES-2 (Fig. 1). Moreover, an N170G substitution was found in GES-3 compared with the amino acid sequence of GES-2, although the G residue at amino acid position 170 was conserved in IBC-1 and GES-1, as well as in GES-3. Therefore, we named this novel class A β -lactamase GES-3, although GES-1 is based on "Guiana extended spectrum" (24).

Antibiotic susceptibilities. The MICs of the β -lactams for parent strain *K. pneumoniae* KG525, transconjugant *E. coli* CSH-2(pKGC525), and transformant *E. coli* XL1-Blue(pKGB525) are listed in Table 3. Parental strain *K. pneumoniae* KG525 was resistant to most β -lactams except the cephamycins and carbapenems. The tranconjugant and transformant were resistant to ceftazidime, and the MICs of the other β -lactams were lower for the tranconjugant and the transformant than for the parent strain. The changes in the MICs of cefotaxime and ceftazidime for parent strain KG525 were apparently observed by the addition of β -lactamase inhibitors, such as clavulanic acid, sulbactam, and tazobactam, while decreases in the MICs of amoxicillin, ampicillin, and piperacillin, as well as cefotaxime and ceftazidime, were observed for the *E. coli* tranconjugant and transformant in the presence of the inhibitors.

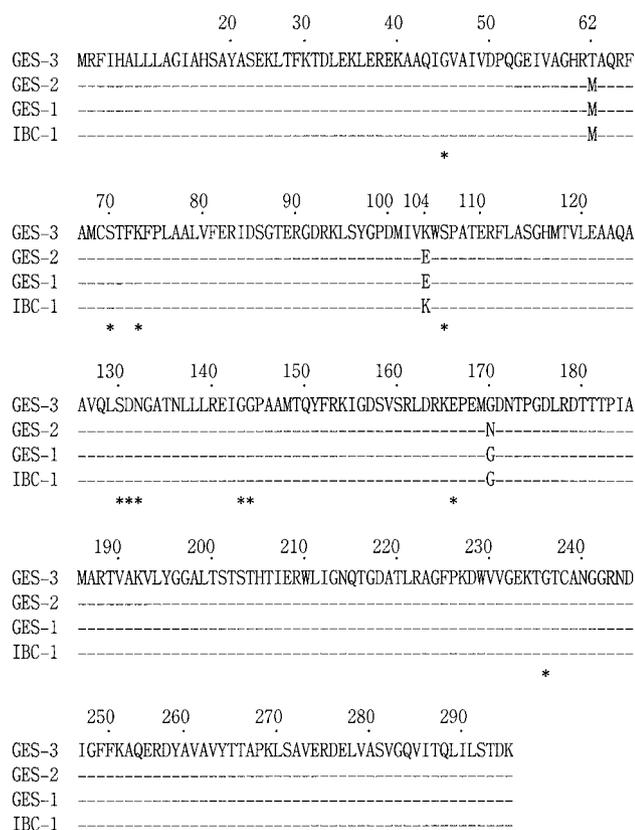


FIG. 1. Comparison of the amino acid sequence of GES-3 with those of the GES-1, GES-2, and IBC-1 β-lactamases. Only the substituted amino acid residues are indicated by the single-letter amino acid code. Dashes represent positions where no amino acid substitution was observed among the four enzymes. The amino acid residues conserved among class A β-lactamases are indicated with asterisks.

Genetic environment of *bla*_{GES-3}. Two distinct BamHI fragments carrying the *bla*_{GES-3} gene were cloned; their sizes were approximately 6.7 and 11.6 kb, respectively, and pKGB525 had the 6.7-kb fragment. Sequencing analysis of the entire insert on pKGB525 revealed that the *bla*_{GES-3} gene was located as a gene cassette within a class 1 integron structure, as was observed in the other GES-type β-lactamase genes, *bla*_{GES-1}, *bla*_{GES-2}, and *bla*_{IBC-1} (Fig. 2). The 59-base element downstream of the *bla*_{GES-3} gene was made up of 110 bp and was different from that of the truncated 59-base element of *bla*_{GES-1} on pTK1 (GenBank accession number AF156486), but shared it 99% nucleotide identity with those of *bla*_{GES-2} on pLAP-1 (GenBank accession number AF326355) and *bla*_{IBC-1} on pHT9-2 (GenBank accession number AF208529).

The second gene cassette adjacent to the *bla*_{GES-3} gene was a fused *aacA1-orfG* gene cassette. The results of the disk diffusion test indicated that the presence of an *aacA1* component, which encodes aminoglycoside-6'-N-acetyltransferase, conferred kanamycin resistance to the transformant *E. coli* XL1-Blue(pKGB525) (data not shown). The nucleotide sequence of this fused gene cassette shared 100% identity with that in a class 1 integron on plasmid pCMXR1 (GenBank accession number AB061794). The function of the product encoded by *orfG* has not been characterized in detail. The third gene cassette is 327 bp and was named *orfA*. The *orfA* gene was

suggested to be a gene cassette by recognition of the features typical of these elements: (i) the presence at the cassette boundaries of 7-bp core site sequences that completely fit the consensus sequence and (ii) the presence of a 59-base element of 78 bp downstream of the *orfA* gene. However, no remarkable similarity between the hypothetical protein encoded by *orfA* and any other known protein sequences was detected in a search performed with the BLAST program. The 3' conserved segment of this integron showed a characteristic organization. The *qacEΔ1* gene was truncated at nucleotide position 114 by the insertion sequence IS26. In the region downstream of IS26, an IS6100 element and two ORFs of unknown function, i.e., *orf5* and *orf6*, were found. The nucleotide sequence of the 1.4-kb region containing IS6100, *orf5*, and *orf6* was identical to that seen downstream of the *sullI* gene of the class 1 integron in the chromosomal multidrug resistance locus of *Salmonella enterica* subsp. *enterica* serovar Typhimurium (GenBank accession number AF261825). Sequencing analysis of pKGM525 carrying the second *bla*_{GES-3} gene on an 11.6-kb fragment was also done, and the *bla*_{GES-3} gene was also found in a class 1 integron structure with the same gene cassette configuration as in pKGB525. The nucleotide sequence of the region from *intI1* to IS26 was the same as that found in pKGB525 carrying a 6.7-kb BamHI fragment.

Construction of IBC-1 by site-directed mutagenesis. Only one amino acid substitution, M62T, was found between the sequences of GES-3 and IBC-1 (Fig. 1). Therefore, to examine whether this amino acid substitution affects the MICs of β-lactams for the *E. coli* clones producing each enzyme, we constructed plasmid pIBC1, which encodes the IBC-1 enzyme under the same promoter as that for GES-3, by site-directed mutagenesis of the *bla*_{GES-3} gene within the parental plasmid, pGES3. However, this single substitution did not markedly influence the MICs for the *E. coli* clones (data not shown).

PCR detection and genotypic comparison. The remaining five nonrepetitive ceftazidime-resistant *K. pneumoniae* strains, strains KG914, KG1116, KG545, KG502, and KG827, were all found to be *bla*_{GES} positive by PCR. The results of PFGE analysis of all six isolates are shown in Fig. 3. Their fingerprinting patterns were very similar but in some cases were distinct. We examined the fingerprinting patterns from 48.5 to 194 kb in detail under other conditions (data not shown). Overall, there were from three to seven band differences among the six strains examined. The ERIC-PCR patterns amplified with the ERIC-2 primer were indistinguishable from one another (data not shown). Taken together with the fact that these isolates were collected over a 1-year period, we speculate that they were genetically related and had probably spread via nosocomial transmission of an endemic clone.

Plasmid profiles and Southern hybridization. The plasmid profiles of the six *bla*_{GES}-positive strains showed the presence of a large plasmid of approximately similar size in five of the six strains (Fig. 4A), while some of them possessed additional plasmids which were smaller and more diverse in size. Hybridization analyses with the probe specific for the GES-type β-lactamase genes, including *bla*_{IBC-1}, revealed that the location of this gene varied among the strains tested. Hybridization signals for large plasmids were detected for strains KG914, KG1116, and KG502 (Fig. 4B). Hybridization signals for both plasmids and chromosomal positions were observed for

TABLE 3. MICs of antimicrobial agents for the parental strain, transconjugant, and transformant

β -Lactam ^a	MIC (μ g/ml)				
	<i>K. pneumoniae</i> KG525	<i>E. coli</i> CSH-2(pKGC525) ^b	<i>E. coli</i> CSH-2	<i>E. coli</i> XL1-Blue(pKGB525) ^c	<i>E. coli</i> XL1-Blue(pBCSK+)
Ampicillin	>128	>128	4	>128	4
Ampicillin + sulbactam	>128	2	2	2	2
Amoxicillin	>128	>128	8	>128	4
Amoxicillin + clavulanate	>128	32	4	32	4
Piperacillin	128	16	1	16	1
Piperacillin + tazobactam	128	0.5	1	0.5	0.5
Cefotaxime	64	2	0.13	2	0.13
Cefotaxime + clavulanate	8	0.06	0.06	0.06	0.06
Cefotaxime + sulbactam	32	0.06	0.06	0.06	0.06
Cefotaxime + tazobactam	64	0.06	0.06	0.06	0.06
Ceftazidime	>1,024	128	0.13	128	0.13
Ceftazidime + clavulanate	256	4	0.13	4	0.06
Ceftazidime + sulbactam	>128	0.25	0.13	0.5	0.13
Ceftazidime + tazobactam	>128	0.5	0.13	0.5	0.13
Cephaloridine	>128	16	2	16	2
Cefminox	8	0.5	0.5	1	0.5
Moxalactam	4	0.25	0.13	0.5	0.13
Cefpirome	>128	1	0.06	2	0.06
Cefepime	16	0.13	0.06	0.25	0.06
Aztreonam	64	4	0.06	4	0.06
Imipenem	0.25	0.5	0.25	0.13	0.13
Gentamicin	2	0.13	0.13	<0.06	<0.06
Amikacin	64	2	0.25	4	0.25
Levofloxacin	<0.06	0.13	0.13	<0.06	<0.06
Ciprofloxacin	<0.06	<0.06	<0.06	<0.06	<0.06

^a Clavulanate, tazobactam, and sulbactam were used at a fixed concentration of 4 μ g/ml each.

^b pKGC525 is a resident plasmid found in *K. pneumoniae* strain KG525, and it carries the *bla*_{GES-3} gene.

^c pKGB525 is a recombinant plasmid that carries a 6.7-kb BamHI insert that mediates the *bla*_{GES-3} gene.

KG525. One of the hybridized plasmids from each of KG914, KG1116, and KG525 were similar in size. A single hybridization signal corresponding to the chromosomal position was detected for each of the strains KG545 and KG827 (Fig. 4C).

pIs of β -lactamases. The pI value of the purified GES-3 enzyme was determined to be 7.0 (Fig. 5A). IEF of crude extracts from six GES-type β -lactamase-producing clinical strains revealed two bands with pIs of 7.6 and 7.0 (Fig. 5B). The band with pI 7.0 was also detected in a GES-3-producing

E. coli transformant which harbored the *bla*_{GES-3} gene of *K. pneumoniae* strain KG525. The band with a pI of 7.6 corresponds to the chromosomally encoded LEN-1 (1) or SHV-type penicillinase of *K. pneumoniae*.

DISCUSSION

Considerable differences in the levels of resistance to various cephalosporins were observed among the *E. coli* clones pro-

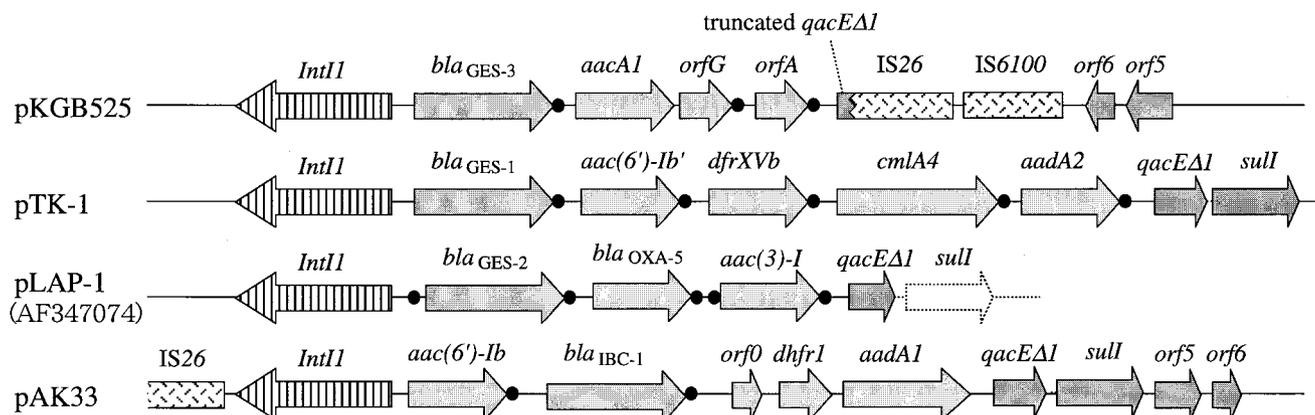


FIG. 2. Schematic comparison of the class 1 integron on pKGB525 with those on pTK1 (GenBank accession number AF156486), pLAP-1 (GenBank accession number AF326355), and pAK33 (34). Filled circles indicate the positions of GTTRRRY (core site) or the 59-base elements around the gene cassettes. pKGM525, which carries the 11.6-kb BamHI insert, was also sequenced; and the nucleotide sequence from *intI1* to IS26 was the same as that found in pKGB525.

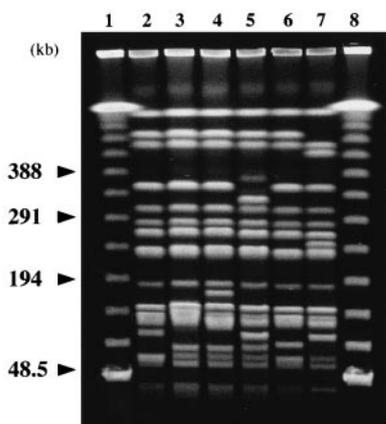


FIG. 3. PFGE analysis of *K. pneumoniae* isolates. Lanes: 1 and 8, PFGE marker; 2, *K. pneumoniae* KG914; 3, *K. pneumoniae* KG1116; 4, *K. pneumoniae* KG525; 5, *K. pneumoniae* KG545; 6, *K. pneumoniae* KG502; 7, *K. pneumoniae* KG827.

ducing GES-1, GES-2, and IBC-1, although the level of production of each enzyme may differ in individual clones. For instance, the MIC of ceftazidime for an *E. coli* clone producing GES-1 was 128 $\mu\text{g/ml}$ (24), while that for an *E. coli* clone producing GES-2 was 8 $\mu\text{g/ml}$ (28). The single amino acid substitution in the Ω loop observed between GES-1 and GES-2, G170N, may well contribute to the difference in the

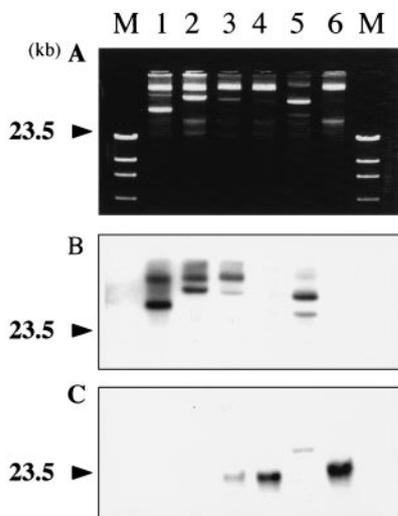


FIG. 4. Plasmid profiles and Southern hybridization analysis. (A) Plasmid profiles of each strain prepared by the method of Kado and Liu (16); (B) hybridization to large plasmids harbored by each strain; (C) hybridization to the chromosomal position of each strain. The photograph of the results of gel electrophoresis of chromosomal DNAs prepared by the method of Stauffer et al. (35) was omitted. The large plasmids and chromosomal DNA were separately extracted by using freshly prepared reagents to avoid cross contamination of nicked or physicochemically amputated DNA fragments. For strains KG545 and KG827, the bla_{GES} gene was suggested to be encoded by the chromosome. In strain KG525, the bla_{GES} gene was suggested to be encoded by both the plasmid and the chromosome. Lanes: M, HindIII-digested DNA marker; 1, *K. pneumoniae* KG914; 2, *K. pneumoniae* KG1116; 3, *K. pneumoniae* KG525; 4, *K. pneumoniae* KG545; 5, *K. pneumoniae* KG502; 6, *K. pneumoniae* KG827.

substrate specificities of these enzymes. On the other hand, the MIC of ceftazidime for an *E. coli* clone producing IBC-1 was $>256 \mu\text{g/ml}$ (11). The only amino acid substitution observed between GES-1 and IBC-1, E104K, might well also be attributed to a higher level of resistance of IBC-1 than that of GES-1 to ceftazidime, cefotaxime, and aztreonam (11, 19, 24). The MIC of ceftazidime for an *E. coli* clone producing GES-3 was 128 $\mu\text{g/ml}$, and GES-3 has a single M62T substitution compared with the sequence of IBC-1, which also confers high-level resistance to ceftazidime in an *E. coli* clone (MIC, $>256 \mu\text{g/ml}$). We investigated whether this one amino acid substitution observed between GES-3 and IBC-1 affects the MICs for *E. coli* clones expressing each enzyme. However, this substitution did not result in significant changes in the MICs for the *E. coli* clones. This finding suggests that the amino acid substitution at position 62 may not play a crucial role in the extended substrate specificity of GES-3 against ceftazidime and that those at positions 104 and 170 would be crucial for extended-spectrum enzyme activity.

In the present study, we also isolated a novel GES-type class A enzyme, GES-3, from *K. pneumoniae* strains which caused neonatal nosocomial infections in 2002 in Japan. Sequence analysis of the genetic environments of the bla_{GES-3} genes on pKGB525 carrying a 6.7-kb insert and pKGM525 carrying a 11.6-kb insert revealed that the bla_{GES-3} genes were located as gene cassettes in class 1 integrons, as observed in other GES-type β -lactamase genes, including bla_{IBC-1} (Fig. 2). Integrons are very sophisticated site-specific recombination systems that capture various gene cassettes, including antibiotic resistance genes, between their 5' and 3' conserved segments (14, 15, 22). The gene cassettes for the GES-type enzymes with a very close phylogenetic relationship might have originated as a single clone and then disseminated worldwide with the help of class 1 integrons possessing very similar genetic organizations. These integrons are mediated by self-transmissible plasmids with a wide host range. Since very similar GES enzymes have so far been found in French Guiana, Greece, South Africa, Portugal, and Japan, these GES-type β -lactamase-producing strains might have been scattered globally by the recent extensive international travel or dissemination of humans, foods, and animals.

In the present study, we analyzed genetic relatedness using PFGE and ERIC-PCR of all six GES-type β -lactamase-producing *K. pneumoniae* strains isolated in a NICU over a 1-year period. Since the fingerprinting patterns obtained by PFGE and ERIC-PCR were very similar, these isolates were suggested to belong to the same genetic lineage that caused the nosocomial spread. The minor differences in the fingerprinting patterns obtained by PFGE might be due to the occurrence of genetic rearrangements over the course of the nosocomial spread. Interestingly, the results of Southern hybridization suggested that the locations of the bla_{GES} genes were multiple and diverse among the six strains studied. By consideration of the results of PFGE, ERIC-PCR, and Southern hybridization, it can be speculated that an endemic strain containing the bla_{GES} genes might have spread within the NICU setting over the 1-year period and might have undergone genetic rearrangements, including translocation and multiplication of the bla_{GES} gene.

The presence of multiple bla_{GES-3} genes in strain KG525 is

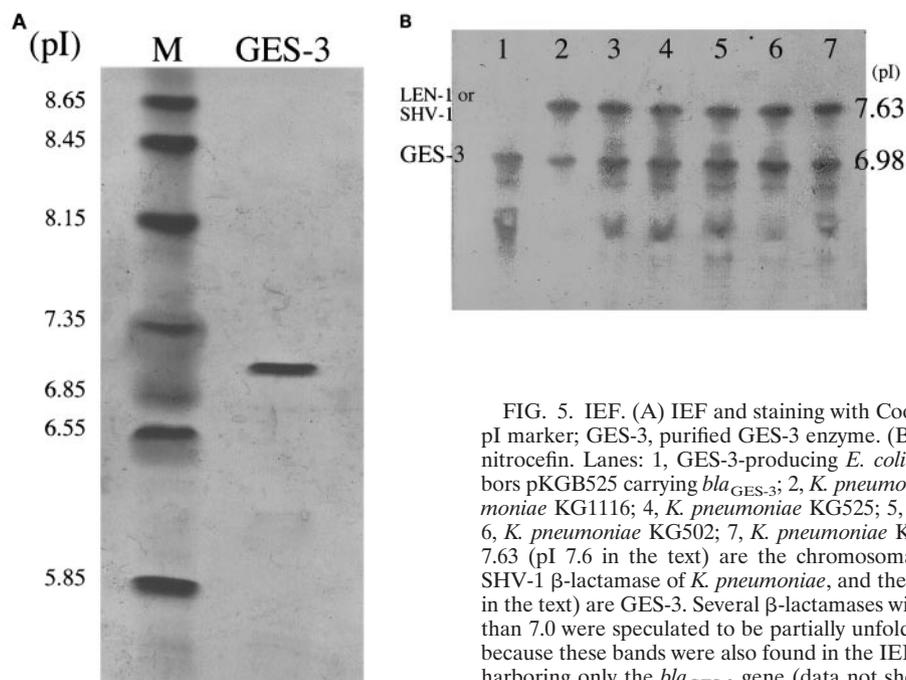


FIG. 5. IEF. (A) IEF and staining with Coomassie blue. Lanes: M, pI marker; GES-3, purified GES-3 enzyme. (B) IEF and staining with nitrocefin. Lanes: 1, GES-3-producing *E. coli* transformant that harbors pKGB525 carrying *bla*_{GES-3}; 2, *K. pneumoniae* KG914; 3, *K. pneumoniae* KG1116; 4, *K. pneumoniae* KG525; 5, *K. pneumoniae* KG545; 6, *K. pneumoniae* KG502; 7, *K. pneumoniae* KG827. The bands of pI 7.63 (pI 7.6 in the text) are the chromosomally encoded LEN-1 or SHV-1 β -lactamase of *K. pneumoniae*, and the bands at pI 6.98 (pI 7.0 in the text) are GES-3. Several β -lactamases with activities at pIs lower than 7.0 were speculated to be partially unfolded GES-3 β -lactamase, because these bands were also found in the IEF gels of an *E. coli* clone harboring only the *bla*_{GES-3} gene (data not shown).

probably the result of these genetic rearrangements. Translocation and multiplication of the *bla*_{GES} gene might be facilitated by mediation of a site-specific recombination system of an integron or a transposon. A similar example of multiple locations of the same antibiotic resistance gene has been reported by Yagi et al. (38). In their study, a single clinical *E. coli* isolate was found to carry three distinguishable Toho-1-like β -lactamase genes, which were later identified as *bla*_{CTX-M-2} by their restriction digestion patterns on the chromosome. These multiple locations of the same β -lactamase gene would be beneficial to bacteria, since they increase the chance of amino acid substitutions necessary for extension of the substrate profiles of β -lactamases as well as the multicopy effect of gene expression. Bradford et al. (3) reported that point mutations leading to ESBLs (ESBLs TEM-1 to TEM-10 and TEM-12) occurred on the plasmids of a single *K. pneumoniae* clinical isolate. A notable finding presented in that report was the distinct hydrolyzing activity between TEM-10 and TEM-12. TEM-10 had hydrolyzing activity against ceftazidime, while TEM-12 also hydrolyzed cefotaxime and aztreonam, in addition to ceftazidime. A variety of susceptibility profiles for cephamycins were also observed among the six *K. pneumoniae* strains in the present study. For instance, strains KG914, KG1116, and KG525 were susceptible to cefmetazole (MICs, 16 μ g/ml), whereas strains KG545, KG502, and KG827 were resistant to this agent (MICs, \geq 128 μ g/ml). In particular, strain KG502 showed high-level resistance to other cephamycins, such as ceftaxime (MIC, >128 μ g/ml), cefminox (MIC, >128 μ g/ml), and moxalactam (MIC, 128 μ g/ml). An evolutionary event similar to that observed in the TEM enzymes (3) might have occurred in these *K. pneumoniae* strains to give them further resistance to a broad range of antibiotics. The MIC of imipenem for strain KG502 was 8 μ g/ml, and this might be due to the hyperproduction of some β -lactamase with an extended

substrate specificity as well as the loss of some outer membrane protein, as reported by Bradford et al. (4). Further molecular characterization of the cephamycin resistance observed in strain KG502 will be undertaken in the next study.

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