TABLE 3. Isolation frequencies of metallo-β-lactamase-producing isolates in 13 laboratories

Laboratory code (type)	No. of isolates collected			No. (%) of MBL-producing isolates[c]		
	2000[a]	2001[b]	Total	2000[a]	2001[b]	Total
A (university hospital)	1,293	1,571	2,864	7 (0.5)	3 (0.2)	10 (0.4)
B (university hospital)	951	1,281	2,232	5 (0.5)	11 (0.9)	16 (0.7)
C (university hospital)	1,139	1,003	2,142	31 (2.7)	24 (2.4)	55 (2.6)
D (university hospital)	965	873	1,838	0 (0)	1 (0.1)	1 (0.1)
E (university hospital)	883	728	1,611	0 (0)	0 (0)	0 (0)
F (general hospital)	482	627	1,109	0 (0)	2 (0.3)	2 (0.2)
G (general hospital)	463	453	916	0 (0)	0 (0)	0 (0)
H (general hospital)	347	421	768	1 (0.3)	2 (0.5)	3 (0.4)
I (general hospital)	304	280	584	1 (0.3)	0 (0)	1 (0.2)
J (general hospital)	255	251	506	4 (1.5)	2 (0.8)	6 (1.2)
K (general hospital)	212	293	505	2 (0.9)	0 (0)	2 (0.4)
L (general hospital)	259	152	411	0 (0)	0 (0)	0 (0)
M (commercial laboratory)	ND[d]	4267	4267	ND[d]	0 (0)	0 (0)

[a] First study period, October 2000 to March 2001.
[b] Second study period, November 2001 to April 2002.
[c] Percentages are no. of MBL-producing isolates/no. of isolates collected × 100.
[d] ND, not determined.

lates were isolated from inpatients with bacterial infections. With respect to the MBL genotypes, 87 (90.6%) isolates carried genes encoding IMP-1-group MBLs, including IMP-1, IMP-3, IMP-6, and IMP-10. Similarly, seven (7.3%) isolates carried genes for IMP-2-group MBLs, such as IMP-2, IMP-8, and IMP-11. Genes encoding VIM-2-group MBLs, such as VIM-2, VIM-3, and VIM-6, were carried by two (2.1%) isolates. Genes for IMP-1-group MBLs were detected in 21 isolates of *P. aeruginosa* at hospitals B, C, and H; 14 isolates of *Acinetobacter* spp. at hospitals A, C, F, H, and J; 44 isolates of *S. marcescens* at hospitals A and C; 4 isolates of *C. freundii* at hospital C; 3 isolates of *Providencia rettgeri* at hospital C; and 1 isolate of *M. morganii* at hospital C. Genes for IMP-2-group MBLs were detected in seven isolates of *Acinetobacter* spp. at hospitals J and K. Genes for VIM-2-group MBLs were detected in one isolate of *P. aeruginosa* at hospital I and one isolate of *E. cloacae* at hospital D. The integrase gene (identified as *intI1*) was detected in all 96 MBL-producing isolates. The *aac (6')-Ib* gene was detected in 37 (38.5%) MBL-positive isolates.

Of 96 MBL-positive isolates, 68 (70.8%), 8 (8.3%), 7 (7.3%), 4 (4.1%), 3 (3.1%), 2 (2.1%), 2 (2.1%), and 2 (2.1%) were recovered from urine, sputum, throats, pus, drains, blood, tracheal tubes, and other samples, respectively. The majority of *A. baumannii* isolates were recovered from respiratory tract specimens, and bacterial species belonging to the family *Enterobacteriaceae* and *P. aeruginosa* were recovered from urine.

RAPD typing with the 272 primer of 22 *P. aeruginosa* isolates from four hospitals identified 11 distinct types. Two or more isolates with the same banding patterns were observed for two of the four hospitals. Of 21 *A. baumannii* isolates from six hospitals, 8 isolates (same ward) from hospital A were found to belong to the same clonal lineage, and 5 isolates (two wards) from hospital J belonged to another clonal lineage. Of 44 *S. marcescens* isolates from hospitals A and C, 2 isolates from hospital A had the same pattern, and two distinct patterns were observed for 42 isolates recovered from hospital C. Forty-one of the isolates from hospital C shared the same pattern, and they had been isolated from eight different wards. At hospital C, the two isolates of *Providencia rettgeri* had the same pattern, and four isolates of *C. freundii* also shared the same pattern. Genetically related isolates, such as those of *P. aeruginosa* in hospital B, *A. baumannii* in hospitals A and J, and *S. marcescens*, *Providencia rettgeri*, and *C. freundii* in hospital C, were isolated from the same ward, suggesting a nosocomial spread of these organisms.

Susceptibility of MBL-producing isolates. The results of susceptibility tests are shown in Table 5. The susceptibilities of the 96 MBL-positive isolates to several antimicrobial agents varied. For the MBL-producing bacterial species belonging to the family *Enterobacteriaceae*, *P. aeruginosa*, and *Acinetobacter* spp., the MICs at which 50% of the isolates were inhibited (MIC_{50}s) of imipenem were 32, 16, and 16, respectively, and the MIC_{90}s of the same agent were >32, 32, and 32 μg/ml, respectively. For species belonging to the family *Enterobacteriaceae*, piperacillin-tazobactam, aztreonam, gentamicin, amikacin, and levofloxacin had relatively high activities. *P. aeruginosa* isolates had similar or lower susceptibilities to non-β-lactam agents than bacterial species belonging to the family *Enterobacteriaceae* or *Acinetobacter* spp. Piperacillin-tazobactam and cefoperazone-sulbactam appeared to have the most potent activities against MBL-producing *Acinetobacter* spp. The MICs for *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 were within the NCCLS control ranges.

DISCUSSION

We investigated the distribution and prevalence of MBL-producing gram-negative rods with the cooperation of 12 clinical laboratories at large-scale general hospitals and one commercial clinical laboratory in the Kinki region of Japan. Such isolates were identified at a rate of 0.5%. In a previous laboratory-based surveillance conducted in 1998 and 2000, MBL-producing isolates were found only in specimens collected by a commercial laboratory (44). However, MBL producers were isolated from 9 of 13 laboratories in the present multi-institutional surveillance study, and the prevalence of MBL-positive isolates ranged from 0 to 2.6%, suggesting that there is a continuous proliferation of MBL producers in Japan. The ma-

TABLE 4. Characteristics of MBL-producing strains and selected clinical data

Species (no. of strains)	RAPD type	MBL type[a] (No. of strains)	No. of *aac(6')-Ib*-a positive strains	Hospital	Ward[b] (no. of strains)	Antibiogram[c] (no. of strains)
P. aeruginosa (22)	a	IMP-1 (5)	4	B	ICU (1), NICU (1), pediatrics 7E (3)	GM, MINO, ST, CP (4), GM, ST, CP (1)
	b	IMP-1 (6)	6	B	Internal medicine 11E (2), internal medicine 13E (1), internal medicine 8E (1), pediatrics 7E (2)	GM, MINO, ST, CP (6)
	c	IMP-1 (1)	0	B	Internal medicine 11E (1)	GM, MINO, LVFX, ST, CP (1)
	d	IMP-1 (1)	1	B	Urology 8W (1)	GM, MINO, ST, CP (1)
	e	IMP-1 (1)	0	B	Urology 8W (1)	MINO, ST, CP (1)
	f	IMP-1 (2)	2	B	Urology 8W (2)	GM, AMK, MINO, LVFX, ST, CP (1), GM, MINO, LVFX, ST, CP (1)
	g	IMP-1 (1)	0	C	Emergency 1S (1)	GM, MINO, LVFX, ST, CP (1)
	h	IMP-1 (1)	0	C	Emergency 1S (1)	GM, AMK, MINO, LVFX, ST, CP (1)
	i	IMP-1 (1)	0	C	Plastic surgery 4C (1)	GM, MINO, LVFX, ST, CP (1)
	j	IMP-1 (2)	2	H	Urology 5W (1), internal medicine MICU (1)	GM, MINO, LVFX, ST, CP (2)
	k	VIM-2 (1)	1	I	Internal medicine 6E (1)	GM, MINO, LVFX, ST, CP (1)
A. baumannii (18)	A	IMP-1 (8)	8	A	Internal medicine 115NS (8)	GM, ST, CP (8)
	B	IMP-1 (1)	1	C	Otolaryngology 7E (1)	GM, CP (1)
	C	IMP-1 (1)	1	F	Cardiac surgery ICU (1)	ST, CP (1)
	D	IMP-1 (1)	1	F	Brain surgery SCU (1)	ST, CP (1)
	E	IMP-1 (1)	1	H	Internal medicine 7E (1)	CP (1)
	F1	IMP-2 (5)	0	J	Surgery W6 (3), brain surgery W7 (2)	ST, CP (5)
	F2	IMP-2 (1)	0	K	Internal medicine 5A (1)	GM, ST, CP (1)
A. junii (1)		IMP-1 (1)	1	C	Otolaryngology 7E (1)	GM, ST (1)
A. calcoaceticus (1)		IMP-1 (1)	0	J	Internal medicine ICU (1)	AMK, ST (1)
A. johnsonii (1)		IMP-2 (1)	0	K	Internal medicine 5A (1)	ST (1)
S. marcescens (44)	1	IMP-1 (2)	0	A	Neurology 75NS (1), urology 65NS (1)	MINO, LVFX, ST, CP (1) MINO, LVFX, CP (1)
	2	IMP-1 (1)	0	C	Urology 7F (1)	MINO, ST, CP (1)
	3	IMP-1 (41)	0	C	Emergency 1S (14), internal medicine CCU (13), urology 7F (5), brain surgery 6F (2), cardiac surgery 4S (2), internal medicine 3S (2), plastic surgery 5C (2), surgery 4F (1)	CP (7), AMK (1), MINO (1), MINO, CP (1)
Providencia rettgeri (3)	1	IMP-1 (2)	2	C	Urology 7F (2)	ST, CP (2)
	2	IMP-1 (1)	1	C	Internal medicine 83 (1)	MINO, LVFX, ST (1)
Citrobacter freundii (4)	1	IMP-1 (4)	4	C	Internal medicine CCU (3), internal medicine 3S (1)	CP (1)
Morganella morganii (1)		IMP-1 (1)	1	C	Urology 7F (1)	MINO, LVFX, ST (1)
Enterobacter cloacae (1)		VIM-2 (1)	0	D	Pediatrics E6 (1)	MINO, ST, CP (1)

[a] MBL types: IMP-1, IMP-1-group MBLs, including IMP-1, IMP-3, IMP-6, and IMP-10; IMP-2, IMP-2-group MBLs, including IMP-2, IMP-8, and IMP-11; VIM-2, VIM-2 MBLs.

[b] ICU, intensive care unit; NICU, neonatal intensive care unit; MICU, medical intensive care unit; SCU, surgical care unit; CCU, coronary care unit.

[c] Antibiogram: AMK, amikacin (64 μg/ml); GM, gentamicin (16 μg/ml); MINO, minocycline (16 μg/ml); LVFX, levofloxacin (8 μg/ml); ST, sulfamethoxazole-trimethoprim (4 μg/ml); CP, chloramphenicol (32 μg/ml).

jority of MBL producers detected in the present study were *S. marcescens*, *P. aeruginosa*, or *Acinetobacter* spp., which was similar to the results of previous studies (33, 37). Moreover, in the present study, several strains of *Providencia rettgeri* and *M. morganii* that produce MBLs were found, suggesting that plasmid-mediated horizontal transfer of the MBL genes is so far likely to occur continuously among gram-negative bacilli, as reported previously (14, 35). This finding gives us an alert on the further dissemination of MBL genes among various gram-negative bacilli. In the present study, the predominant type of MBL in Japan was found to be the IMP-1 group, but MBLs belonging to the IMP-2 group and the VIM-2 group were also detected. MBL genes encoding bla_{IMP-2} and bla_{VIM-2} have been reported in Italy (21, 33), France (32), Korea (22), Taiwan (45, 46), Portugal (7), and Greece (25). Recently, a genetic classification of the MBLs detected in Japan was reported (37), but the frequency of isolation of MBL producers from clinical specimens was not described. The present study provides the

TABLE 5. Susceptibility of MBL-producing isolates to various antimicrobial agents

Antimicrobial agent[a]	MIC (μg/ml) for organism								
	Enterobacteriaceae (n = 53)			*P. aeruginosa* (n = 22)			*Acinetobacter* spp. (n = 21)		
	Range	MIC_{50}	MIC_{90}	Range	MIC_{50}	MIC_{90}	Range	MIC_{50}	MIC_{90}
Ceftazidime	16–>128	>128	>128	64–>128	128	>128	128–>128	>128	>128
Piperacillin	8–>128	16	>128	4–>128	16	>128	8–>128	32	128
Piperacillin-tazobactam[a]	≤1–>128	8	16	2–>128	8	>128	≤1–32	≤1	4
Cefepime	2–>128	64	128	32–>128	64	>128	64–>128	128	128
Cefoperazone-sulbactam	32–>128	>128	>128	64–>128	64	>128	≤1–16	2	4
Aztreonam	≤1–64	16	32	2–>128	8	32	4–32	16	32
Cefmetazole	>128–>128	>128	>128	>128–>128	>128	>128	64–>128	>128	>128
Latamoxef	64–>128	>128	>128	>128–>128	>128	>128	>128–>128	>128	>128
Meropenem	1–>32	>32	>32	16–>32	32	>32	8–>32	32	>32
Imipenem	1–>32	32	>32	1–>32	16	32	8–>32	16	32
Gentamicin	≤1–4	≤1	2	2–>8	>8	>8	≤1–>8	8	>8
Amikacin	≤4–>32	16	32	≤4–>32	16	32	≤4–>32	8	16
Minocycline	≤4–>8	≤4	>4	>8–>8	>8	>8	≤4–≤4	≤4	≤4
Levofloxacin	≤2–>4	≤2	4	≤2–>4	4	>4	≤2–4	≤2	≤2
Sulfamethoxazole-trimethoprim	≤0.5–>2	≤0.5	>2	>2–>2	>2	>2	≤0.5–>2	>2	>2
Chloramphenicol	≤8–>16	16	>16	>16–>16	>16	>16	≤8–>16	>16	>16

[a] Tazobactam was tested at a fixed concentration of 4 μg/ml.

first reported data on the prevalence of bacteria carrying the genes for MBLs, including IMP-1, IMP-2, and VIM-2 MBLs, in the western portion of Japan.

A total of 96 MBL-positive isolates were typed by RAPD analysis to determine the stabilities of the strain genotypes. The RAPD typing results are summarized in Table 4. The 16 isolates of *P. aeruginosa* from hospital B yielded seven different RAPD patterns and originated from six different wards. Eight *A. baumannii* isolates from hospital A had the same RAPD pattern and were from the same ward. The five *A. baumannii* isolates carrying the genes for IMP-2-group MBLs, isolated from two wards of hospital J, appeared to be of the same clonal lineage. This is the first report of nosocomial spread of *A. baumannii* isolates carrying genes for IMP-2-group MBLs in Japan. Of the 41 *S. marcescens* isolates from hospital C, 13 isolates from the internal medicine coronary care unit had the same RAPD pattern and were isolated within a 5-month period, suggesting that there was probable nosocomial spread within the same ward. Thus, the same or closely related isolates were identified repeatedly by PCR fingerprinting by RAPD analysis from five hospitals, suggesting the nosocomial spread of these organisms in each hospital. Furthermore, long-term cross-transmission of plasmids that carry MBL genes among different bacterial strains and species could result in the current complicated features of MBL producers, especially in hospital B.

The 2-MPA test, which is a simple test that was first described by Arakawa et al. (2), is a useful method for the routine laboratory detection of MBLs (45). Moreover, in the present study, all isolates that tested positive in the 2-MPA test were subsequently confirmed to be positive for the MBL gene by PCR. However, the growth inhibition zones of bla_{VIM-2}-positive *E. cloacae* isolates were weak and ambiguous, possibly due to the excessive production of AmpC and/or a change in membrane permeability. The production of some extended-spectrum β-lactamases as well as the excessive production of the chromosomal AmpC cephalosporinase could be responsible for the characteristics of these strains that were previously reported for *E. cloacae* (2, 7). In such cases, imipenem and meropenem disks would be better than ceftazidime disks for the detection of MBL production because imipenem and meropenem are essentially not hydrolyzed by extended-spectrum β-lactamases and class C cephalosporinases.

With respect to antimicrobial susceptibilities, various β-lactam antimicrobial agents such as ureidopenicillin, cephalosporins, cephamycins, and carbapenems had high MICs for most MBL-positive isolates, whereas monobactam and piperacillin typically had low MICs for MBL producers. Low MICs of cefepime, meropenem, and imipenem were observed for several isolates, even though MBLs can hydrolyze these agents. The production of MBLs in these isolates could be cryptic or suppressed in strains showing low-level carbapenem resistance (14). It is also possible that IMP-3 and IMP-6 MBLs, which have low-level hydrolytic activities against these agents (15, 47), are produced in such isolates. The increased ability of active efflux systems and decreased outer membrane permeabilities have been reported to contribute to β-lactam resistance in *P. aeruginosa* (23, 24). Therefore, the low-level MICs of piperacillin, cefepime, and carbapenems for some isolates may be due to higher permeability coefficients or less efficient efflux pumps in the bacterial membranes in addition to the molecular mechanisms described above.

The MICs of monobactam and piperacillin for MBL producers were relatively low compared to those of oximinocephalosporins, cephamycins, and carbapenems (35, 36); however, this finding does not necessarily reflect their clinical efficacy against MBL producers because most gram-negative rods have the intrinsic ability to produce chromosomal AmpC cephalosporinases, which can hydrolyze monobactam and piperacillin (17). Although the administration of high doses of aztreonam or tazobactam-piperacillin was reported to be useful for the reduction of MBL-producing strains in rats suffering from experimental pneumonia (3), it is possible that the induction of intrinsic chromosomal AmpC production in MBL producers may promote the emergence of multiple-β-lactam-resistant gram-negative rods in clinical settings.

In the present study, MICs of tazobactam-piperacillin and cefoperazone-sulbactam were generally low for MBL-positive *Acinetobacter* isolates. Strains producing IMP-1 or VIM-2 usually show high-level resistance to oximinocephalosporins and cephamycins, but the MIC of piperacillin for these strains is usually lower than those of oximinocephalosporins and cephamycins (9, 30, 32). Because the activities of MBLs are not reduced significantly by β-lactamase inhibitors, such as sulbactam and tazobactam (5), the observations for *Acinetobacter* isolates suggested that the phenotypes related to these combination drugs may depend mainly on the intrinsic production of AmpC cephalosporinase (4, 10) as well as the low-level production of MBLs and alterations in membrane permeability. Thus, the low MIC levels of tazobactam-piperacillin and cefoperazone-sulbactam for MBL-producing *Acinetobacter* isolates could be an intrinsic feature of this bacterial genus.

In conclusion, plasmid-mediated MBL-producing gram-negative rods were first described approximately 13 years ago in Japan, and in the present study, such isolates were found to have disseminated to many hospitals in the Kinki region of Japan. It is conceivable that several isolates have spread nosocomially among a number of hospitals. The results of the present study should be considered when health care facilities develop policies and strategic practices to prevent and address the emergence and spread of MBL-producing gram-negative microorganisms in clinical environments.

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Nosocomial Transmission of CTX-M-2 β-Lactamase-Producing *Acinetobacter baumannii* in a Neurosurgery Ward

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Three strains of cefotaxime (CTX)-resistant *Acinetobacter baumannii*, FM0209680, FM0300106, and FM0301433, were isolated from transtracheal aspirate cultures of three patients with probable nosocomial infections in a neurosurgery ward in Japan. The CTX MICs for these isolates were greater than 128 μg/ml but were drastically reduced in the presence of 4 μg of clavulanic acid per ml. These strains were also resistant to ceftriaxone, cefpodoxime, and aztreonam but were susceptible to ceftazidime and imipenem. The profile of resistance to various broad-spectrum β-lactams was transferred by conjugation. Strain FM0209680 was not eradicated from case patient 1 by administration of imipenem, ceftazidime, and levofloxacin, even after a 6-month hospitalization period. Strains FM0300106 and FM0301433 were isolated from case patients 2 and 3 during the sixth week following admission, respectively, and then each patient was colonized for 3 weeks. Eradication of FM0300106 was successfully obtained from case patient 2 by imipenem treatment, while administration of imipenem was continued to prevent pneumonia. Prophylactic antimicrobial therapy was discontinued in case patient 3 because of the lack of pneumonic symptoms, and FM0301433 disappeared after the discontinuation of antimicrobial chemotherapy. All three strains carried the $bla_{CTX-M-2}$ gene, and the appearance of colonies in the growth-inhibitory zones around disks of CTX and aztreonam in double-disk synergy tests suggested inducible β-lactamase production in these *A. baumannii* strains. The ribotyping investigation suggested that all these strains belong to the same clonal lineage. The plasmids harbored by *A. baumannii* had the same restriction profile as those harbored by *Proteus mirabilis* strains previously isolated in a urology ward of the Funabashi Medical Center.

Acinetobacter species, including *Acinetobacter baumannii*, had been regarded as one of the important groups of opportunistic pathogens implicated in various infections such as pneumonia, urinary tract infection, endocarditis, surgical site infection, meningitis, and septicemia, particularly in immunocompromised patients (4). *A. baumannii* has recently been rerecognized as an important causative pathogen of nosocomial infections (2). Patients admitted to intensive care units tend to become the main victims of this nosocomial pathogen, which occurs worldwide (12, 22, 29, 54). Increasing therapeutic difficulties due to the acquisition of a profile of multidrug resistance to major groups of antimicrobial agents by various bacterial species have been becoming a serious clinical concern (1, 6, 14, 21, 23, 31, 51). A wide variety of molecular mechanisms for resistance to broad-spectrum β-lactams have been elucidated, i.e., β-lactamase production, mutations of penicillin-binding proteins, and alterations to membrane permeability as well as augmented functioning of the active efflux system (4, 6, 20, 27). *Acinetobacter* species are renowned for their characteristic nature of readily accepting foreign DNA in order to survive in hazardous environments (15, 36). Various β-lactamases demonstrating broad-spectrum substrate specificities that allow *A. baumannii* to cope with broad-spectrum β-lactams, such as OXA-type class D β-lactamases (19, 39, 53), metallo-β-lactamases belonging to class B β-lactamases (45, 51, 57), and AmpC-type class C β-lactamases (7), have been found in *A. baumannii* so far. Moreover, PER-1 and VIB-1 class A β-lactamases demonstrating broad-spectrum substrate specificities have also recently been detected among nosocomially isolated *A. baumannii* strains in Turkey (52) and were then detected in France (40, 41) and Korea (26).

Since June 2002, the medical microbiology laboratory of the Funabashi Medical Center, Chiba, Japan, isolated three cefotaxime-resistant *A. baumannii* strains from inpatients in a neurosurgery ward. In the present study, we characterized the molecular mechanism of cefotaxime resistance in *A. baumannii* strains associated with a nosocomial infection episode.

MATERIALS AND METHODS

Bacterial strains. Three cefotaxime-resistant *A. baumannii* strains, FM0209680, FM0300106, and FM0301433, were isolated from cultures of transtracheal aspirates from three different inpatients in the neurosurgery ward of the Funabashi Medical Center since June 2002. Biochemical identification of the isolates was performed with the API 20NE system (bioMérieux, Marcy l'Etoile, France) combined with a complementary test for the ability to grow at 44°C. Alternatively, sequencing of the 16S rRNA gene was performed by the method described by Sasaki et al. (46). β-Lactamase testing was performed on the basis of acidometry by using a commercial product (P Case test; Nissui Pharmaceutical, Tokyo, Japan). The bacterial strains were stored in Casitone medium (Eiken Chemical, Tokyo, Japan) at room temperature until they were used.

MIC determinations. MICs were determined by a microdilution broth method with a WalkAway-96 SI system (NEG Combo 5J and NEG MIC 5J panels; Dade Behring, Sacramento, Calif.) with an inoculum of 10^5 CFU per well. Suscepti-

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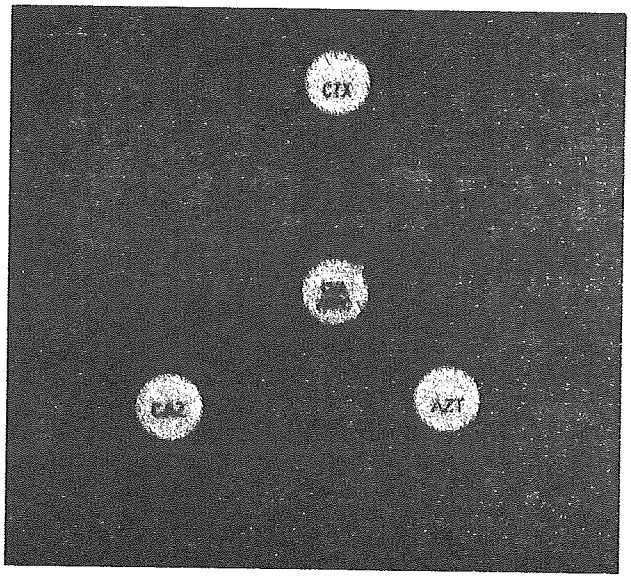

FIG. 1. Double-disk synergy test with CTX-M-2 ESBL-producing *A. baumannii* FM0209680. Disks: CTX, cefotaxime at 30 μg; CAZ, ceftazidime at 30 μg; AZT, aztreonam at 30 μg; CVA · AMPC, clavulanic acid at 10 μg and amoxicillin at 20 μg.

bility categories were determined according to the criteria of the National Committee for Clinical Laboratory Standards (35).

The ESBL plus panel (Dade Behring) with an inoculum of 10^4 CFU per well was used as complementary test for MIC measurements. The panel was incubated for 18 h at 35°C, and then the results were assessed visually.

β-Lactamase study. The double-disk synergy test was used to screen for extended-spectrum β-lactamase (ESBL)-producing strains. Kirby-Bauer disks containing cefotaxime (30 μg), ceftazidime (30 μg), aztreonam (30 μg), and amoxicillin-clavulanic acid (20 μg-10 μg) for tests on Mueller-Hinton agar (BBL Microbiology Systems, Cockeysville, Md.) were obtained from Nissui Pharmaceuticals. The distance between the disks was adjusted so that synergy could be detected correctly (48), as shown in Fig. 1.

The inducibility of AmpC β-lactamase production was tested by disk antagonism tests (30). Disks containing an inducing agent, cefoxitin at 30 μg or ceftazidime at 30 μg, were placed on Mueller-Hinton agar plates (BBL Microbiology Systems). The distance between the disks was adjusted so that the blunting phenomenon of the ceftazidime zone could be detected correctly.

PCR amplification and *bla* gene sequencing. The oligonucleotides used as primers for amplification and sequencing are shown in Table 1. A search for the bla_{TEM} (49), bla_{SHV} (41), $bla_{CTX\text{-}M\text{-}1}$ (3), $bla_{CTX\text{-}M\text{-}2}$ (24), and $bla_{CTX\text{-}M\text{-}9}$ (32) genes in the clinical isolates was performed by PCR amplification, as described previously (34). Detection of the *ampC* gene was performed as described by Bou and Martínez-Beltrán (7).

A resistance plasmid carrying the $bla_{CTX\text{-}M\text{-}2}$ gene was prepared from the *Escherichia coli* CSH2 (F⁻ *metB*, with resistance to both nalidixic acid and rifampin [18]) transconjugant. The 902-bp fragments corresponding to the main frame of the $bla_{CTX\text{-}M\text{-}2}$ gene were amplified by PCR with a set of primers (43) and were used for sequencing. The PCR products were labeled with fluorescent materials by using a BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Foster City, Calif.). Both strands of the DNA sequences were analyzed with an ABI PRISM 377 XL sequencer analyzer (Applied Biosystems). The nucleotide and deduced amino acid sequences were analyzed and compared with sequences in a database by using the FASTA analysis programs available at the National Institute of Genetics (Mishima, Japan) website (http://www.nig.ac.jp/section/service.html).

Plasmid preparation and restriction endonuclease analysis. The resident plasmids of three *A. baumannii* strains and previously isolated *Proteus mirabilis* strains (34) were prepared by a conventional protocol (38). The plasmids were digested with EcoRI, and the restriction fragment profiles were compared by agarose gel electrophoresis.

Automated ribotyping and analysis. Because pulsed-field gel electrophoresis analysis failed to give clear results due to the persistent degradation of DNA, we used automated ribotyping for analysis of the genetic relatedness of the three clinical isolates. Ribotyping was performed with the restriction enzyme EcoRI and a RiboPrinter (Qualicon Inc., Wilmington, Del.), as described previously (13, 33). Briefly, a single colony from a 5% sheep blood agar plate was suspended in a sample buffer and heat treated at 80°C for 15 min. After addition of lysis buffer to release the DNA, the sample was loaded into the RiboPrinter system. Further processing including EcoRI digestion, agarose gel separation, transfer to a nylon membrane, and hybridization with a chemiluminescence-labeled DNA probe containing the rRNA operon from *E. coli*, which was carried out by automated instruments in 8 h. Output ribotype patterns with similarity coefficients of >0.93 were considered a single ribogroup and were automatically given a code number. Computerized ribotypes were finally exported for analysis in text files and imported into BioNumerics software (version 3.0; Applied Maths, Sint-Martens-Latem, Belgium) by using the Qualicon macro. Clustering analysis was performed by the unweighted pair group with arithmetic averages method (48), based on the Dice coefficient (16) for band matching, with a position

TABLE 1. Nucleotide sequences of the oligonucleotides used for PCR amplification and DNA sequencing

Procedure and gene	Primer	Expected size of PCR amplicon (bp)	Reference
Amplification			
$bla_{TEM\text{-}1}$	5′-CCG TGT CGC CCT TAT TCC-3′ 5′-AGG CAC CTA TCT CAG CGA-3′	824	50
$bla_{SHV\text{-}1}$	5′-ATT TGT CGC TTC TTT ACT CGC-3′ 5′-TTT ATG GCG TTA CCT TTG ACC-3′	1,051	42
$bla_{CTX\text{-}M\text{-}1}$	5′-CGG TGC TGA AGA AAA GTG-3′ 5′-TAC CCA GCG TCA GAT TAC-3′	354	3
$bla_{CTX\text{-}M\text{-}2}$	5′-ACG CTA CCC CTG CTA TTT-3′ 5′-GCT TTC CGC CTT CTG CTC-3′	780	24
$bla_{CTX\text{-}M\text{-}9}$	5′-GCA GAT AAT ACG CAG GTG-3′ 5′-CGG CGT GGT GGT GTC TCT-3′	393	32
bla_{AmpC}	5′-ACT TAC TTC AAC TCG CGA CG-3′ 5′-TAA ACA CCA CAT ATG TTC CG-3′	663	7
Sequencing, $bla_{CTX\text{-}M\text{-}2}$	5′-TTA ATG ATG ACT CAG AGC ATT C-3′ 5′-GAT ACC TCG CTC CAT TTA TTG-3′	902	43

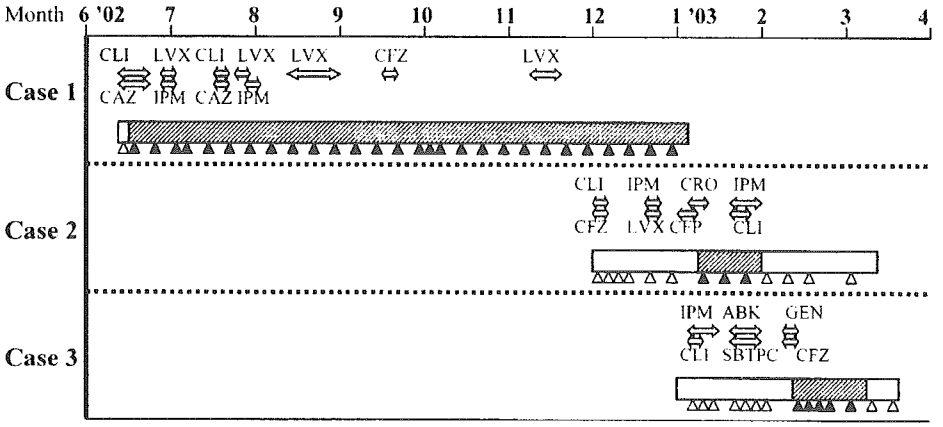

FIG. 2. Sequence of isolation of cefotaxime-resistant *A. baumannii* strains as well as the antimicrobial agents prescribed for three patients. Rectangles, periods of hospitalization; black triangles, times of isolation of cefotaxime-resistant *A. baumannii* from transtracheal aspirate cultures; open triangles, times that cefotaxime-resistant *A. baumannii* strains from transtracheal aspirate cultures were negative; hatched rectangles, times of probable colonization or infection with CTX-M-2 producers; double-headed arrows, prescription periods during which each antimicrobial agent was prescribed: CLI, clindamycin; LVX, levofloxacin; CFZ, cefazolin; CAZ, ceftazidime; IPM, imipenem-cilastatin; CRO, ceftriaxone; CFP, cefoperazone-sulbactam; ABK, arbekacin; GEN, gentamicin; SBTPC, sultamicillin.

tolerance setting of 1.0% (default values are 1% position tolerance and 0.5% optimization). Bands for analysis with the Dice coefficient were assigned manually, according to densitometric curves and the accompanying hard-copy photograph.

RESULTS

Identification of *A. baumannii* isolates and their clinical associations. Strain FM0209680 was derived from the culture of a transtracheal aspirate from a 24-year-old male patient (case patient 1) from whom the first cefotaxime-resistant *A. baumannii* strain was isolated on 19 June 2002. This patient was admitted to the neurosurgery ward on 14 June with a pneumonia-related condition and had a history as a patient in the same ward in October 1999 due to hemorrhaging of the brain caused by underlying cerebral arteriovenous malformation. Until his hospital discharge, on 6 January 2003, cefotaxime-resistant *A. baumannii* isolates were recurrently and predominantly detected in cultures of transtracheal aspirates from the patient. These data point out the great difficulty in eradicating the organisms. Strain FM0300106 was then recovered from the culture of a transtracheal aspirate from a 62-year-old female patient (case patient 2) who had been hospitalized in the neurosurgery ward due to brainstem infarction on 24 November 2002. The cefotaxime-resistant *A. baumannii* strain was first and predominantly detected in the specimen for culture taken on 6 January 2003, although it had not been found in a total of six samples for culture taken up to that time. Strain FM0301433 was recovered from the culture of a transtracheal aspirate from a 65-year-old male patient (case patient 3) who had been hospitalized in the neurosurgery ward due to cerebral hemorrhage on 27 December 2002. As was noted for case patient 2, the cefotaxime-resistant *A. baumannii* strain was first detected predominantly in the specimen for culture taken on 12 February 2003, although it had not been found in a total of seven specimens for culture obtained up to that time. In both case patients 2 and 3, the eradication of the cefotaxime-resistant *A. baumannii* strains was achieved within 3 weeks after the strains were first detected. Imipenem therapy was used for case patient 2 because a complication of pneumonia was a serious concern and the organisms disappeared. The cefotaxime-resistant *A. baumannii* strain was isolated from case patient 3 during prophylactic antimicrobial therapy with gentamicin and cefazolin. Administration of cefazolin may have induced colonization with this organism in case patient 3, in view of the fact that strain FM0301433 was resistant to cefazolin. The prophylactic therapy was then discontinued because no pneumonic clinical findings were noted, and, fortunately, the organism disappeared after 3 weeks.

The history of antimicrobial therapy before isolation of cefotaxime-resistant *A. baumannii* was untraceable for case patient 1 because this patient was transferred from another hospital to the Funabashi Medical Center. Sultamicillin, cefazolin, ceftriaxone, imipenem-cilastatin, cefoperazone-sulbactam, levofloxacin, clindamycin, gentamicin, and arbekacin were used prior to the isolation of *A. baumannii* in case patients 2 and 3. The sequence of isolation of the three cefotaxime-resistant *A. baumannii* strains, as well as the antimicrobial agents prescribed for the three case patients, are summarized in Fig. 2.

Three isolates, FM0209680, FM0300106, and FM0301433, which shared the same biochemical profile code, 0041073, by use of the API NE20 system, combined with a positive result for the ability to grow at 44°C as a complementary test, were eventually identified as *A. baumannii*. The 16S rRNA nucleotide sequences of these microorganisms shared 99% identity with that of *A. baumannii* type strain ATCC 19606 deposited in the EMBL database (accession number Z93435).

Antibiotic susceptibilities. The antibiotic susceptibility profiles of all three *A. baumannii* strains are shown in Table 2. These strains were broadly resistant to penicillins, cephalosporins, cephamycins, and monobactam but were highly susceptible to carbapenems. For all strains, addition of 4 μg of clavulanic acid per ml to the medium for antimicrobial susceptibility testing significantly decreased the MICs of cefotaxime from

TABLE 2. Antibiotic susceptibilities of *A. baumannii* clinical isolates

Antibiotic[a]	MIC (μg/ml)[b]		
	FM0209680	FM0300106	FM0301433
Ampicillin	>16	>16	>16
Amoxicillin-CLA	8/4	8/4	16/8
Piperacillin	>64	>64	>64
Cefazolin	>16	>16	>16
Cefotiam	>16	>16	>16
Cefoperazon-SUL	≤16/8	≤16/8	32/16
Cefotaxime	>128	>128	>128
Cefotaxime-CLA	2	8	4
Ceftazidime	8	8	8
Ceftazidime-CLA	1	4	2
Ceftriaxone	>64	>64	>64
Cefpirome	>16	>16	>16
Cefepime	>32	>32	>32
Cefozopran	>16	>16	>16
Cefaclor	>16	>16	>16
Cefpodoxime	>64	>64	>64
Cefoxitin	>32	>32	>32
Cefmetazole	32	32	>32
Cefotetan	32	32	16
Flomoxef	16	16	16
Imipenem	≤0.5	≤0.5	≤0.5
Meropenem	1	1	1
Aztreonam	>64	>64	>64
Gentamicin	>8	>8	2
Amikacin	32	>32	16
Minocycline	4	4	4
Levofloxacin	4	4	2
Fosfomycin	>16	>16	>16

[a] CLA, clavulanic acid at a fixed concentration of 4 μg/ml; SUL, sulbactam.
[b] NEG Combo 5J and NEG MIC 5J panels and ESBL plus panel were used for MICs determinations.

>128 μg/ml to 2 to 8 μg/ml, while the ceftazidime MICs decreased modestly from 8 μg/ml to 1 to 4 μg/ml.

β-Lactamase production. The production of β-lactamase was initially judged by the P/Case test (37), which can discriminate between penicillinase (with 1.5 mg of benzylpenicillin as the substrate) and cephalosporinase (with 1.5 mg of cephaloridine and 75 μg of clavulanic acid as the substrate). According to the instructions of the manufacturer, the bacterial colonies are rubbed onto two indicator disks containing benzylpenicillin and cephaloridine with clavulanic acid, respectively. When the strain produces any class A β-lactamase, such as TEM- or SHV-derived β-lactamases or CTX-M-type enzymes, a disk containing benzylpenicillin turns yellow, while the other disk containing cephaloridine with clavulanic acid remains purple, because hydrolysis of cephaloridine by the class A β-lactamases is blocked in the presence of clavulanic acid. If the strain produces class C β-lactamases, the disk containing cephaloridine with clavulanic acid turns yellow, because these enzymes are not blocked by clavulanic acid. The results of the test suggested that all three *A. baumannii* strains produce both class A and class C β-lactamases.

The isolates suggested to produce class A β-lactamases were investigated further to determine whether they produce ESBLs by the double-disk synergy test. The presence of an ESBL was suggested by the synergy between amoxicillin-clavulanic acid and cefotaxime, ceftazidime, and aztreonam against all three strains. However, growth of scattered colonies was also noted within the expanded growth-inhibitory zones around the cefotaxime and aztreonam disks (Fig. 1). Enhanced production of β-lactamases as well as alterations in membrane protein composition might be implicated in this phenomenon. A significant decrease in the MICs of cefotaxime from >128 μg/ml to 2 to 8 μg/ml in the presence of 4 μg of clavulanic acid per ml (Table 2) suggested the production of some CTX-M-type class A β-lactamases by these isolates.

As described above, the P/Case test suggested that these isolates produce an AmpC cephalosporinase, and the *ampC* gene (7) was actually detected as a 662-bp PCR amplicon in all three strains, although no obvious inducibility of AmpC β-lactamases was detected in any of them by cefoxitin-ceftazidime disk antagonism tests (data not shown).

No amplicons for the bla_{TEM}, bla_{SHV}, $bla_{CTX\text{-}M\text{-}1}$, and $bla_{CTX\text{-}M\text{-}9}$ genes were detected in any of the strains except strain FM0301433, which produced an 824-bp amplicon for the bla_{TEM} gene.

Sequencing of $bla_{CTX\text{-}M\text{-}2}$ gene and plasmid analysis. In a preliminary search by PCR, 780-bp amplification products specific for the $bla_{CTX\text{-}M\text{-}2}$ genes were observed in all three *A. baumannii* strains. The nucleotide sequence of the 902-bp amplicon encoding a full-length *bla* gene, which was amplified with a set of PCR primers (43), was exactly the same as that of the $bla_{CTX\text{-}M\text{-}2}$ gene (GenBank accession number X92507), suggesting that all these strains certainly harbor the $bla_{CTX\text{-}M\text{-}2}$ gene. The nucleotide sequences of the $bla_{CTX\text{-}M\text{-}2}$ gene harbored by the *A. baumannii* strains were exactly the same as that of a *P. mirabilis* strain previously isolated in the urology ward of the Funabashi Medical Center (34). Moreover, the $bla_{CTX\text{-}M\text{-}2}$-bearing plasmids prepared from three *A. baumannii* strains were digested into four different EcoRI fragments of approximately 6.6, 6.4, 4.1, and 3.8 kb (data not shown). The restriction fragment profiles of the plasmids were exactly the same as those of plasmids previously isolated from *P. mirabilis* strains (data not shown).

Ribotyping. In order to investigate the genetic relationship among FM0209680, FM0300106, and FM0301433, the isolates were analyzed by automated ribotyping. Figure 3 shows the results of a clustering analysis performed with the ribotype patterns of the three isolates and some collection strains of the genus *Acinetobacter*, including strains with patterns related to those of European outbreak clones I and II (11, 17) and a newly described clone, clone III (S. Brisse, personal communication). The three *A. baumannii* strains shared the same fingerprint pattern, indicating a clonal lineage. Moreover, the patterns of the three strains showed a high degree of similarity (92%) with those of a clone II strain. Nevertheless, the patterns of all three strains were different from those of the European clones, which, it has been suggested (11), are probably widespread worldwide.

DISCUSSION

This report describes the probable nosocomial transmission of CTX-M-2 β-lactamase-producing *A. baumannii* strains in a neurosurgery ward, where the majority of inpatients are represented by immunocompromised hosts who have undergone respiratory manipulations, such as a tracheotomy or mechanical ventilation, and where careful and strict monitoring is

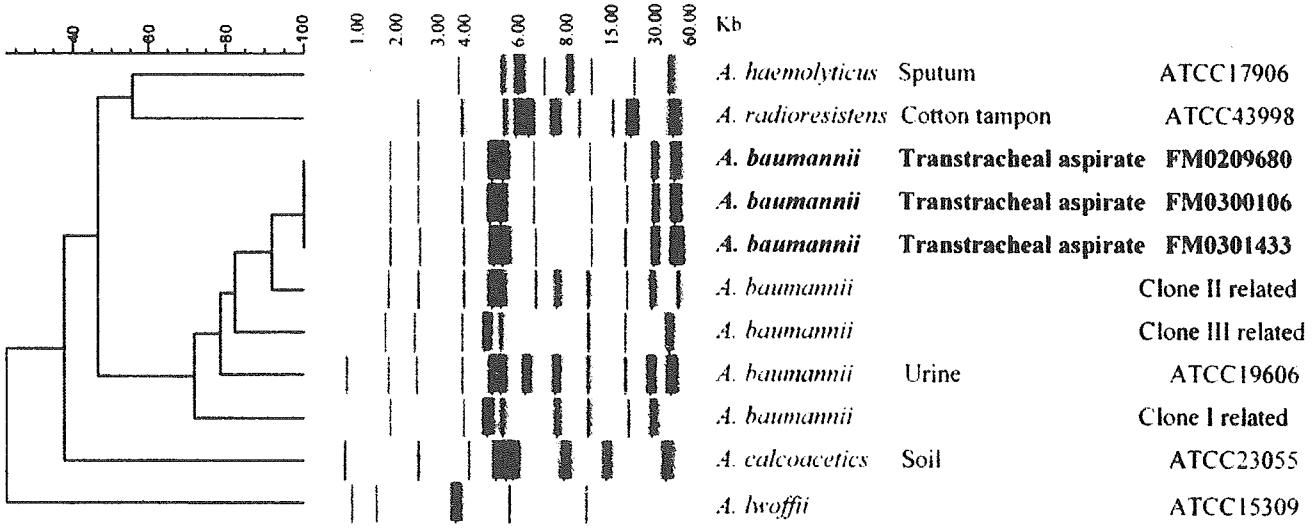

FIG. 3. EcoRI ribotypes of the three cefotaxime-resistant *A. baumannii* clinical isolates. A comparative analysis of these isolates with some collection strains of the genus *Acinetobacter*, including European outbreak clones I, II, and III of *A. baumannii*, is shown. Clustering was performed by the unweighted pair group method with arithmetic averages, and similarity analysis was based on the use of the Dice coefficient.

provided. For case patient 1, a CTX-M-2-producing *A. baumannii* strain was first isolated on day 5 after admission. During the 6-month hospitalization of this patient, cefotaxime-resistant *A. baumannii* strains were detected continuously, despite the administration of imipenem, ceftazidime, and levofloxacin, which consequently failed to eradicate the microorganism. For case patients 2 and 3, CTX-M-2-producing *A. baumannii* strains were detected at the sixth week after admission, and the patients were then colonized for 3 weeks. Imipenem treatment was used to eradicate *A. baumannii* from case patient 2 because a high risk of pneumonia was a serious concern in this patient. On the other hand, prophylactic therapy was discontinued in case patient 3 because no pneumonic clinical findings appeared and the microorganism consequently disappeared. As shown in Fig. 2, it seems very likely that an *A. baumannii* strain harboring the $bla_{CTX-M-2}$ gene was persistently transmitted from case patient 1 to case patient 3 via case patient 2 over a period of 9 months in the same neurosurgery ward.

The three *A. baumannii* strains, strains FM0209680, FM0300106, and FM0301433, shared the same ribotype pattern, suggesting a clonal lineage. For all three patients, multiple bacterial species other than *A. baumannii* were frequently coisolated throughout the periods of hospitalization, but fortunately, none was a CTX-M-2 producer. The genes for CTX-M-type β-lactamases are often encoded on plasmids and are easily transmitted among gram-negative bacilli, including members of the family *Enterobacteriaceae* (5, 38), and glucose nonfermenters, such as *Pseudomonas aeruginosa* (8). Since no CTX-M-2 ESBL-producing bacterial isolates, including *A. baumannii*, were previously detected in the neurosurgery ward, it is likely that case patient 1 already carried a CTX-M-2-producing *A. baumannii* isolate before his rehospitalization. However, in the Funabashi Medical Center, a previous outbreak of nosocomial infections in the urology ward, located on a different floor from the neurosurgery ward, involved 19 inpatients and was related to CTX-M-2-producing *P. mirabilis* (34). Agarose gel electrophoresis demonstrated that the $bla_{CTX-M-2}$-bearing plasmids found in both bacterial species had the same EcoRI digestion pattern. Therefore, this finding strongly suggests the possibility of the lateral transfer of the plasmids carrying the $bla_{CTX-M-2}$ gene between *P. mirabilis* and *A. baumannii* in the clinical setting described here.

A. baumannii strains which produce plasmid-mediated CTX-M-type β-lactamases or TEM- and SHV-derived ESBLs are still very rare. The class A β-lactamases PER-1 and VEB-1, which are genetically distant from the predominant TEM- and SHV-derived ESBLs and CTX-M-type enzymes, were first reported among nosocomial *A. baumannii* isolates in Turkish and French hospitals (41, 52). Similar strains have subsequently been isolated in France (40) and Korea (26). Among the CTX-M-type β-lactamases, the CTX-M-5 gene has been found in *A. baumannii* (GenBank accession number AF462635); however, no details have been published to date. *A. baumannii* usually produces a chromosomally encoded AmpC cephalosporinase, but this kind of enzyme generally cannot hydrolyze oxyiminocephalosporins, cephamycins, or carbapenems. Therefore, acquisition of plasmid-mediated enzymes with broad- and extended-spectrum substrate specificities could well allow this bacterial species to survive in present clinical environments. *A. baumannii* has become one of the major groups of bacteria that causes respiratory infections, especially among patients in intensive care units. Thus, the emergence of CTX-M-2-producing *A. baumannii* strains could become a serious clinical problem in Japan, because CTX-M-2-producing as well as CTX-M-1-producing microorganisms have already been frequently found among clinical isolates from humans (5, 25, 55) and cattle (47).

Early outbreaks due to β-lactamase-producing strains resulted in epidemics caused by isolates with only a single β-lactamase. However, more complex situations involving multiple-β-lactamase-producing microorganisms have been documented in recent nosocomial outbreaks (7, 9, 10, 56). The three *A. baumannii* strains reported in the present study pro-

duced two β-lactamases, the CTX-M-2 enzyme as well as the AmpC β-lactamase. Moreover, the third strain also produced a TEM-type penicillinase. In the present study, the induction of β-lactamase production in *A. baumannii* strains was suggested by the growth of colonies within the growth-inhibitory zones around cefotaxime and aztreonam disks. Such inducible class C β-lactamase production by *A. baumannii* was not detected by previously reported disk antagonism tests with cefoxitin and ceftazidime disks (30), although the presence of the *ampC* gene was confirmed by PCR. Thus, it is likely that some of the colonies appearing in the growth-inhibitory zone around the cefotaxime and aztreonam disks might be mainly constitutive CTX-M-2 producers. It is also possible that these colonies corresponded to AmpC-hyperproducing mutants in which the regulatory system of AmpC production was disrupted through mutations in the *ampC* promoter region or in genes implicated in the regulation of ApmC production, such as *ampR*, *ampG*, and *ampD* (28). These mutations might be easily induced in *A. baumannii* in the presence of cefotaxime, aztreonam, and clavulanic acid. Moreover, all three *A. baumannii* strains characterized in this study demonstrated resistance to cephamycins, such as cefmetazole, cefoxitin, and cefotetan. This may depend on a continuous and moderate level of production of chromosomal AmpC cephalosporinase as well as alterations in bacterial membrane permeability (44). Functional analysis of the *Acinetobacter* AmpC cephalosporinase and further characterization of the nature of the bacterial cells appearing around cefotaxime and aztreonam disks will be continued.

A. baumannii tends to accept foreign DNA as a means to adapt to environments hazardous for bacterial growth (15, 36) and has a high survival capacity in any environment with damp conditions and low temperatures. This inherent ability of *A. baumannii* may facilitate the development of a multidrug resistance profile with the widespread use of antimicrobial agents through the selection of strains with ever accumulating antibiotic resistance profiles in hospital environments. Thus, the emergence of CTX-M-2-producing *A. baumannii* strains not only may confer the potential for epidemics but also could serve as reservoirs for the plasmid-dependent CTX-M-2 enzyme for dispersal of the $bla_{CTX\text{-}M\text{-}2}$ gene among different gram-negative bacterial species. Indeed, although it might be difficult to detect the coproduction of class A β-lactamases like CTX-M-type enzymes in intrinsically cephalosporin-resistant *A. baumannii*, knowledge of the presence of these burdensome strains would seem to be very important in achieving suitable infection control measures in hospitals on a daily basis.

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Molecular Characterization of a Cephamycin-Hydrolyzing and Inhibitor-Resistant Class A β-Lactamase, GES-4, Possessing a Single G170S Substitution in the Ω-Loop

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The nosocomial spread of six genetically related *Klebsiella pneumoniae* strains producing GES-type β-lactamases was found in a neonatal intensive care unit, and we previously reported that one of the six strains, strain KG525, produced a new β-lactamase, GES-3. In the present study, the molecular mechanism of cephamycin resistance observed in strain KG502, one of the six strains described above, was investigated. This strain was found to produce a variant of GES-3, namely, GES-4, which was responsible for resistance to both cephamycins (cefoxitin MIC, >128 μg/ml) and β-lactamase inhibitors (50% inhibitory concentration of clavulanic acid, 15.2 ± 1.7 μM). The GES-4 enzyme had a single G170S substitution in the Ω-loop region compared with the GES-3 sequence. This single amino acid substitution was closely involved with the augmented hydrolysis of cephamycins and carbapenems and the decreased affinities of β-lactamase inhibitors to GES-4. A cloning experiment and sequencing analysis revealed that strain KG502 possesses duplicate bla_{GES-4} genes mediated by two distinct class 1 integrons with similar gene cassette configurations. Moreover, the genetic environments of the bla_{GES-4} genes found in strain KG502 were almost identical to that of bla_{GES-3} in strain KG525. From these findings, these two phenotypically different strains were suggested to belong to a clonal lineage. The bla_{GES-4} gene found in strain KG502 might well emerge from a point mutation in the bla_{GES-3} gene harbored by its ancestor strains, such as strain KG525, under heavy antibiotic stress in order to acquire extended properties of resistance to cephamycins and carbapenems.

Over the past decade, a number of new plasmid-mediated β-lactamases with wide substrate specificities have appeared mainly in gram-negative bacilli belonging to the family *Enterobacteriaceae* (11, 19, 20, 27). In particular, the emergence of bacteria producing TEM- and SHV-derived extended-spectrum β-lactamases (ESBLs) has made chemotherapy for bacterial infections more complex than ever (13). Furthermore, non-TEM- and non-SHV-type ESBLs, such as the CTX-M-type (23, 31), GES-type (8, 21, 25, 28, 29), and VEB-type (3, 22) β-lactamases, have also been identified in these gram-negative bacilli. Generally, the β-lactamases described above are often plasmid encoded and can hydrolyze oximino-cephalosporins and monobactams as well as penicillins but not 7-α-methoxy-cephalosporins, the so-called cephamycins. Carbapenems are also very stable against these enzymes. Therefore, at present cephamycins and carbapenems are potent agents for the treatment of infections caused by the gram-negative bacilli that produce these new class A β-lactamases with wide substrate specificities. Among the various β-lactamase genes described above, the genes encoding the GES-type β-lactamases as well as the VEB-type β-lactamases are often located in integrons as gene cassettes (8, 17, 21, 22, 25, 29). Integrons have been described to play a sophisticated role in the accumulation and expression of genes responsible for antibiotic resistance as well as their dissemination among gram-negative bacilli (9, 10).

Among the GES-type β-lactamases, GES-1 was first reported from a *Klebsiella pneumoniae* clinical isolate in France in 1998 (21); and then two other GES-type β-lactamases, IBC-1 and GES-2, were found in *Enterobacter cloacae* and *Pseudomonas aeruginosa*, respectively (8, 25). GES-2 has an amino acid substitution (glycine to asparagine at position 170) compared to the sequence of GES-1 and shows a higher imipenem-hydrolyzing activity than GES-1.

We found that the high-level ceftazidime resistance of six genetically related *K. pneumoniae* clinical strains, which had been isolated from a neonatal intensive care unit (NICU) over a 1-year period, depended on the production of GES-type β-lactamases, and one of the six isolates was found to produce the GES-3 β-lactamase (30). The bla_{GES-3} gene encoding GES-3 was located as a gene cassette in a class 1 integron, as has been observed for the GES-type β-lactamase genes found in Europe. GES-3 production does not affect the level of cephamycin resistance in the *Escherichia coli* host, as has been reported for the other Ambler class A β-lactamases, including ESBLs. However, the levels of resistance to cephamycins varied widely among the six GES-type β-lactamase-producing strains. Among these, the highest MICs of the carbapenems as well as the cephamycins were seen for strain KG502 (30), which also showed an inhibitor resistance phenotype. The goal of this study was to elucidate the molecular mechanism responsible for resistance to cephamycins and carbapenems in strain KG502, as well as its inhibitor-resistant nature.

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Characteristics	Source or reference
K. pneumoniae KG502	Clinical isolate, resistant to cephamycins, carrying bla_{GES-4}	This study
K. pneumoniae KG525	Clinical isolate, susceptible to cephamycins, carrying bla_{GES-3}	30
E. coli CSH-2	*metB* F⁻ nalidixic acid^r rifampin^r	T. Sawai, Chiba University
E. coli XL1-Blue	*supE44 recA1 endA1 gyrA96 thi hsdR17* (r_K^- m_K^+) *relA1 lac* [F′ *proAB*⁺ *lacIqZ*ΔM15::Tn*10* (Tet^r)]	Stratagene
E. coli BL21(DE3)pLysS	F⁻ *ompT hsdS*$_B$ (r_B^- m_B^-) *gal dcm* (DE3) pLysS (Cam^r)	Invitrogen
Plasmids		
pKGL502	Recombinant plasmid carrying a 6.6-kb BamHI fragment containing bla_{GES-4} of *K. pneumoniae* KG502	This study
pKGS502	Recombinant plasmid carrying a 6.0-kb BamHI fragment containing bla_{GES-4} of *K. pneumoniae* KG502	This study
pKGB525	Recombinant plasmid carrying a 6.7-kb BamHI fragment containing bla_{GES-3} of *K. pneumoniae* KG525	30
pET-GES4	Recombinant plasmid containing PCR-amplified bla_{GES-4} ligated to pET29a(+)	This study
pET-GES3	Recombinant plasmid containing PCR-amplified bla_{GES-3} ligated to pET29a(+)	This study
pBCSK+	Cloning vector, chloramphenicol^r	Stratagene
pCR2.1	Cloning vector, ampicillin^r kanamycin^r	Invitrogen
pET29a(+)	Expression vector; kanamycin^r	Novagen

MATERIALS AND METHODS

Bacterial strains and plasmids. The strains and plasmids used in this study are listed in Table 1. *K. pneumoniae* strain KG502 was isolated in May 2002 from the pus of a neonate under treatment in the NICU of a general hospital in Japan. This strain was resistant to oximino-cephalosporins and cephamycins. GES-3-producing strain KG525 was isolated in the same NICU where strain KG502 was isolated.

Susceptibility testing. MICs were determined by the agar dilution method with Mueller-Hinton agar (Becton Dickinson, Cockeysville, Md.), according to the guidelines in National Committee for Clinical Laboratory Standards document M7-A5 (18). *E. coli* ATCC 25922 and ATCC 35218 were purchased from the American Type Culture Collection (ATCC) and served as control strains for MIC determinations. The double-disk synergy test for the detection of ESBL production and an inhibitory test with thiol compounds for the detection of metallo-β-lactamase producers were carried out by the methods described elsewhere (1, 6, 12), with the modification that 2-mercaptopropionic acid was replaced with sodium mercaptoacetic acid.

Transfer of β-lactam resistance genes. Conjugation experiments were performed by the filter mating method with rifampin- and nalidixic acid-resistant *E. coli* CSH-2 as the recipient. Transconjugants were detected on Luria-Bertani (LB) agar supplemented with rifampin (100 μg/ml), nalidixic acid (100 μg/ml), and either ceftazidime (4 μg/ml) or cefminox (2 μg/ml). Transformation of *E. coli* XL1-Blue with the large plasmids of the parental strain *K. pneumoniae* KG502 was performed by electroporation. Transformants were selected on LB agar containing ceftazidime (4 μg/ml) or cefminox (2 μg/ml).

PCR amplification, cloning, and sequencing of β-lactamase gene. To amplify the bla_{GES} gene, PCR was performed with the primers under the conditions described elsewhere (30). The cloning experiment was carried out as follows: total DNA prepared from strain *K. pneumoniae* KG502 was digested with BamHI, and the resultant fragments were ligated to vector pBCSK+ (Stratagene, La Jolla, Calif.), which had been digested with the same enzyme. Transformants carrying recombinant plasmids were selected on LB agar plates containing chloramphenicol (30 μg/ml) and either ceftazidime (4 μg/ml) or cefminox (2 μg/ml). Both strands of the DNA fragments inserted into the recombinant plasmids (pKGL502 and pKGS502) were sequenced.

Purification of β-lactamase. β-Lactamases were purified by exactly the same protocol described elsewhere (30). In brief, β-lactamases were overproduced with the pET system, extracted by use of a French press, and cleared by ultracentrifugation. After ultracentrifugation the supernatant was loaded onto a Hi-Load 16/60 Superdex 200 and anion-exchange Hitrap Q HP column (Pharmacia Biotech, Uppsala, Sweden). The purity of the enzyme was checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis.

β-Lactamase assay. Substrate hydrolyses by GES-4 and GES-3 were assayed at 30°C in phosphate buffer (50 mM; pH 7.0) by use of an autospectrophotometer (V-550; Nihon Bunko Ltd., Tokyo, Japan). The molar extinction coefficients (Δε values) used were as follows: for benzylpenicillin (232 nm), 1.077 mM^{-1} cm^{-1}; for ampicillin (235 nm), 1.121 mM^{-1} cm^{-1}; for cephaloridine (300 nm), 0.384 mM^{-1} cm^{-1}; for cefotaxime (264 nm), 5.725 mM^{-1} cm^{-1}; for ceftazidime (274 nm), 6.123 mM^{-1} cm^{-1}; for cefpirome (290 nm), 4.057 mM^{-1} cm^{-1}; for cefoxitin (293 nm), 0.325 mM^{-1} cm^{-1}; for cefminox (298 nm), 1.878 mM^{-1} cm^{-1}; for imipenem (297 nm), 8.061 mM^{-1} cm^{-1}; and for aztreonam (315 nm), 0.68 mM^{-1} cm^{-1}. Fifty percent inhibitory concentrations (IC_{50}s) were determined with benzylpenicillin as the substrate and the inhibitors clavulanic acid, sulbactam, tazobactam, and imipenem. Purified enzyme and various concentrations of these inhibitors were preincubated in 50 mM phosphate buffer (pH 7.0) at 30°C for 5 min. Purified GES-4 and GES-3 β-lactamases and nonpurified extracts of 50-ml cultures of strain KG502 were subjected to isoelectric focusing (IEF) analysis with an Immmobiline Drystrip (pH 3 to 10; Pharmacia Biotech) and an IPGphor electrophoresis system (Pharmacia Biotech).

Nucleotide sequence accession numbers. The nucleotide sequences described in this work appear in the GenBank nucleotide database under accession numbers AB116260 and AB116723.

RESULTS

Properties of *K. pneumoniae* isolate KG502. *K. pneumoniae* KG502 was isolated from the pus of a neonate in May 2002. This strain was one of the six GES-type β-lactamase-producing strains that we reported previously (30). Strain KG502 exhibited resistance to oximino-cephalosporins and the cephamycins and intermediate susceptibility to carbapenems. No synergy between an amoxicillin-clavulanic acid disk and a ceftazidime and/or a cefotaxime disk was detectable against this strain. The lack of production of metallo-β-lactamases was also suggested by the results of inhibition testing with sodium mercaptoacetic acid. Preliminary PCR detection of some class A β-lactamase and metallo-β-lactamase genes was performed as we reported in our previous study (30), and all PCRs gave negative results.

Transfer of β-lactam resistance by conjugation and transformation. Our previous Southern hybridization experiment with a digoxigenin-labeled bla_{GES}-specific probe demonstrated that the GES-type β-lactamase genes of strain KG502 are located on two distinct plasmids. Therefore, we performed conjugation by filter mating in an attempt to transfer these plasmids to *E. coli* CSH-2, as well as electroporation to introduce them directly into *E. coli* XL1-Blue. However, the transfer of these plasmids into *E. coli* was unsuccessful, despite repeated attempts.

Cloning and sequencing of the β-lactamase gene. Sequencing of the DNAs of the PCR products obtained with the primers specific for the GES-type β-lactamase gene revealed the presence of a variant of the bla_{GES-3} gene in strain KG502. Cloning was performed by standard procedures to determine the entire nucleotide sequences of this new gene. Two distinct recombinant plasmids, one of which carried a 6.6-kb BamHI

```
                    20        30        40        50        62
GES-4  MRFIHALLLAGIAHSAYASEKLTFKTDLEKLEREKAAQIGVAIVDPQGEIVAGHRTAQRF
GES-3  ----------------------------------------------------T----
GES-2  ----------------------------------------------------M----
GES-1  ----------------------------------------------------M----

          70        80        90       100 104   110       120
GES-4  AMCSTFKFPLAALVFERIDSGTERGDRKLSYGPDMIVKWSPATERFLASGHMTVLEAAQA
GES-3  -------------------------------------K----------------------
GES-2  -------------------------------------E----------------------
GES-1  -------------------------------------E----------------------

         130       140       150       160 164   170       179
GES-4  AVQLSDNGATNLLLREIGGPAAMTQYFRKIGDSVSRLDRKEPEMSDNTPGDLRDTTTPIA
GES-3  ------------------------------------------G----------------
GES-2  ------------------------------------------N----------------
GES-1  ------------------------------------------G----------------

         190       200       210       220       230       240
GES-4  MARTVAKVLYGGALTSTSTHTIERWLIGNQTGDATLRAGFPKDWVVGEKTGTCANGGRND
GES-3  ------------------------------------------------------------
GES-2  ------------------------------------------------------------
GES-1  ------------------------------------------------------------

          250       260       270       280       290
GES-4  IGFFKAQERDYAVAVYTTAPKLSAVERDELVASVGQVITQLILSTDK
GES-3  -------------------------------------------------
GES-2  -------------------------------------------------
GES-1  -------------------------------------------------
```

FIG. 1. Amino acid alignments of GES-4, GES-3, GES-2, and GES-1 β-lactamases. Hyphens indicate identical amino acids, and the Ω-loop region of β-lactamase is underlined.

fragment and the other of which carried a 6.0-kb BamHI fragment, were obtained independently. The nucleotide sequences of both genetic determinants for β-lactam resistance were the same and differed by a glycine (G)-to-alanine (A) mutation at position 509 compared with the sequence of bla_{GES-3}, so they were designated bla_{GES-4}. The deduced amino acid sequence of GES-4 had an amino acid substitution of G to serine (S) at position 170 (G170S) within the Ω-loop region of the Ambler class A β-lactamase compared with the sequence of GES-3 (Fig. 1). Among the GES-type β-lactamases, a similar amino acid substitution was reported at position 170, G to asparagine (N), leading to the conversion from GES-1 to GES-2 (25).

Sequencing of bla_{GES-4} flanking region. The inserts in recombinant plasmids pKGL502 and pKGS502 were sequenced, which revealed that both fragments commonly contained bla_{GES-4} gene in the class 1 integron separately, followed by an *aacA1-orfG* fused gene, as the first and second gene cassettes, respectively. The integron in pKGL502 differed from that in pKGS502, in that a third gene cassette, *orfA*, was present (Fig. 2). The product encoded by *orfA* had no significant homology with any other known protein at the amino acid sequence level, so the function of the product could not be presumed. Moreover, the backbone genetic structure surrounding the integron containing the bla_{GES-4} gene from strain KG502 was otherwise identical to that surrounding the integron containing the bla_{GES-3} gene from strain KG525, except that it lacked the outer 128-bp nucleotide sequences, including the 25-bp terminal repeat (IRt) at the left end of IS*6100*, as shown in Fig. 2.

Susceptibilities to various β-lactams. The MICs of β-lactams for *K. pneumoniae* strain KG502 and *E. coli* XL1-Blue harboring recombinant plasmid pKGL502 are listed in Table 2. GES-4 β-lactamase-producing strain KG502 exhibited resistance to cefminox, moxalactam, and cefmetazole and intermediate susceptibility to imipenem and meropenem, whereas GES-3-producing strain KG525 was susceptible to all these agents. These resistance trends were also observed in each of the *E. coli* clones harboring pKGL502 or pKGB525, but the overall resistance levels of the clones were lower than those of the parent strains.

Two notable differences were detected between the MICs for a GES-4-producing *E. coli* clone (pKGL502) and those for a GES-3-producing *E. coli* clone (pKGB525), expressed under the same promoters located within *intI1*. One was a difference in the levels of resistance to cephamycins. The MICs of cephamycins, such as cefminox, cefoxitin, moxalactam, and cefmetazole, were much higher for the GES-4-producing clone than the GES-3-producing clone. In addition, the meropenem MIC for the GES-4 producer was 16-fold higher than that for the GES-3 producer.

The other major differences were the inhibition profiles obtained when the β-lactamase inhibitors clavulanic acid, sulbactam, and tazobactam were added. The MICs of ampicillin, amoxicillin, piperacillin, ceftazidime, and cefotaxime for the GES-4-producing *E. coli* clone were decreased a maximum of only 8-fold in the presence of β-lactamase inhibitors, whereas those for the GES-3-producing *E. coli* clone decreased at least 32- to 512-fold.

IEF analysis. IEF analysis of the crude extract of parent strain KG502 revealed the presence of two major bands with β-lactamase activities corresponding to pIs of 6.9 and 7.6, respectively. The band with a pI of 7.6 was likely the chromosomally encoded

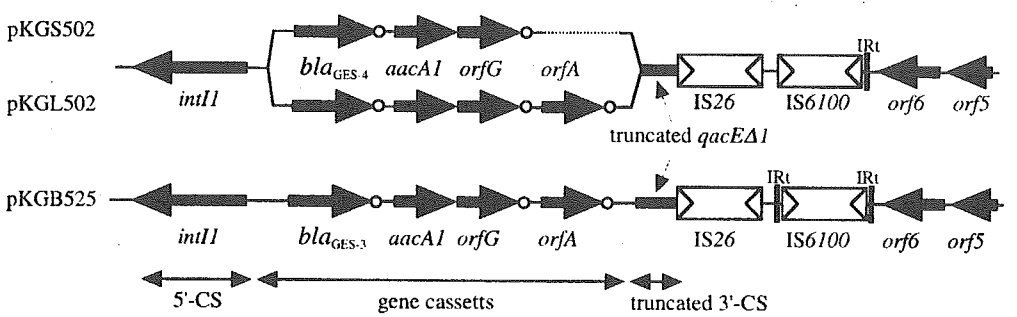

FIG. 2. Schematic comparison of the genetic environments of three class 1 integrons mediating bla_{GES-4} on pKGL502 and pKGS502 and bla_{GES-3} on pKGB525. Open circles represent the positions of the 59-base element. CS, conserved segment.

TABLE 2. MICs of β-lactams

β-Lactam[a]	MIC (μg/ml)				
	K. pneumoniae KG502(GES-4)	*K. pneumoniae* KG525(GES-3)	*E. coli* XL1-Blue pKGL502(GES-4)	*E. coli* XL1-Blue pKGB525(GES-3)	*E. coli* XL1-Blue pBCSK+
Ampicillin	>128	>128	>128	>128	4
Ampicillin + sulbactam	>128	>128	>128	2	2
Amoxicillin	>128	>128	>128	>128	4
Amoxicillin + clavulanate	>128	>128	>128	32	4
Piperacillin	128	128	64	16	1
Piperacillin + tazobactam	64	128	16	0.5	1
Cefotaxime	16	64	1	2	0.13
Cefotaxime + clavulanate	16	8	0.25	0.06	0.06
Cefotaxime + sulbactam	16	32	1	0.06	0.06
Cefotaxime + tazobactam	16	64	1	0.06	0.06
Ceftazidime	1,024	>1,024	64	128	0.13
Ceftazidime + clavulanate	512	256	8	4	0.06
Ceftazidime + sulbactam	>128	>128	32	0.25	0.13
Ceftazidime + tazobactam	>128	>128	32	0.5	0.13
Cephaloridine	>128	>128	64	16	2
Cefminox	>128	8	16	1	0.5
Cefoxitin	>128	128	>128	8	4
Moxalactam	128	4	16	0.5	0.13
Cefmetazole	>128	16	128	2	0.5
Cefpirome	128	>128	1	2	0.06
Cefepime	8	16	0.25	0.25	0.06
Aztreonam	32	64	2	4	0.06
Imipenem	8	0.25	0.25	0.13	0.13
Meropenem	8	0.5	0.25	0.015	0.015

[a] Clavulanate, tazobactam, and sulbactam were each used at a fixed concentration of 4 μg/ml.

SHV-type β-lactamase of *K. pneumoniae*. The estimated pI of 6.9 was identical to those of the purified GES-3 and GES-4 enzymes.

Kinetic studies. The kinetic parameters of the GES-4 and GES-3 β-lactamases for representative β-lactams are given in Table 3. The hydrolyzing efficiencies (k_{cat}/K_m) of GES-4 for the penicillins were about twice as high as those of GES-3, although GES-2 showed less efficient hydrolysis but lower K_m values for cephaloridine and penicillin. On the other hand, GES-3 hydrolyzed ceftazidime and cefotaxime more efficiently than GES-4 did. GES-4 measurably hydrolyzed cefoxitin, cefminox, and imipenem, which accounted for the increases in the MICs of these agents for the clone harboring pKGL502, but no measurable hydrolysis of these agents as substrates by GES-3 was observed under the same experimental conditions used in the present study. No measurable hydrolysis was observed for aztreonam as the substrate with each type of β-lactamase. The IC_{50}s measured with benzylpenicillin as the substrate are listed in Table 4. GES-2 was reported to be inhibited by lower concentrations of clavulanic acid and tazobactam; but GES-4 was inhibited 10-fold less by clavulanic acid, 16-fold less by sulbactam, 8-fold less by tazobactam, and 21-fold less by imipenem than GES-3 was. These results corroborate the inhibitor-resistant nature of GES-4.

DISCUSSION

GES-4 had a single G170S substitution within the Ω-loop region of class A β-lactamases compared with the sequence of

TABLE 3. Kinetic parameters of GES-4, GES-3, and GES-2

Substrate	GES-4			GES-3			GES-2[c]		
	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1} s^{-1}$)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1} s^{-1}$)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1} s^{-1}$)
Benzylpenicillin	160 ± 10	130 ± 10	7.8×10^5	33 ± 7	15 ± 1	4.5×10^5	4	0.4	9.6×10^4
Ampicillin	62 ± 4	19 ± 11	3.1×10^5	120 ± 30	23 ± 4	1.9×10^5	NP[e]	NP	NP
Cephaloridine	2,200 ± 400	490 ± 30	2.3×10^5	2,300 ± 100	270 ± 10	1.2×10^5	7.7	0.5	6.5×10^4
Cefotaxime	700 ± 200	17 ± 6	2.4×10^4	1,100 ± 100	120 ± 10	1.1×10^5	890	2.2	2.5×10^3
Ceftazidime	1,500 ± 400	2.5 ± 0.5	1.7×10^3	990 ± 130	23 ± 2	2.3×10^4	>3,000	ND[d]	ND
Cefpirome	340 ± 70	3.6 ± 0.4	1.1×10^4	550 ± 30	36 ± 2	6.6×10^4	NP	NP	NP
Cefoxitin	810 ± 70	85 ± 3	1.1×10^5	NH	NC	NC	—[f]	—	—
Cefminox	370 ± 70	7.7 ± 2.0	2.0×10^4	NH	NC	NC	NP	NP	NP
Imipenem	4.7 ± 0.7	0.38 ± 0.07	8.1×10^4	NH	NC	NC	0.45	0.004	9.0×10^3
Aztreonam	NH[a]	NC[b]	NC	NH	NC	NC	—	—	—

[a] NH, no measurable hydrolysis was detected after 2 h of incubation.
[b] NC, not calculated.
[c] Data for GES-2 were reported in reference 25.
[d] ND, not determined due to very high K_m values.
[e] NP, not provided in reference 25.
[f] —, not hydrolyzed (the initial rate of hydrolysis was less than 0.001 $\mu M^{-1} s^{-1}$).

TABLE 4. Inhibition profiles of GES-type β-lactamases

Inhibitor	IC_{50} (μM)				
	GES-4	GES-3	GES-2[a]	GES-1[a,b]	IBC-1[c]
Clavulanic acid	15 ± 1.7	1.5 ± 0.15	1.0 ± 0.5	5.0	1.1
Sulbactam	15 ± 0.9	0.91 ± 0.10	—[d]	—	—
Tazobactam	1.4 ± 0.31	0.19 ± 0.03	0.5 ± 0.2	2.5	0.12
Imipenem	2.1 ± 0.16	0.10 ± 0.01	8 ± 2	0.1	0.06

[a] Data were reported in reference 25.
[b] Data were reported in reference 21.
[c] Data were reported in reference 8.
[d] —, data not provided in the references.

GES-3. Replacement of the side chain (—H) of the glycine residue with that (—CH_2—OH) of the serine residue may indeed contribute to the acceleration of cephamycin hydrolysis as well as the inhibitor resistance profile. The GES-2 β-lactamase, identified as a variant of GES-1, had a substitution from glycine to asparagine at position 170, which is the same position leading to the conversion from GES-3 to GES-4. In comparison with GES-1, GES-2 showed an extended substrate specificity for imipenem and a lower affinity for β-lactamase inhibitors (25), as was seen with GES-4. However, the obvious increases in the MICs of cephamycins and meropenem seen for GES-4 were not detected for GES-2. These findings suggest that a single amino acid substitution at position 170, the center of the Ω-loop region, would play a key role in the expansion of the substrate specificities among GES-type β-lactamases. To elucidate the nature of GES-4, molecular modeling analysis as well as X-ray crystallographic analysis will be undertaken in the next study.

Although amino acid substitutions in the Ω-loop region, which influence hydrolyzing activities against oximino-cephalosporins and carbapenems, have also been observed in several class A β-lactamases, such as those of the TEM type (5, 16), SHV type (2, 15), CTX-M type (23), and GES type (25). Disruption of the salt bridge between R164 and D179 was suggested to be mainly involved in the expansion of substrate specificity for oxyimino-cephalosporins in these enzymes. However, substitutions resulting in increased cephamycin resistance have not been reported in class A β-lactamases so far. To our knowledge, this is the first report of a class A β-lactamase with cephamycin-hydrolyzing ability as a result of a single amino acid substitution in the center of the Ω-loop region. Poyart et al. (26) also reported a similar phenomenon in a TEM-type β-lactamase (TEM-52), in which significant decreases in vitro susceptibilities to some cephamycins were not due to an amino acid substitution in the Ω-loop region. The same investigators reported, however, that the combination of three amino acid substitutions E104K, M182Y, and G238S (on the basis of the sequence of TEM-1) in TEM-25 was responsible for the elevated MICs of moxalactam and cefotetan.

The G170S substitution found in the GES-4 β-lactamase affected not only cephamycin and carbapenem resistance but also inhibitor resistance. The IC_{50}s of clavulanic acid, sulbactam, tazobactam, and imipenem for GES-4 were considerably higher than those of GES-3. Since 1990 IRT β-lactamases derived from TEM-type β-lactamases have been reported to be inhibitor resistance class A β-lactamases. The IRT β-lactamases differ from parental enzyme TEM-1 or TEM-2 by several amino acid substitutions at different locations. The IC_{50}s of clavulanic acid and tazobactam for GES-4 (15.2 and 1.43 μM, respectively) were similar to those of some IRT β-lactamases, including IRT-7 (23 and 0.9 μM, respectively), IRT-8 (25 and 1 μM, respectively), and IRT-14 (22.5 and 1.48 μM, respectively), while the IC_{50} of sulbactam for GES-4 was much lower than those for IRTs (4). GES-4 seems to be a very characteristic enzyme, because it has a strong inhibitor-resistant nature like IRT enzymes, while it maintains the capacity to hydrolyze cephamycins and carbapenems.

As with the other bla_{GES} genes, the bla_{GES-4} gene was located in the class 1 integron as a gene cassette. Strain KG502 was unique, in that it possessed two distinct class 1 integrons which carried similar gene cassette configurations, including the bla_{GES-4} gene cassette. The coexistence of class 1 integrons with similar gene cassette arrays might result from the duplication of a region containing one original class 1 integron by mobile elements, such as transposons in strain KG502. It was speculated that in this strain a region containing one original class 1 integron with the bla_{GES-4}, *aacA1-orfG*, and *orfA* gene cassettes was first duplicated in the bacterium. Next, one of the class 1 integrons might have excised the *orfA* gene cassette by a site-specific recombination mechanism catalyzed by some recombinases, including integrases or transposases. Consequently, strain KG502 might have come to have two class 1 integrons with very similar backbone structures.

The entire genetic structure of the flanking region containing bla_{GES-4} on pKGL502 was almost identical to that containing bla_{GES-3} on pKGB525. Taken together with the facts that both the bla_{GES-3} and the bla_{GES-4} genes were found in genetically related *K. pneumoniae* strains and that the genetic environments of these two genes are almost the same, it is probable that the bla_{GES-4} gene emerged from the point mutation in the bla_{GES-3} gene under conditions of antibiotic stress in order to acquire resistance to additional groups of drugs, i.e., the cephamycins and carbapenems.

We characterized here for the first time a novel class A β-lactamase, GES-4, which acquired extended substrate specificity for the cephamycins through a single amino acid substitution within the Ω-loop region. This finding indicates that β-lactamases which are capable of hydrolyzing cephamycins are not limited to the Ambler class B and class C β-lactamases. The emergence of a cephamycin-hydrolyzing class A β-lactamase might complicate treatment in clinical settings, because cephamycins have generally been considered stable to class A β-lactamases and to retain good efficacies for the treatment of infectious diseases caused by organisms producing class A β-lactamases. Moreover, the inhibitor resistance of the GES-4 β-lactamase may introduce confusion during the routine laboratory detection of class A β-lactamase-producing strains, including ESBL producers. The much higher IC_{50} of clavulanic acid for GES-4 hampered the detection of GES-4-producing clinical isolates by conventional double-disk synergy testing.

Since GES-type β-lactamase-producing gram-negative bacteria have been identified worldwide and nosocomial outbreaks caused by these microorganisms have been reported worldwide (7, 14, 24), due consideration must be given to the possible emergence of variants of GES-type β-lactamases like GES-4 which have acquired several amino acid substitutions to

expand their substrate specificities to cope with the extensive use of broad-spectrum β-lactams in clinical settings.

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Inhibitor-Sensitive AmpC β-Lactamase Variant Produced by an *Escherichia coli* Clinical Isolate Resistant to Oxyiminocephalosporins and Cephamycins

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***Escherichia coli* HKY28, a ceftazidime-resistant strain isolated from a urine specimen in Japan, produced an inhibitor-sensitive AmpC β-lactamase variant. The deduced amino acid sequence of the enzyme contained a number of substitutions and a tripeptide deletion (Gly286-Ser287-Asp288) compared with the sequence of native AmpC of *E. coli*. When the deletion was reverted by a 9-base insertion at the relevant site of *ampC* in the clone, the typical inhibitor-resistant phenotype of AmpC was restored, while at the same time the levels of resistance to ceftazidime, cefpirome, and cefepime were reduced eightfold or more. Molecular modeling studies indicated that a structural change took place in the H-10 helix as a result of the deletion, and this change caused an alteration of the substrate binding site, leading to a unique phenotype analogous to that of inhibitor-sensitive class A extended-spectrum β-lactamases. The degree of inhibition was greater with sulbactam and tazobactam than with clavulanic acid. To our knowledge, this is the first report to have characterized an *E. coli ampC* that encodes chromosomal AmpC β-lactamase sensitive to the available β-lactamase inhibitors.**

The principal and most prevalent mechanism of resistance to β-lactam agents among pathogenic gram-negative bacteria is the production of β-lactamases (3, 17). One approach to overcoming the problem has been the development of β-lactams resistant to the hydrolytic activities of these enzymes. The other has been the development of β-lactamase inhibitors, which protect β-lactams from hydrolysis by β-lactamases when the inhibitors are used in combination with β-lactams (28). At present, three β-lactamase inhibitors, clavulanic acid, sulbactam, and tazobactam, are available for clinical use in combination with a number of penicillins. These inhibitors mainly target Ambler class A β-lactamases and inactivate their active-site serines, thus potentiating the actions of β-lactamase-sensitive compounds. Clavulanic acid and sulbactam are generally not effective in inhibiting the activities of AmpC β-lactamases, although some are known to be moderately inhibited by tazobactam (4, 14).

In 1994, we isolated an *Escherichia coli* clinical strain, HKY28, which produced a chromosomal AmpC β-lactamase that had an inhibitor-sensitive and extended-spectrum activity profile similar to those of class A extended-spectrum β-lactamases (ESBLs). However, the results of PCR experiments with representative TEM- and SHV-derived ESBLs and CTX-M-type β-lactamases were negative. In the present study we conducted genetic, biochemical, and molecular modeling analyses of this unique AmpC β-lactamase variant.

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MATERIALS AND METHODS

Bacterial strains, plasmids, and media. *E. coli* HKY28 was isolated from a culture of urine from an inpatient in Japan in 1994. *E. coli* XL1-Blue (Stratagene, La Jolla, Calif.) was used as the recipient strain for plasmids. *E. coli* BMH71-18mutS and *E. coli* MV1184 (Takara Bio Inc., Ohtsu, Japan) were used as the hosts in a site-directed mutagenesis experiment. Plasmid vectors pBCKS+ (Stratagene) and pKF18k (Takara Bio) were used for the cloning and site-directed mutagenesis experiments, respectively. For enzyme purification, *ampC*-deficient *E. coli* CS14-2 (7) was used as the host to avoid background AmpC production. Bacteria were grown in Luria-Bertani (LB) broth supplemented with the appropriate antibiotics, unless specified otherwise.

Antibiotics and susceptibility testing. The following β-lactam antibiotics and β-lactamase inhibitors were obtained from the indicated sources: aztreonam, Eizai Co., Ltd., Tokyo, Japan; ampicillin, amoxicillin, and cefminox, Meiji Seika Kaisha, Ltd., Tokyo, Japan; cefepime, Bristol Pharmaceuticals K. K., Tokyo, Japan; cefmetazole and chloramphenicol, Sankyo Co., Ltd., Tokyo, Japan; cefotaxime and cefpirome, Aventis Pharma, Ltd., Tokyo, Japan; cefoxitin and imipenem, Banyu Pharmaceutical Co., Ltd., Tokyo, Japan; ceftazidime and clavulanic acid, GlaxoSmithKline K. K., Tokyo, Japan; cephaloridine and moxalactam, Shionogi & Co., Ltd., Osaka, Japan; sulbactam, Pfizer Pharmaceuticals Inc., Tokyo, Japan; and tazobactam, Taiho Pharmaceutical Co., Ltd., Tokyo, Japan.

MICs were determined by the agar dilution method by the protocol recommended by the National Committee for Clinical Laboratory Standards (18).

PCR amplification. To amplify broad-spectrum β-lactamase genes from HKY28, PCR analysis was performed with sets of primers for various β-lactamases, including TEM- and SHV-derived ESBLs as well as CTX-M-1-, CTX-M-2-, and CTX-M-9-type β-lactamases, as described previously (27).

Transfer of ceftazidime resistance. Conjugation experiments were conducted with *E. coli* CSH2 as the recipient by broth mating and filter mating methods (7). Transconjugants were selected on LB agar supplemented with rifampin (50 μg/ml), nalidixic acid (50 μg/ml), and ceftazidime (4 μg/ml).

Cloning and sequencing of β-lactamase gene. The basic recombinant DNA manipulations were carried out as described by Sambrook et al. (24). The

genomic DNA of HKY28 was prepared and digested with EcoRI. The resultant fragments were ligated with plasmid vector pBCKS+, and electrocompetent *E. coli* XL1-Blue was transformed with these recombinant plasmids. Transformants were selected for resistance to chloramphenicol (30 μg/ml) and ceftazidime (4 μg/ml). For determination of the MICs and use of the transformants for site-directed mutagenesis, the *ampC* gene of HKY28 was amplified with oligonucleotide primers *ampC*-U (5′-CGG **AAT TCG** GTT TTC TAC GGT CTG GC-3′) and *ampC*-L (5′-CGG **GAT CCG** ATG ACA GCA AGG AAA AG-3′), which contained EcoRI and BamHI cleavage sites (indicated in boldface), respectively, at their 5′ ends, by using Pyrobest DNA polymerase (Takara Bio). The EcoRI-BamHI fragment containing the *ampC* gene of *E. coli* HKY28 was ligated with pBCKS+ to yield pBE28W, which was then used to transform *E. coli* XL1-Blue and *E. coli* CS14-2. The coding sequences of the cloned fragments were determined by using custom sequencing primers as well as a BigDye Terminator Cycle Sequencing Ready Reaction kits and an ABI 3100 DNA sequencer (Applied Biosystems, Foster City, Calif.). The enzymes used for gene manipulations were purchased from Nippon Gene Co. Ltd. (Tokyo, Japan) or New England Biolabs, Inc. (Beverly, Mass.).

Reversion of AmpC deletion. Site-directed mutagenesis was performed to revert the 9-nucleotide deletion in the cloned *ampC* gene of *E. coli* HKY28 corresponding to a tripeptide deletion at positions 286 to 288 in AmpC. The reagents and strains contained in the Mutan-Express Km mutagenesis kit (Takara Bio) were used according to the procedures based on the oligonucleotide-directed dual Amber method (9) provided by the manufacturer. The following mutagenic primer containing the 9-nucleotide insertion (in boldface) was used: 5′-CCA GTG CAA TTT TAT TGT **CAC TGC CGT** TAA TGA TGA TGT CAG G-3′. After mutagenesis, the EcoRI-BamHI fragment containing the revertant *ampC* was ligated with pBCKS+ to yield pBE28R, which was then used to transform *E. coli* XL1-Blue and *E. coli* CS14-2.

Enzyme purification. *E. coli* CS14-2 harboring pBE28W or pBE28R was cultured overnight in 2 liters of LB broth supplemented with 30 μg of chloramphenicol per ml. Cells were harvested by centrifugation and washed with and then suspended in 3 ml of 50 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS) buffer (pH 6.0). The cells were frozen and thawed twice and were then ultracentrifuged at 100,000 × *g* for 4 h at 4°C. For gel filtration, the supernatant containing β-lactamase was chromatographed through a HiLoad 16/60 Superdex 200 prepgrade (Pharmacia Biotech, Uppsala, Sweden) column preequilibrated with 50 mM MOPS buffer (pH 6.0). For cation-exchange chromatography, fractions with activity were then applied to a HiTrap SP HP column (Pharmacia Biotech) preequilibrated with the same buffer. The enzymes were eluted with a linear gradient of 0 to 0.5 M NaCl in the same buffer. The purity of the enzymes was checked by a sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Enzyme assays. Purified AmpC enzymes were assayed against various β-lactam substrates at 37°C in 50 mM phosphate buffer (pH 7.0) by using an autospectrophotometer (V-550; Nihon Bunko Ltd., Tokyo, Japan). The specific activity of the enzymes was defined as the activity that hydrolyzed 1 μmol of cephaloridine per min. K_m and k_{cat} values were obtained by a direct-weight fit to the Michaelis-Menten equation by using KaleidaGraph software (Hulinks, Tokyo, Japan). The concentrations of inhibitors giving a 50% reduction in hydrolysis of cephaloridine (IC_{50}) were measured after 10 min of preincubation of the enzymes with the inhibitors at 37°C and cephaloridine as the substrate at 1 mM. The affinities of the enzymes for the inhibitors (K_is) were measured by competition procedures with cephaloridine in the same buffer with no preincubation of the enzyme or the inhibitor. To determine the isoelectric points, 10 μl of enzyme solution was loaded onto an Immobiline DryStrip (pH 3 to 10 and 6 to 11; Pharmacia Biotech), and electrophoresis was carried out with an IPGphor electrophoresis system (Pharmacia Biotech).

Modeling of substrate-enzyme complex structures. The crystal structure of the AmpC β-lactamase (Protein Data Bank accession number 2BLS) was used as the reference to build a model of the AmpC enzyme of *E. coli* HKY28. The tripeptide at the H-10 helix was deleted by the loop search method of the Homology module installed in Insight II software (version 2000; Molecular Simulations Inc., San Diego, Calif.). An initial structure of the enzyme was optimized by use of molecular dynamics calculations at 298 K by the cell multipole method, a distance-dependent dielectric constant, and a time step of 1 fs for 100 ps by sampling the conformation every 1 ps by use of Discover 3 software (version 98.0; Molecular Simulations Inc.). One hundred conformations were minimized until the final root-mean square deviation became less than 0.1 kcal/mol/Å, and the lowest energy conformation was selected for the substrate-docking study. The substrates were roughly docked into the ligand-binding cleft with the guidance of a hydrogen bond of a β-lactam carbonyl oxygen at the oxyanion hole as well as a hydrogen bond of the carboxylate oxygen with Tyr150 (12). The initial complex model was minimized, and then the substrate-binding site was covered by water molecules (sphere thickness, 20 Å). The structure consisted of the substrate and the residues within 10 Å from the substrate, which were energy optimized in the presence of the water molecules by the molecular dynamics and minimization procedure described above. The lowest-energy structures were selected as energy-refined complex models.

Nucleotide sequence accession number. The nucleotide sequence encoding AmpC characterized in this study appears in the EMBL/GenBank/DDBJ databases under accession number AB108683.

RESULTS

Susceptibility of parental strain. The MICs of β-lactams for parental strain *E. coli* HKY28 are shown in Table 1. Strain HKY28 was resistant to ampicillin, amoxicillin, cephaloridine, cefminox, and cefoxitin. It was also resistant to ceftazidime (MIC, 32 μg/ml) but remained susceptible to aztreonam and imipenem. Interestingly, the MIC of ampicillin was reduced by at least 8-fold when it was combined with sulbactam, and the MICs of cefotaxime were reduced by 16- and 8-fold when it was combined with sulbactam and tazobactam, respectively. Addition of sulbactam reduced the MIC of ceftazidime by eightfold. Overall, the reductions in the MICs were the greatest with sulbactam, followed by tazobactam and clavulanic acid.

PCR analysis of β-lactamase genes. By PCR *E. coli* HKY28 was negative for the genes for the TEM-, SHV-, CTX-M-1-, CTX-M-2- and CTX-M-9-type β-lactamases, which are the prevalent types of ESBLs in Japan.

Transfer of ceftazidime resistance. The ceftazidime resistance of *E. coli* HKY28 could not be transferred to recipient *E. coli* strain CSH2 by conjugation, despite repeated attempts.

Cloning and sequencing of resistance gene. A 6-kb EcoRI fragment containing a ceftazidime resistance determinant was cloned into the vector pBCKS+ and was termed pE753. Nucleotide sequencing analysis revealed a chromosomal locus of *E. coli* containing *ampC* flanked by *frdD* and *blc* but without any other β-lactamase gene. PCR-generated recombinant plasmid pBE28W containing *ampC* of *E. coli* HKY28 was found to possess an *ampC* gene identical to that of pE753 and conferred resistance to ceftazidime. The deduced amino acid sequence contained seven amino acid substitutions and three amino acid deletions (Gly286, Ser287, and Asp288) of the AmpC product compared with the sequence of *E. coli* K-12 (10) (Fig. 1). The promoter region of the *ampC* gene contained three mutations (a C-to-T change at position −73, a C-to-T change at position +6, and a G-to-A change at position +34) and a T insertion between positions −14 and −13 compared with the sequence of the corresponding region of the *E. coli* K-12 genome. Recombinant plasmid pBE28R, generated by site-directed mutagenesis, was confirmed to possess *ampC* of *E. coli* HKY28, except for the insertion of the 9-nucleotide sequence designed to restore the tripeptide deleted from *ampC* of *E. coli* HKY28.

Susceptibilities of clones to β-lactams. Both *E. coli* XL1-Blue harboring pBE28W (the HKY28 clone) and that harboring pBE28R (the revertant clone) displayed resistance or reduced susceptibilities to all β-lactams except cefpirome, cefepime, and imipenem; but the degree of resistance varied significantly between the two clones. The cefotaxime and ceftazidime MICs were fourfold or more higher for the HKY28 clone than for the revertant clone. The cefpirome and cefepime MICs were 64-fold higher for the HKY28 clone than