

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 \times 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. morgani* 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 (MIC₅₀s) of imipenem were 32, 16, and 16, respectively, and the MIC₉₀s of the same agent were >32, 32, and 32 μ g/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 <i>aac(6')-Ib</i> -a positive strains	Hospital	Ward ^b (no. of strains)	Antibiogram ^c (no. of strains)
<i>P. aeruginosa</i> (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)
	<i>A. baumannii</i> (18)	k	VIM-2 (1)	1	I	Internal medicine 6E (1)
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)
		IMP-1 (1)	1	C	Otolaryngology 7E (1)	GM, ST (1)
		IMP-1 (1)	0	J	Internal medicine ICU (1)	AMK, ST (1)
<i>A. junii</i> (1)		IMP-1 (1)	0	K	Internal medicine 5A (1)	ST (1)
		IMP-2 (1)	0	K	Internal medicine 5A (1)	MINO, LVFX, ST, CP (1)
<i>A. calcoaceticus</i> (1)		IMP-1 (1)	0	A	Neurology 75NS (1), urology 65NS (1)	MINO, LVFX, CP (1)
<i>A. johnsonii</i> (1)		IMP-1 (1)	0	C	Urology 7F (1)	MINO, ST, CP (1)
<i>S. marcescens</i> (44)	1	IMP-1 (2)	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)
	2	IMP-1 (1)	0	C	Urology 7F (2)	ST, CP (2)
	3	IMP-1 (41)	0	C	Internal medicine 83 (1)	MINO, LVFX, ST (1)
<i>Providencia rettgeri</i> (3)	1	IMP-1 (2)	2	C	Internal medicine CCU (3), internal medicine 3S (1)	CP (1)
<i>Citrobacter freundii</i> (4)	1	IMP-1 (4)	4	C	Urology 7F (1)	MINO, LVFX, ST (1)
<i>Morganella morganii</i> (1)		IMP-1 (1)	1	C	Pediatrics E6 (1)	MINO, ST, CP (1)
<i>Enterobacter cloacae</i> (1)		VIM-2 (1)	0	D		

^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).

majority 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 ($\mu\text{g/ml}$) for organism								
	<i>Enterobacteriaceae</i> (n = 53)			<i>P. aeruginosa</i> (n = 22)			<i>Acinetobacter</i> 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	8->32	≤ 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 $\mu\text{g/ml}$.

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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Drug resistance of *Enterococcus faecium* clinical isolates and the conjugative transfer of gentamicin and erythromycin resistance traits

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Abstract

Drug resistance and the transferability of resistance were examined in 218 *Enterococcus faecium* clinical isolates obtained from inpatients of a Japanese university hospital between 1990 and 1999. One hundred and sixty one isolates (73.9%) were drug-resistant and 127 (58.2%) isolates were resistant to two or more drugs. Vancomycin resistant *E. faecium* (VRE) was not isolated. The transferability of drug-resistance to an *E. faecium* strain was examined by broth or filter mating. Six (12.5%) of the 48 gentamicin resistance traits, and fifty (50%) of the 101 erythromycin resistance traits were transferred by filter mating. The gentamicin resistance traits of five isolates and the erythromycin resistance traits of four isolates were transferred to the recipient strains by both broth mating and filter mating at a frequency of about 10^{-6} and 10^{-5} per donor cell, respectively. The five gentamicin resistant strains were shown to harbor pMG1-like plasmids on the basis of their Southern hybridization with pMG1 (65.1 kbp, Gm^r), which transfers efficiently between enterococci by broth mating. Each of the four erythromycin resistant transconjugants obtained by broth mating harbored a large conjugative plasmid (more than 100 kbp). The plasmids showed no homology with well-characterized enterococcal conjugative plasmids such as pAD1, pPD1, pAMβ1, pIP501 and pMG1 by Southern hybridization. Of the erythromycin resistance traits that transferred only by filter mating, it was found that the erythromycin resistance trait was conferred by a 47-kbp transposable element that transferred from the chromosome of the donor strain to different sites within the pheromone responsive plasmid pAD1 (60 kbp) of the recipient strain, suggesting that the erythromycin resistance trait was encoded on a conjugative transposon, which was named Tn950.

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1. Introduction

Enterococcus strains have become a significant cause of nosocomial infections and are the third most commonly isolated genus in clinical isolates [1–5]. Of the strains belonging to the enterococcus genus, *Enterococcus*

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faecalis and *Enterococcus faecium* are common isolates from human. The enterococcus strains isolated from clinical infection have multiple-drug resistance traits. The multiple-drug resistance traits of the enterococci provide these organisms with a selective advantage in the hospital environment. In particular, vancomycin (glycopeptide)-resistant enterococci (VRE or GRE) can cause serious problems for hospitalized patients due to the limited options for treatment of VRE infection [5–7].

Selective pressure by drugs or a genetic transfer system is essential for the increase or spread of drug resistant organisms. *E. faecium* clinical isolates are usually multiple-resistant and VanA type vancomycin resistant enterococci (VRE) are predominately isolated from *E. faecium* [6,7]. Transferable plasmids or mobile genetic elements encoding drug resistance traits have been reported in *E. faecium*. The VanA-type resistance determinant is encoded on transposon Tn1546, which is borne by non-conjugative or conjugative plasmids that transfer among enterococci by mating on a solid surface (filter mating) [8–10]. The VanB determinant of *E. faecium* is encoded on a large mobile genetic element on a conjugative transposon [11–13].

Little is known about the systems of efficient plasmid transfer in *E. faecium*. Previously, we reported the first isolation of a new type of conjugative plasmid, named pMG1 [14–16], which transfers efficiently among enterococcus strains during broth mating, carries gentamicin resistance trait, and was isolated from an *E. faecium* clinical isolate in Japan. Conjugative gentamicin resistance pMG1-like plasmids are widely disseminated in vancomycin resistant *E. faecium* clinical isolates in the US [15], and are thought to contribute to the spread of other resistance traits, including vancomycin resistance in enterococci [15,16]. In this report, we describe the drug resistance of *E. faecium* clinical isolates from a Japanese hospital, and the transferabilities of the drug resistance traits.

2. Materials and methods

2.1. Bacteria, plasmid and media

A total of 218 *E. faecium* clinical isolates were used in this study. These isolates were obtained from different patients who had been admitted to Gunma University Hospital, Maebashi, Japan, between January 1990 and December 1999. A total of 41 *E. faecium* isolates from the feces of different healthy students were used as control strains. The laboratory strains and plasmids used in this study were *E. faecalis* FA2-2 (Rif^r, Fus^r) [17], JH2SS (Str^r, Spc^r) [18], *E. faecium* BM4105RF (Rif^r, Fus^r) [15], BM4105SS (Str^r, Spc^r) [15], plasmid pMG1 (gentamicin resistances) (65.1 kbp) [14], pAD1 [19–25], pPD1 [26–28], pAM373 [29], pAMβ1 [30], pIP501

[31,32] and pAM120 [33]. The media used in this study were Todd-Hewitt broth (THB) and agar plates (Difco Laboratories, Detroit, Mich.). The MIC of the antimicrobial drugs was determined according to the criteria of the National Committee for Clinical Laboratory Standards (NCCLS) using Mueller-Hinton agar. The antibiotic concentrations used in the selective plates were as follows (in micrograms per milliliter): ampicillin, 25; fosfomycin, 25; erythromycin, 25; streptomycin, 500; spectinomycin, 500; kanamycin, 500; gentamicin, 500; chloramphenicol, 25; tetracycline, 3; vancomycin, 3; rifampin, 25; and fusidic acid, 25.

2.2. Mating procedures

Broth mating and filter mating were performed as previously described [34–36] with a donor/recipient ratio of 1:10.

2.3. Isolation and manipulation of plasmid DNA

Plasmid DNA was isolated by the alkaline lysis method [37,38].

2.4. Pulsed-field gel electrophoresis of chromosomal DNA

Pulsed-field gel electrophoresis (PFGE) of chromosomal DNA isolated from the *E. faecium* strain was performed as previously described [3]. The gels were electrophoresed with a clamped homogeneous electric field (CHEF-DR II; Bio-Rad Laboratories, Richmond, CA).

2.5. Southern hybridization

Southern hybridization was performed with the digoxigenin-based non-radioisotope system of Boehringer GmbH (Mannheim, Germany), and all procedures were based on the manufacturer's manual and standard protocols [15,37].

3. Results and discussion

3.1. Drug resistance and the conjugative transfer of high-level erythromycin and gentamicin resistance

As shown in Table 1, 161 (73.9%) isolates were drug resistant and the remaining 57 (26.1%) isolates were drug susceptible. Tetracycline, erythromycin, and ampicillin resistant strains were isolated at relatively high frequencies of about 50–40% when compared to other drug resistances, and 48 (22.0%) of the isolates had a high level of resistance to gentamicin (more than 500 µg/ml MIC). No vancomycin resistant isolates were obtained.

Table 1
Isolation of drug resistance of *E. faecium*

Drug resistance	Number of drug resistant strain (%)	
	Clinical isolates	Isolates from feces of healthy students
Ampicillin (Ap)	97 (44.5%)	
Chloramphenicol (Cm)	8 (3.7%)	
Erythromycin (Em)	101 (46.3%)	1 (2.4%)
Gentamicin (Gm)	48 (22.0%)	3 (7.3%)
Kanamycin (Km)	82 (37.6%)	1 (2.4%)
Streptomycin (Sm)	41 (18.8%)	5 (12.2%)
Tetracyclin (Tc)	120 (55.0%)	
Vancomycin (Vm)	0 (0%)	33 (80.5%)
Drug susceptible	57 (26.1%)	
Total number of strains tested	218 (100%)	41 (100%)

The drug resistance levels (MICs) of ampicillin, chloramphenicol, erythromycin, gentamicin, kanamycin, streptomycin, tetracyclin, and vancomycin were equal to or greater than 16, 16, 16, 64, 1024, 512, 8 and 64 µg/ml, respectively.

Drug resistance among the isolates showed many different patterns and there was no predominant drug resistant pattern. Approximately 30% of the isolates were resistant to more than four drugs (data not shown).

To examine the transferability of the high-level erythromycin (more than 100 µg/ml MIC) and gentamicin resistances, broth mating or filter mating experiments were performed between each drug resistant isolate and the recipient strain *E. faecium* BM4105RF. Of the 48 gentamicin resistant isolates, five were transferred by broth mating and filter mating, and one was transferred by filter mating only. Of the 101 erythromycin resistant isolates, four were transferred by broth mating and filter mating, and 46 were transferred only by filter mating.

3.2. Gentamicin resistance conjugative plasmid

Each of the five gentamicin resistant strains, which were obtained by broth mating, were designated as GF112, GF113, EFG13, EFG16, and EFG17 and their drug resistance patterns were Em Gm Km Sm Tc, Ap Gm Sm Tc, Ap Em Gm Km Sm Tc, Ap Gm Km Sm Tc, and Ap Em Gm Km Sm Tc, respectively. Each gentamicin resistance trait was transferred to an *E. faecium* or *E. faecalis* strain at a frequency of 10^{-5} – 10^{-7} per donor cell by broth mating or more than 10^0 per donor cell by filter mating. The transfer frequency more than 10^0 per donor cell resulted from that plasmid of all donor cells could transfer to recipient cell and plasmid of the transconjugant could re-transfer to recipient cell. The resistance of ten transconjugants of each strain was examined in detail. All of the transconjugants were resistant only to gentamicin. The gentamicin resistance conjugative plasmid pMG1 (65.1 kbp) was originally isolated from *E. faecium* GF113 [14]. The plasmids isolated from GF112, EFG13, EFG16, and EFG17 exhibited *Eco*RI restriction profiles almost identical to pMG1

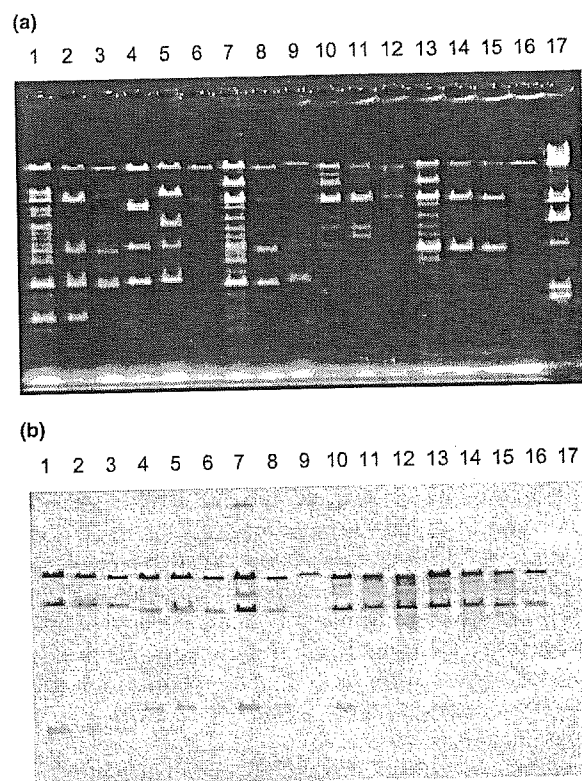


Fig. 1. Agarose gel electrophoresis of restriction endonuclease-digested plasmid DNAs of *E. faecium* isolates harboring a pMG1-like plasmid, and Southern hybridization with pMG1. (a) Agarose gel electrophoresis of *Eco*RI-digested plasmid DNA isolated from gentamicin-resistant *E. faecium* clinical isolates, the gentamicin-resistant *E. faecium* BM4105RF transconjugants and *E. faecalis* FA2-2 transconjugants. (b) The gel was Southern blotted and hybridized to pMG1. Lanes 1, 4, 7, 10, 13: *Eco*RI-digested plasmid DNA isolated from the clinical isolates GF112, GF113, EFG13, EFG16, and EFG17, respectively; lanes 2, 5, 8, 11, 14: *Eco*RI-digested plasmid DNA isolated from BM4105RF transconjugants of strains GF112, GF113, EFG13, EFG16, and EFG17, respectively; lanes 3, 6, 9, 12, 15: *Eco*RI-digested plasmid DNA isolated from FA2-2 transconjugants of strains GF112, GF113, EFG13, EFG16, and EFG17, respectively; lane 16: *Eco*RI-digested pMG1; lane 17: *Hind*III-digested lambda DNA.

and hybridized to pMG1 DNA (Fig. 1). These data implied that these isolates harbored pMG1-like plasmids.

Analysis by pulsed field gel electrophoresis of *Sma*I-digested chromosomal DNA from GF112, GF113, EFG13, EFG16 and EFG17 showed four different patterns (data not shown). Although the isolation frequency of gentamicin resistant strains harboring pMG1-like plasmids was still low, the pMG1-like plasmid could also disseminated to different strains in Japanese clinical isolates. EFG13 and EFG17 had identical restriction endonuclease digestion patterns. EFG13 and EFG17 were isolated from patients in the same ward who had overlapping periods of hospitalization, which implied that nosocomial transmission of the strain had occurred (data not shown).

3.3. Highly efficient transfer of high-level erythromycin resistance and the conjugative plasmid

There was a possibility that each of the four strains designated as EFG114, EFG115, EFG132 and EFG160 that transferred the high-level erythromycin resistance by broth mating were harboring the erythromycin resistance transferable plasmid. Each erythromycin resistance trait was transferred at a frequency of around 10^{-4} – 10^{-5} per donor cell in broth, or around 10^{-2} – 10^{-1} per donor cell in filter mating between *E. faecium* BM4105 strains.

The plasmid DNA was prepared from each erythromycin resistant (Em^r) transconjugant of BM4105RF and analyzed by agarose gel electrophoresis. Agarose gel electrophoresis produced many faint bands of *Eco*RI restriction fragments of each plasmid, which implied that the molecular sizes of the plasmid DNAs were relatively large and these were estimated to be more than 100 kbp (Fig. 2(a)). It is probable that the conjugative erythromycin resistance plasmids are related to each other with respect to their DNA similarity. The plasmid DNA isolated from the EFG132 transconjugant was labeled for use as a probe in Southern hybridization (Fig. 2(b)). The plasmid DNAs isolated from each of Em^r transconjugants showed a similar *Eco*RI restriction profile with respect to the DNA fragments that hybridized to the EFG132 plasmid DNAs by Southern analysis. In particular, two plasmids isolated from the transconjugants of EFG132 and EFG160 showed an identical *Eco*RI restriction profile. The plasmid DNAs of the EFG132 transconjugant showed no homology with pMG1 (Fig. 2, lane 5). Southern analysis was also performed using the plasmid DNAs of the EFG132 transconjugant and the pheromone-responsive plasmids pAD1, pPDI, broad-host-range conjugative plasmids pAM β 1, pIP501, and conjugative transposon Tn916. It showed no homology with other plasmids or the conjugative transposon (data not shown), indicating that the plasmids might be another type of *E. faecium* conjuga-

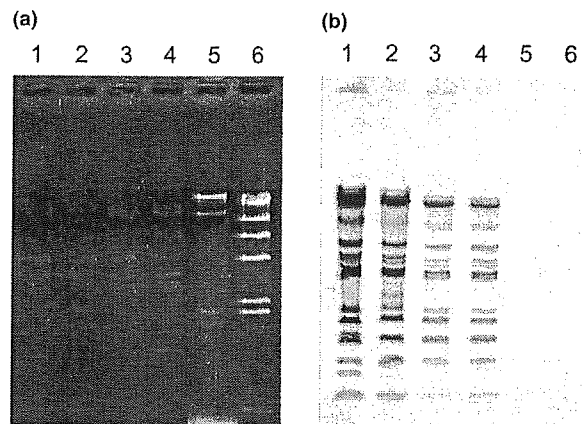


Fig. 2. Agarose gel electrophoresis of restriction endonuclease-digested plasmid DNAs of erythromycin resistant *E. faecium* BM4105RF transconjugants isolated from erythromycin resistant *E. faecium* isolates, and Southern hybridization with the erythromycin-resistant plasmid of EFG132. (a) Agarose gel electrophoresis of *Eco*RI-digested plasmid DNA isolated from erythromycin-resistant BM4105RF transconjugants isolated from erythromycin resistant isolates. (b) The gel was Southern blotted and hybridized with the plasmid DNA isolated from the transconjugant of EFG132. Lanes 1–4: *Eco*RI-digested plasmid DNAs isolated from the transconjugants of EFG114, EFG115, EFG132, EFG160, respectively; lane 5: *Eco*RI-digested pMG1; lane 6: *Hind*III-digested lambda DNA.

tive plasmids. The high-level erythromycin resistances (more than 100 μ g/ml MIC) of *E. faecium* are generally determined by *erm* genes such as *ermB* and *ermA* [39]. The genes encode 23S rRNA methyl transferase and locate on the mobile elements such as transposon.

3.4. Conjugative transfer of chromosome borne high-level erythromycin resistance

The erythromycin resistance of *E. faecium* T383 was transferred to the recipient strains *E. faecalis* FA2-2 and *Enterococcus hirae* ATCC9790RF at a frequency of 8.6×10^{-8} and 5.7×10^{-6} per donor cell by filter mating, respectively. The erythromycin resistance of each of the transconjugants was transferred between *E. faecalis* strains, between *E. hirae* and *E. faecalis* strains, and between *E. hirae* strains by filter mating at a frequency of approximately 10^{-4} – 10^{-5} per donor cell. The parent strain *E. faecium* T383 contained several plasmids (data not shown). Of the erythromycin resistant *E. faecalis* FA2-2 transconjugants, one transconjugant that was named *E. faecalis* KT1 was found to be devoid of plasmid after repeated transfer experiments. The erythromycin resistance of *E. faecalis* KT1 was also transferred among enterococcus strains during filter mating at a frequency of around 10^{-5} – 10^{-6} per donor cell. These results implied that the erythromycin resistance determinant might reside on a transposable element that is located on the bacterial chromosome and is capable of subsequent transfer by filter mating.

The plasmid pAD1 (hemolysin/bacteriocin, 60 kbp) is an *E. faecalis* pheromone-responsive plasmid that transfers at high frequency to the *E. faecalis* recipient strain during broth mating at a frequency 10^{-2} – 10^0 [17,20]. Filter mating experiments were performed using the erythromycin resistant transconjugant of *E. faecalis* JH2SS as a donor and *E. faecalis* FA2-2 harboring pAD1 plasmid as a recipient. Erythromycin resistant transconjugants of *E. faecalis* FA2-2 (pAD1) were obtained at a frequency of around 10^{-5} per donor cell. If the erythromycin resistance transposable element was transposed to pAD1 and was linked with the plasmid, the resistance would be transferred to the recipient *E. faecalis* strain at a relatively high frequency as the pheromone responsive plasmid pAD1 in subsequent broth mating experiments. About 10% of the erythromycin resistant *E. faecalis* FA2-2 transconjugants was found to transfer erythromycin resistance to the recipient JH2SS strains at the relatively high frequency of 10^{-2}

per donor cell by broth mating, indicating linkage of the resistance determinant to the plasmid. Four erythromycin resistant *E. faecalis* FA2-2 (pAD1) transconjugants, which transferred the erythromycin resistance at relatively high frequency by subsequent broth mating, were chosen as representative strains.

3.5. Agarose gel electrophoresis analysis of pAD1 borne erythromycin resistance

The agarose gel electrophoresis profile of *EcoRI*-digested plasmid DNA from each of the four strains differed from that of pAD1, which consists of nine *EcoRI* fragments designated A to I with corresponding molecular sizes of 19.3, 15.4, 12.0, 4.1, 3.4, 2.7, 1.5, 1.2 and 0.2 kbp (Fig. 3(a)). Of the plasmids isolated from the four strains, one plasmid (pMG3001, Fig. 3(a), lane 3) did not have the 1.2 kbp fragment corresponding to *EcoRI* fragment H of pAD1 and the other three

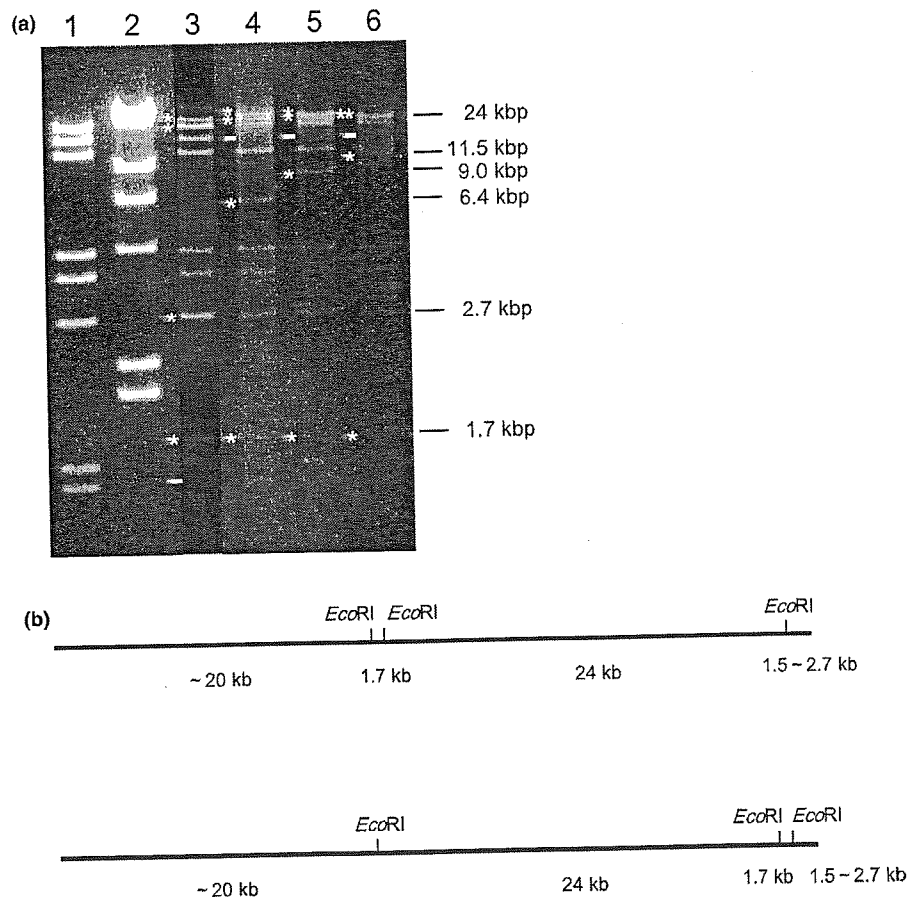


Fig. 3. Agarose gel electrophoresis of *EcoRI*-digested DNAs of pAD1 borne erythromycin resistance and the possible structure of the erythromycin resistance transposable element (Tn950). (a) *EcoRI*-digested plasmid DNA isolated from erythromycin-resistance pAD1. Lane 1: *EcoRI* digested pAD1; lane 2: *HindIII*-digested lambda DNA; lanes 3–6: *EcoRI*-digested plasmid DNAs of pMG3001, pMG3002, pMG3003 and pMG3004, respectively. Symbols: the star and small bar on the left side of the agarose gel for each plasmid indicates the new band and missing band, respectively. (b) Two putative physical maps of the erythromycin resistance 47 kbp transposable element (Tn950). There are three *EcoRI* sites in the transposon and two internal *EcoRI* fragments (24 and 1.7 kbp).

plasmids (i.e., pMG3002 (lane 4), pMG3003 (lane 5), pMG3004 (lane 6)) did not have the 15.4 kbp fragment corresponding *EcoRI* fragment B of pAD1. Each of four plasmids gave rise to four new fragments. The molecular sizes of the new fragments of pMG3001 were 24, 20, 2.7 and 1.7 kbp. The total molecular size of the four new fragments of pMG3001 was about 48.4 kbp. These results implied that *EcoRI* fragment H (1.2 kbp) of pAD1 had an insertion with molecular size of about 47 kbp, and this 48.2 kbp fragment gave rise to new four *EcoRI* fragments. The molecular sizes of the new fragments of pMG3002 were 30, 24, 6.4 and 1.7 kbp (Fig. 3(a), lane 4). The molecular sizes of the new fragments of pMG3003 were 28, 24, 9.0 and 1.7 kbp (Fig. 3(a), lane 5). The molecular sizes of the new fragment of pMG3004 were 25, 24, 11.5 and 1.7 kbp (Fig. 3(a), lane 6). The total molecular size of the four new fragments of each of these plasmids was about 62.5 kbp. These data indicated that *EcoRI* fragment B (15.4 kbp) of pAD1 had an insertion with molecular size of about 47 kbp, and that the 62.5 kbp fragment gave rise to new four *EcoRI* fragments in each of the three plasmids. Of the four new *EcoRI* fragments observed in each plasmid, two fragments with a molecular size of 24 and 1.7 kbp were found in each of the four plasmids. The other two *EcoRI* fragments varied in size depending on which of the four plasmids they originated from, indicating that each of the two fragments contained either one fragment that separated *EcoRI* fragment H (1.2 kbp) of pAD1 by insertion of the 47 kbp element in case of pMG3001, and separated *EcoRI* fragment B (15.4 kbp) of pAD1 by insertion of the 47 kbp element in case of pMG3002, pMG3003 and pMG3004, respectively. These results implied that the approximately 47 kbp element encoding erythromycin resistance had transposed from the chromosomal DNA to different sites within pAD1 by conjugation. The 47 kbp element, which will be subsequently referred to as conjugative transposon Tn950, had three *EcoRI* sites and the two internal *EcoRI* fragments were 1.7 and 24 kbp in size. The putative *EcoRI* sites of Tn950 are shown in Fig. 3(b).

3.6. DNA–DNA hybridization

The conjugative transposon Tn916 (16 kbp, *tetM*) was originally isolated from *E. faecalis* DS16 and is a representative conjugative transposon. The transposon Tn917 (5 kbp, *MLS^F* (macrolide-lincosamide-streptogramin B resistance)) was also originally isolated from *E. faecalis* DS16 and is a member of the Tn3 family. Tn917 encodes *ermB* gene conferring the high-level erythromycin resistance and Tn917-like transposon is widespread in enterococcus species. The plasmid pMG3005, that is another pAD1 derivative carrying an erythromycin resistance transposable element within the *EcoRI* fragment B, was studied for homology with Tn917 and

Tn916. pAM225 is the pBR332-derived vector pBR325 carrying the pAD1 *EcoRI* fragment H::Tn917. pAM120 is the pBR322-derived vector pGL101 carrying the pAD1 *EcoRI* fragment F::Tn916. The plasmids pAM225 and pAM120 were used as probes for Tn917 and Tn916, respectively. pAM120 only hybridized to a 2.7 kbp *EcoRI* fragment of pMG3005 corresponding to *EcoRI* fragment F of pAD1, which is contained in pAM210 (Fig. 4(b)). These results suggested that the erythromycin resistance transposable element did not contain any sequence homologous with Tn916. pAM225 hybridized to a 1.2-kbp *EcoRI* fragment of pMG3005 corresponding to *EcoRI* fragment H of pAD1, which is contained in pAM225, and also hybridized to the 24-kbp *EcoRI* fragment of pMG3005, which corresponds to the new *EcoRI* fragment of pMG3005, and the hybridization band was relatively faint (Fig. 4(c)). The results suggested that the erythromycin resistance transposable element did not contain significant sequence homologous with Tn917 and the faint signal might indicate the similarity with the erythromycin resistance determinant (*ermB* gene) encoded on the transposon.

Conjugative transposons are genetic elements that move from the genome of a donor cell to the genome of a recipient bacterial cell by conjugation with cell to cell contact [36,40,41]. Conjugative transposons are widespread in gram-positive bacteria and play a significant role in the dissemination of antibiotic resistance and are particularly common in streptococci and enterococci [42]. Tn916 (18kbp) and Tn1545 (25.3 kbp) from

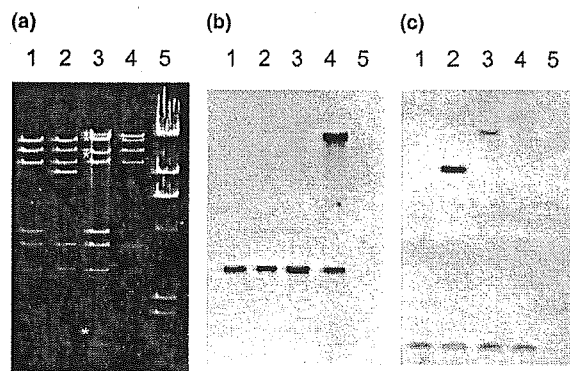


Fig. 4. Southern hybridization analysis of the 47 kbp transposable element (Tn950) that carries erythromycin resistance with the Tn916 probe (b) and Tn917 probe (c). (a) Agarose gel electrophoresis of *EcoRI*-digested plasmid DNAs of pAD1 and the derivatives. Symbols: the star and small bar on the left side of the agarose gel of lane 3 indicates the new band and missing band, respectively. (b) Southern hybridization analysis using the labeled plasmid DNA of pAM120 as a Tn916 probe. (c) Southern hybridization analysis using the labeled whole plasmid DNA of pAM225 as a Tn917 probe. Lanes 1: pAD1; lane 2: pAM307 (pAD1::Tn917); lane 3: pMG3005 (pAD1::Tn950, Tn950 inserted into *EcoRI* fragment B of pAD1); lane 4: pAM210 (pAD1::Tn916); lane 5: *HindIII*-digested lambda DNA.

E. faecalis and *Streptococcus pneumoniae*, respectively, are the best-studied examples of conjugative transposons in gram-positive bacteria and are representative of the conjugative transposons. They and many other conjugative transposons confer tetracycline resistance on their host strains by the *tetM* determinant that encodes a ribosome binding protein [42]. The conjugative transposons are excised and integrated via a reciprocal recombination mechanism using the *Int* and *Xis* proteins, which belong to lambda integrase family and are analogous to lambda *Xis* protein, respectively.

The 47 kbp transposable element did not contain any sequence homologous with that of the representative conjugative transposon Tn916. Although the nature of intercellular transfer of the 47 kbp element was unknown, these data implied that the 47 kbp element might be different type of conjugative transposon from the Tn916-like transposon.

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CFE-1, a Novel Plasmid-Encoded AmpC β -Lactamase with an *ampR* Gene Originating from *Citrobacter freundii*

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A clinical isolate of *Escherichia coli* from a patient in Japan, isolate KU6400, was found to produce a plasmid-encoded β -lactamase that conferred resistance to extended-spectrum cephalosporins and cephamycins. Resistance arising from production of a β -lactamase could be transferred by either conjugation or transformation with plasmid pKU601 into *E. coli* ML4947. The substrate and inhibition profiles of this enzyme resembled those of the AmpC β -lactamase. The resistance gene of pKU601, which was cloned and expressed in *E. coli*, proved to contain an open reading frame showing 99.8% DNA sequence identity with the *ampC* gene of *Citrobacter freundii* GC3. DNA sequence analysis also identified a gene upstream of *ampC* whose sequence was 99.0% identical to the *ampR* gene from *C. freundii* GC3. In addition, a fumarate operon (*frdABCD*) and an outer membrane lipoprotein (*blc*) surrounding the *ampR-ampC* genes in *C. freundii* were identified, and insertion sequence (IS26) elements were observed on both sides of the sequences identified (forming an IS26 composite transposon); these results confirm the evidence of the translocation of a β -lactamase-associated gene region from the chromosome to a plasmid. Finally, we describe a novel plasmid-encoded AmpC β -lactamase, CFE-1, with an *ampR* gene derived from *C. freundii*.

The AmpC β -lactamase produced by gram-negative bacteria such as *Citrobacter* spp., *Enterobacter* spp., *Serratia* spp., and *Morganella* spp. can hydrolyze several β -lactam antibiotics, including cephamycins and extended broad-spectrum cephalosporins (30).

The regulation of AmpC β -lactamase expression is intimately linked to cell wall recycling and involves at least three genes: *ampR*, which encodes a transcriptional regulator of the LysR family; *ampG*, which encodes a transmembrane permease; and *ampD*, which encodes a cytosolic *N*-acetyl-anhydromuramyl-L-alanine amidase that hydrolyzes 1,6-anhydromuropeptides (16, 21, 23, 31). AmpR has been shown to bind to a 38-bp sequence within the intercistronic region between *ampR* and *ampC*. In the absence of a β -lactam inducer, AmpR represses the synthesis of β -lactamase 2.5-fold, whereas expression is induced 10- to 200-fold in the presence of a β -lactam inducer (26). Mutations in the specific site of *ampR* work as an activator of *ampC* and result in the constitutive hyperproduction of AmpC β -lactamase (3, 4, 24). Deletion mutation of the *ampR* gene results in a slightly higher level of basal expression of the *Citrobacter freundii* β -lactamase, but enzyme synthesis can no longer be induced. Knockout mutations in the *ampD* gene result in constitutive hyperproduction of the AmpC β -lactamase even in the absence of a β -lactam inducer (6, 18).

In recent years *ampC* genes have been found on conjugative plasmids, mainly among *Klebsiella pneumoniae* isolates but also

occasionally among *Escherichia coli* isolates. Some of these plasmid-encoded genes have DNA and amino acid sequences very similar to those of the chromosome-encoded AmpC β -lactamases of *C. freundii* (CMY-2, CMY-4, and LAT-1) (1, 5, 42, 43), *Enterobacter cloacae* (MIR-1 and ACT-1) (11, 33), and *Morganella morganii* (DHA-1 and DHA-2) (13, 14), although the phylogenies of the various enzymes (FOX-1, MOX-1, and CMY-9) (12, 15, 19) remain unclear (Fig. 1).

Until recently, plasmid-encoded *ampC* genes were considered noninducible because they lack the regulator gene *ampR* (35). However, this generalization is no longer valid: three inducible plasmid-encoded AmpC β -lactamases, DHA-1, DHA-2, and ACT-1, have been described; and all of these carry the *ampR* and the *ampC* genes (2, 13, 37). The mechanism by which plasmid-encoded AmpC β -lactamase was generated from the chromosomal gene has not yet been discovered.

Compared with plasmid-encoded class A extended-spectrum β -lactamases, these plasmid-encoded AmpC β -lactamases (except for ACC-1) are active against cephamycins and are also effective against oxyimino-cephalosporins, such as cefotaxime, ceftazidime, and aztreonam, a monobactam. The *in vitro* activities of these AmpC β -lactamases are not inhibited by clavulanic acid. Genes encoding these enzymes are now found on plasmids at increasing frequencies (34).

A plasmid-encoded AmpC β -lactamase which confers resistance to cephamycins and expanded-spectrum cephalosporins was detected in Japan in a clinical isolate of *E. coli*. In this report, we characterize a novel plasmid-encoded AmpC β -lactamase, CFE-1, and analyze the nucleotide sequences of the *ampC*, *ampR*, and surrounding genes to compare with those of chromosome-encoded and plasmid-encoded AmpC β -lactamases.

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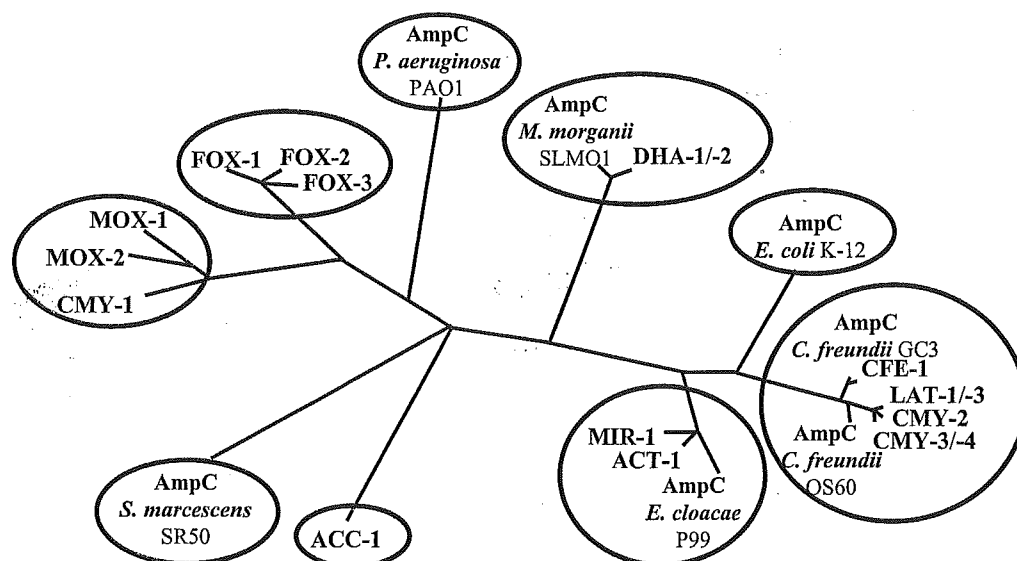


FIG. 1. Dendrogram for chromosomal and plasmid-encoded AmpC β-lactamases calculated by the Clustal V program by using the neighbor-joining method (38).

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1. Clinical strain *E. coli* KU6400 was isolated at a hospital in Japan in 1997 and was found to contain plasmid pKU601. *E. coli* K-12 ML4947 (AmpD wild type) and ML4953 (AmpD mutant) strains were used as recipients of the plasmid (24, 27). Plasmid pHSG398 is a vector plasmid that confers resistance to chloramphenicol.

Antibacterial agents. Reference samples of the antibacterial agents listed below were used in this study and were provided as powders of known potencies by the respective manufacturers. Piperacillin (Toyama Chemical, Toyama, Japan) was used as a representative penicillin, while cephalothin (Shionogi, Osaka, Japan), cefpodoxime (Sankyo, Tokyo, Japan), cefmetazole (Sankyo), cefotaxime (Nippon Hoechst Marion Roussel, Tokyo, Japan), and cefepime (Bristol-Myers Squibb, Tokyo, Japan) were used as representative cepheims. Other β-lactam agents, including imipenem (Banyu Pharmaceutical, Tokyo, Japan) as well as chloramphenicol (Sankyo) and rifampin (Sigma Chemical, St. Louis, Mo.), were also used. Clavulanic acid (SmithKline Beecham Pharmaceuticals, Tokyo, Japan) was used as a β-lactamase inhibitor.

Drug susceptibility assay. The susceptibility profiles were determined by the agar dilution method with sensitivity disk agar (Eiken Chemical, Tokyo, Japan) according to the guidelines of NCCLS (29).

Transconjugation. Conjugation was carried out by a broth method as described previously (20). Exponential-phase Luria broth cultures of donor strain KU6400 and recipient strain ML4947 were mixed at a ratio of 1:10 (by volume).

This mating mixture was incubated for 2 h at 35°C. The transconjugants were selected on sensitivity disk agar containing rifampin at 64 μg/ml and cefpodoxime at 4 μg/ml.

Assay for β-lactamase. Crude extraction of AmpC β-lactamase was performed as described previously (32). Cells were harvested by centrifugation (1,700 × g, 10 min), resuspended in 3 ml of 50 mM potassium phosphate buffer (pH 7.0), and sonicated. After centrifugation at 14,000 × g for 10 min at 4°C, β-lactamase activity was measured by determination of the protein content of the extract, and the protein contents of the cultures were compared. β-Lactamase activity was determined by spectrophotometry (UV2000; Shimadzu, Tokyo, Japan) at 30°C in 50 mM phosphate buffer (pH 7.0). Protein content was determined by a protein assay (Bio-Rad Laboratories, Hercules, Calif.) (10). One unit of β-lactamase activity was defined as the amount of β-lactamase that hydrolyzed 1 μmol of cephalothin in 1 min at 30°C. Cefoxitin (10 μg/ml) was used as the inducer. Induction was allowed to proceed for 60 min (4).

Cloning of the ampC and ampR genes. DNA extraction, restriction enzyme digestion, recombinant DNA manipulation, and transformations of plasmid DNA were performed as described by Sambrook et al. (40). Restriction enzymes and T4 DNA ligase were purchased from Takara Shuzo (Kyoto, Japan) and Nippon Gene (Tokyo, Japan), respectively. Plasmid pKU601 DNA was isolated from *E. coli* ML4947(pKU601) by the alkaline lysis method (8). The DNA was digested with BamHI and BglII and ligated into the BamHI site of pHSG398. The recombinant plasmid was designated pKU611 and was introduced into *E. coli* ML4947 by electroporation with a gene pulse controller unit (Bio-Rad

TABLE 1. Bacterial strains and plasmids used in this study

Bacterial strain or plasmid	Characteristics ^a
Bacterial strains	
<i>E. coli</i>	
ML4947.....	F ⁻ <i>galk2 galT22 hsdR hsdM lacY1 metB1 relA supE44 Rif^r</i> (AmpD wild type)
ML4953.....	F ⁻ <i>galk2 galT22 hsdR hsdM lacY1 metB1 relA supE44 Rif^r ampD9</i> (AmpD mutant)
KU6400.....	Clinical isolate carrying pKU601
<i>C. freundii</i> GC3.....	Clinical isolate from Japan which produces AmpC β-lactamase
Plasmids	
pKU601.....	Conjugative plasmid encoding CFE-1 isolated from KU6400
pKU611.....	14-kb fragment containing <i>bla</i> _{CFE-1} gene and <i>ampR</i> gene from pKU601 cloned into pHSG398
pKU612.....	Recombinant plasmid containing <i>ampC</i> gene of pKU611 in pHSG398
pHSG398.....	Cloning vector, purchased from Nippon Gene (Tokyo); Chl ^r

^a Characteristics include designation of markers, source, or derivation. Rif, rifampin; Chl, chloramphenicol.

TABLE 2. MICs of selected antibiotics for *E. coli* strains

Strain	MIC ($\mu\text{g/ml}$) ^a									
	PIP	CPD	CEF	CTX	CTX-CLA ^b	CAZ	CMZ	ATM	FEP	IPM
KU6400	>256	>256	>256	64	64	64	64	8	0.25	0.25
ML4947(pKU601)	>256	>256	>256	256	256	>256	256	64	1	0.5
ML4953(pKU601)	>256	>256	>256	256	256	>256	256	64	1	1
ML4947(pKU611)	64	>256	>256	16	16	8	16	2	0.25	0.25
ML4947(pKU612)	8	32	256	4	2	4	2	0.5	<0.063	0.25
ML4947	2	0.5	8	<0.063	<0.063	0.25	0.5	<0.063	<0.063	<0.063

^a Antibiotics: PIP, piperacillin; CEF, cephalothin; CPD, cefpodoxime; CTX, cefotaxime; CLA, clavulanic acid; CAZ, ceftazidime; CMZ, cefmetazole; ATM, aztreonam; FEP, cefepime; IPM, imipenem.

^b MICs were determined in the presence of clavulanic acid (5 $\mu\text{g/ml}$).

Laboratories). Transformants (containing *ampC* and *ampR*) were selected on the basis of resistance to cefpodoxime (4 $\mu\text{g/ml}$) and chloramphenicol (25 $\mu\text{g/ml}$) after overnight incubation at 37°C and were further characterized by analysis of their antibiotic susceptibility patterns. The size of the insert in the plasmid was estimated by restriction enzyme digestion and electrophoresis in 1.2% agarose gels.

To construct a plasmid containing only the *ampC* gene, plasmid pKU612, pKU611 was digested with SacI and ligated into the SacI site of pHSG398. The resulting plasmid was used to transform *E. coli* ML4947, and the plasmid from which the fragment containing the *ampR* sequence was deleted was identified from the plasmid DNA size and by DNA sequencing.

DNA sequencing and sequence comparisons. Sequencing of both strands of DNA was carried out as described by Sanger et al. (41) with a BigDye terminator cycle sequencing kit and an ABI 310 DNA sequencer (Applied Biosystems, Foster City, Calif.). Sequence analysis and comparison with other known sequences were performed with the BLAST and FAST programs at the National Center for Biotechnology Information.

Nucleotide sequence accession number. The nucleotide sequence data presented in this report appear in the DDBJ, EMBL, and GenBank nucleotide databases under accession number AB107899.

RESULTS

Susceptibilities to antibiotics. The MICs of selected β -lactam antibiotics for clinical isolate *E. coli* KU6400 and the transconjugants that acquired pKU601 by conjugation at a frequency of 10^{-5} are given in Table 2. KU6400 was highly resistant to piperacillin, cefotaxime, the combination of cefotaxime and clavulanic acid, aztreonam, and all cephalosporins except cefepime. The MICs of a variety of different cephalosporins were increased for the transconjugants.

Cloning of the *ampC* and *ampR* genes and gene expression in the *E. coli* recipient. We selected seven *E. coli* transformants resistant to β -lactams in a manner similar to that of *E. coli* ML4947 that acquired pKU601 by conjugation (Table 1). These transformants were resistant to cefpodoxime, cefotaxime, and chloramphenicol.

All transformants were found to produce an AmpC β -lactamase; they harbored a recombinant plasmid (pKU611) with an insert of 14 kb from pKU601. *E. coli* isolates harboring pKU611 showed a resistance profile similar to that of pKU601 (Table 2). Addition of clavulanic acid did not modify the resistance pattern in any transformant.

Characterization of the *bla*_{CFE-1} gene. Both strands of the entire 14-kb insert from recombinant plasmid pKU611 were sequenced. Analysis of this insert for coding regions revealed two open reading frames (ORFs) (Fig. 2). The first consisted of 1,137 bp encoding a putative protein of 378 amino acids (Fig. 3). This ORF had an ATG start codon at position 1008 and a stop codon at position 2151. Database searches with this ORF

identified similarities with several chromosome- and plasmid-encoded AmpC β -lactamases, particularly the chromosome-encoded AmpC β -lactamase of *C. freundii* GC3 (99.8% sequence identity) (17). The deduced amino acid sequence carried catalytic residues S-X-X-K, with the initial serine at position 64 (which is typical of AmpC β -lactamases); the motif Y-S-N at position 150; and the K-T-G motif at position 315.

The second ORF, which contained 876 nucleotides was transcribed in the opposite orientation and was located in the 5' direction from the *ampC* structural gene. It began with an ATG start codon at nucleotide 876 and had a stop codon at nucleotide 3. By analogy with the *ampC* and *ampR* genes of the AmpC β -lactamase, this ORF may correspond to the regulatory gene *ampR*. A sequence corresponding to transcriptional regulators of the LysR family, particularly the AmpR proteins of the family *Enterobacteriaceae*, was deduced. The DNA sequence of the corresponding gene from *C. freundii* GC3 was 99.0% identical to the sequence of this ORF. The deduced protein sequence showed only one difference, at position 135 (Ala for Asp), compared to the sequence of *C. freundii* GC3.

The 131-bp region between the *ampR* and *ampC* start codons contained overlapping putative promoters. This region was 97% identical to the corresponding region of *C. freundii* GC3.

Sequences surrounding *ampR* and *ampC* regions. In addition to identifying the sequences in the regions surrounding the *ampR* and *ampC* sequences, sequence analysis indicated that the *frdABCD* operon of *C. freundii* (7) was located upstream from the *ampC* gene; and a part of the ORF contained the sequence for the outer membrane lipoprotein encoded by the *blc* gene of *C. freundii* (GenBank accession nos. D85910 and U21727), which was located immediately downstream of the *ampC* gene (Fig. 4). Furthermore, two IS26 elements were observed to surround the *ampR* and *ampC* genes and were directed in the same orientation, forming an IS26 composite transposon. One was inserted in the *frdA* gene (as detected by PCR), and another was inserted in the *blc* gene.

β -Lactamase activities. The β -lactamase activities encoded by the plasmids are shown in Table 3. *E. coli* ML4947 (pKU601) and ML4953(pKU601) produced large amounts of β -lactamase (10.9 and 14.4 U/mg of protein, respectively). When *E. coli* ML4953 (AmpD mutant) was used as the host, the β -lactamase activities encoded by pKU601 were slightly higher than those detected when *E. coli* ML4947 (AmpD wild type) was used as the host. The β -lactamase activities of these strains in the presence of ceftiofur were slightly increased com-

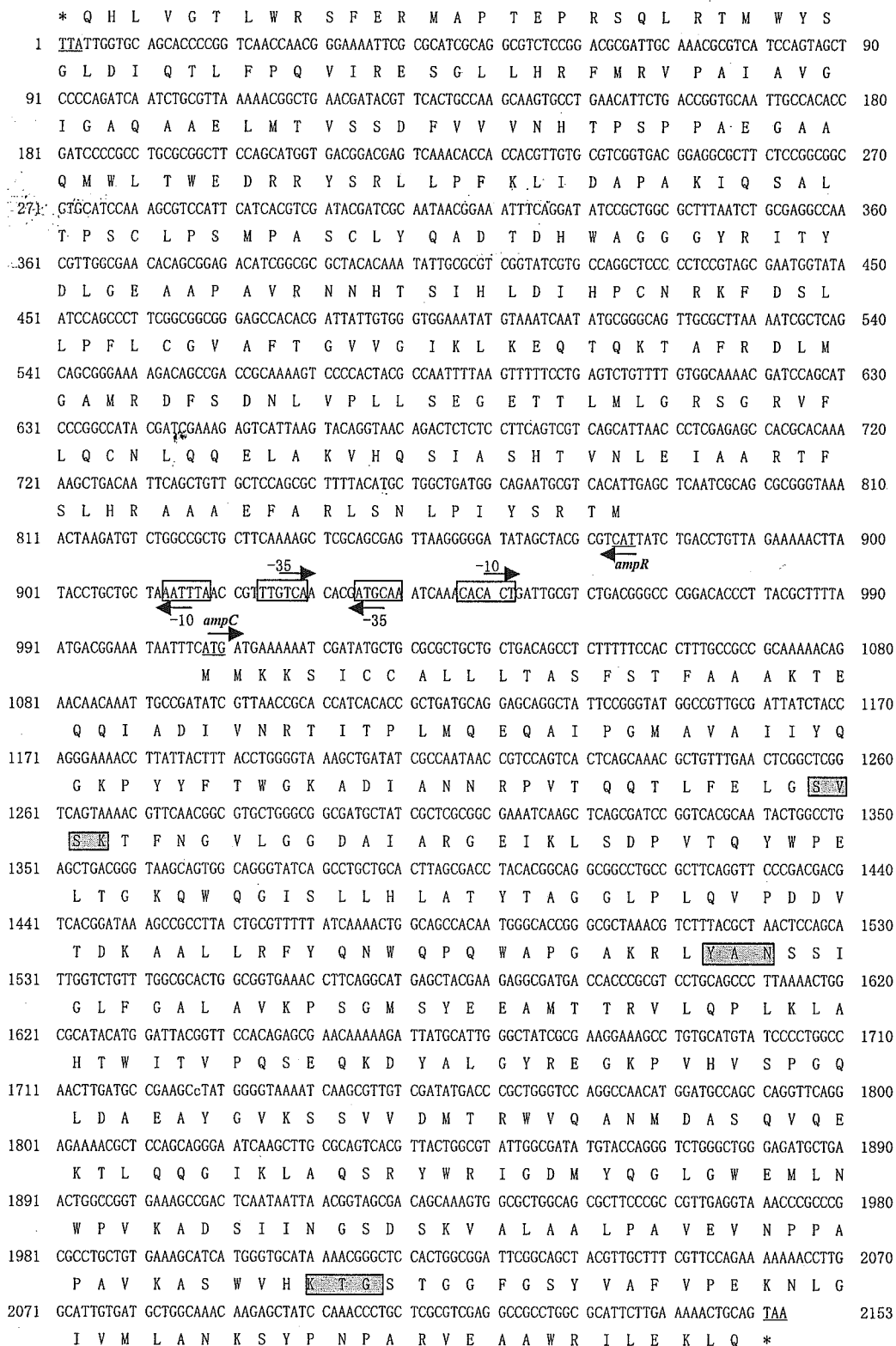


FIG. 2. Nucleotide sequence of the 2,153-bp fragment of pKU611 containing the *ampC*- and *ampR*-coding regions. Deduced amino acid sequences are designated in single-letter code. Putative promoter sequences are represented by the -35 and -10 regions (boxed). The start and stop codons of these genes are underlined. Additionally, conserved residues among class C β -lactamases are shown in shaded boxes.

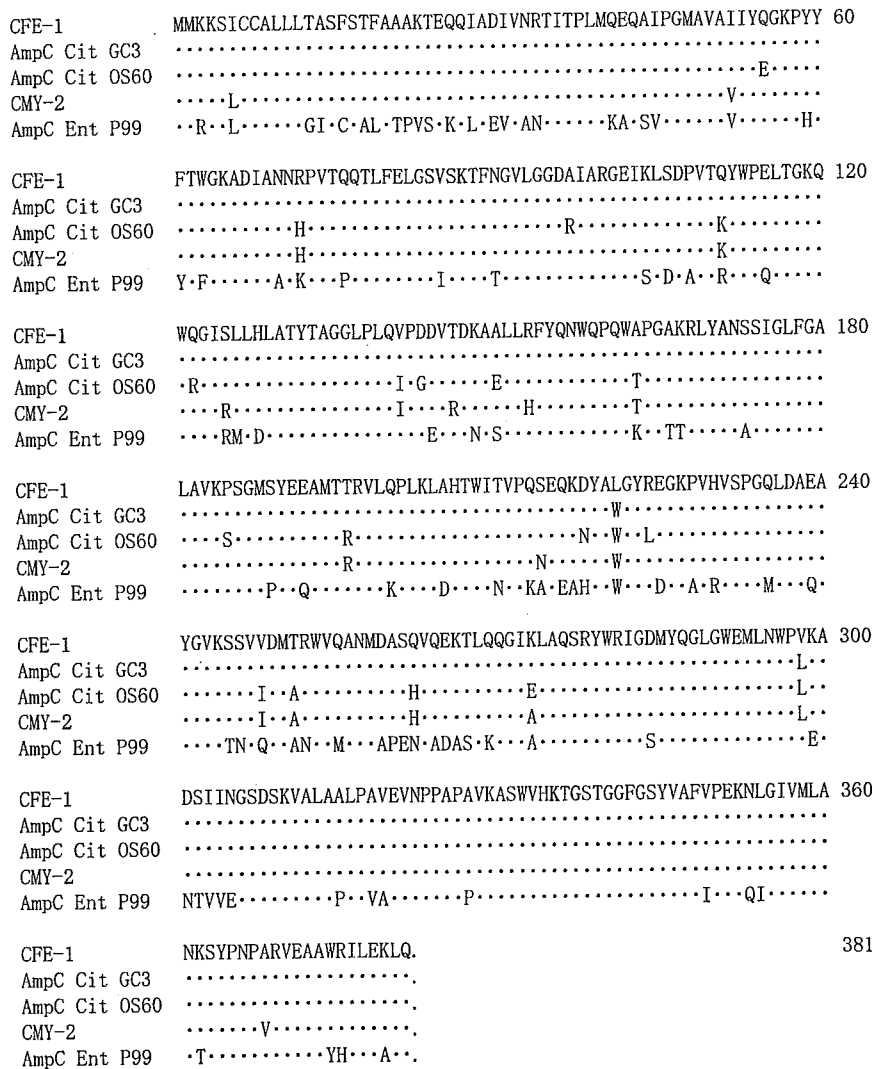


FIG. 3. Derived amino acid sequence of the CFE-1 β-lactamase compared to the sequences of other selected class C β-lactamases. The alignments of the deduced amino acid sequence of the CFE-1 β-lactamase with the AmpC β-lactamases of *C. freundii* GC3 (AmpC Cit GC3), *C. freundii* OS60 (AmpC Cit OS60), and *E. cloacae* P99 (AmpC Ent P99) and with the CMY-2 β-lactamase are shown. Dots indicate identical amino acids at that residue.

pared with the basal level, but the increases were not significantly different. This suggests that the *bla*_{CFE-1} gene may produce the enzyme constitutively.

The activity of CFE-1 was not inhibited by clavulanic acid, a characteristic confirming the close resemblance of CFE-1 to the AmpC β-lactamase, as mentioned in the description of the nucleotide sequence.

pKU611, which encoded the *ampR* and *ampC* genes, and recombinant plasmid pKU612, which encoded only the *ampC* gene (i.e., it lacked the *ampR* gene), were introduced into *E. coli* ML4947; and the β-lactamase activities of each construct were analyzed (Table 3). The specific enzyme activity of *E. coli* ML4947(pKU611) was 1.4 U/mg of protein, while that of *E. coli* ML4947(pKU612) was 0.2 U/mg of protein, a markedly lower level of expression.

DISCUSSION

In addition to previous reports concerning the MOX-1 and CMY-9 plasmid-encoded AmpC β-lactamases, which were detected in an *E. coli* clinical isolate in Japan (12, 19), in the present study we characterized a novel AmpC β-lactamase gene, *bla*_{CFE-1}, in a Japanese *E. coli* clinical isolate. This is the first report in East Asia of a plasmid-encoded AmpC β-lactamase, CFE-1, carrying an *ampR* gene derived from the *C. freundii* chromosome.

Our findings depict the organization of sequences surrounding the *ampR-ampC* region, including *bla*_{CFE-1}, which is seen in various enterobacterial species. Most plasmid-encoded AmpC β-lactamases, like CMY-2, CMY-4, and LAT-1, lack the *ampR* gene. *Citrobacter* spp. and *Enterobacter* spp. possess *ampR* and *ampC* genes, the fumarate operon *frdABCD* immediately

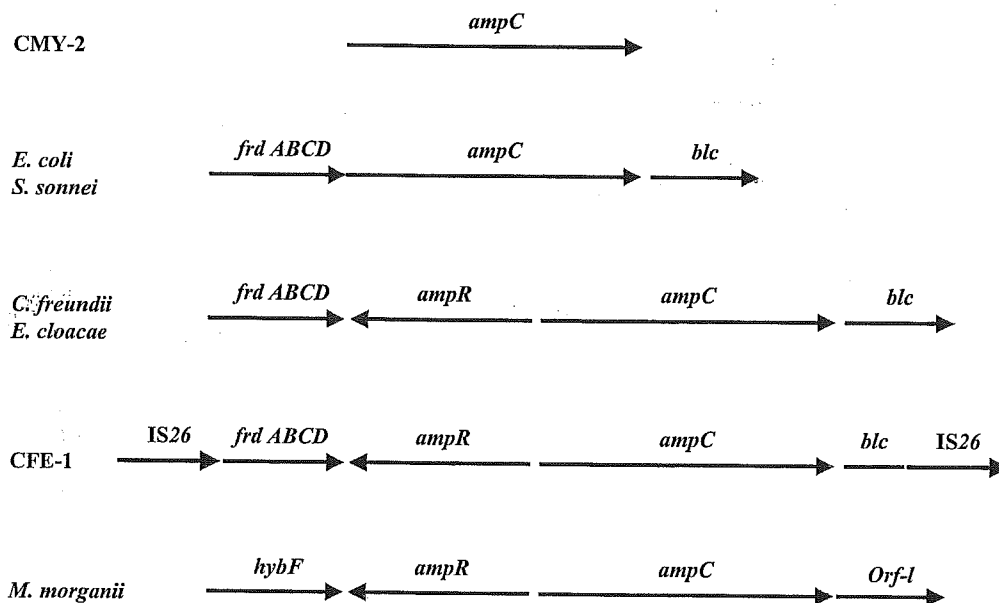


FIG. 4. Organization of the sequences surrounding *ampC* in various enterobacterial species. The positions of the fumarate operon (*frdABCD*), *blc*, *hybF*, *orf-1*, *IS26*, *ampC*, and *ampR* genes are shown, with directions indicated by arrows.

downstream of the *ampR* gene, and also outer membrane lipoprotein *blc* immediately downstream of the *ampC* gene (7, 9). In contrast, *M. morgani* possesses the *ampR* and *ampC* genes but not the fumarate operon (36); *hybF* is substituted for the fumarate operon upstream from the *ampC* gene (Fig. 4).

Analysis of the *bla*_{CFE-1} gene revealed that it has a very close relationship to the chromosomal gene that encodes the AmpC β -lactamase in *C. freundii* (1) (Table 4). The amino acid sequence of CFE-1 showed 99.5% identity with that of *C. freundii* GC3 isolated from clinical specimens in Japan (17), differing only at position 221 (Leu for Trp) and position 298 (Val for Leu) (Fig. 3). This identity was greater than that with *C. freundii* OS60 (95.0%) (26). The amino acid sequence of AmpR showed 99.0% identity with that of *C. freundii* GC3, differing only at position 135 (Ala for Asp). This similarity strongly suggests that the *bla*_{CFE-1} gene is derived from the chromosomal *ampC* gene of *C. freundii* GC3 (1). This hypothesis is supported by the finding that *bla*_{CFE-1} has both the *ampR* gene and the *ampC* gene, as well as the *frdABCD* gene operon and the *blc* gene, which were found to surround the *ampR* and *ampC* genes, as in *C. freundii*.

In addition, two *IS26* elements were detected on plasmid pKU601; one was located immediately upstream from the *frdA* gene, while the other was located immediately downstream of the *ampC* gene and was inserted in the *blc* gene (Fig. 4). These were directed in the same orientation as that seen for the *IS26* composite transposon (25). Some plasmid-encoded β -lactamase genes form part of transposons frequently flanked by insertion sequence elements, such as *IS26* (SHV-2a and ACC-1) (22, 28) and *ISEcp1* (CTX-M5) (39). These results present direct evidence that the *bla*_{CFE-1} gene translocated to a plasmid from the chromosome of *C. freundii* strain GC3 by using the *IS26* function(s). Further studies are continuing to determine whether the *bla*_{CFE-1} gene is capable of translocation.

As shown in Tables 2 and 3, an *E. coli* strain harboring pKU601 encoding *bla*_{CFE-1} expressed β -lactamase constitutively in the presence or absence of a β -lactam inducer and in the AmpD wild type (ML4947) or AmpD mutant (ML4953). *E. coli* ML4947(pKU612), which lacks the *ampR* gene, showed a decrease in β -lactamase activity compared with that of *E. coli* ML4947(pKU611); nevertheless, the AmpC β -lactamases of *C. freundii*, *E. cloacae*, and *M. morgani* with an *ampR* deletion showed increased levels of β -lactamase expression. This result

TABLE 3. β -Lactamase activities of *E. coli* strains

Strain	Relative β -lactamase activity (U/mg of protein) ^a	
	Noninduced	Induced ^b
KU6400	7.7	7.9
ML4947(pKU601)	10.9	11.1
ML4953(pKU601)	14.4	16.3
ML4947(pKU611)	1.4	1.7
ML4947(pKU612)	0.2	ND ^c

^a β -Lactamase activities are the geometric mean determinations for three independent cultures. The standard deviations were within 10%.

^b Cefoxitin (10 μ g/ml); was used as the inducer.

^c ND, not done.

TABLE 4. Identity of the CFE-1 amino acid sequence to those of other AmpC β -lactamases

β -Lactamase	% Identity with:				
	CFE-1	GC3	OS60	CMY-2	P99
CFE-1	100	99.5	95.0	95.3	74.5
<i>C. freundii</i> GC3 AmpC		100	95.5	95.8	74.5
<i>C. freundii</i> OS60 AmpC			100	95.8	73.2
CMY-2				100	75.1
<i>E. cloacae</i> P99 AmpC					100

indicates that AmpR of pKU601 seems to function as regulator of the constitutive expression of *bla*_{CFE-1}. Bartowsky and Normark (3, 4) have reported that the activation of *ampC* transcription in *C. freundii* is dependent on the conversion of AmpR into a transcriptional activator. The AmpR mutants of *C. freundii*, which have Glu instead of Gly at position 102 or Tyr instead of Asp at position 135, express β -lactamase at high levels. Increased levels of β -lactamase expression have been reported (24) when the Arg-86 and Asp-135 mutations are present in *E. cloacae* AmpR. In the *ampR* gene of pKU601, the amino acid at position 135 was Ala, whereas it was Asp in the wild type. These results may indicate that overexpression of β -lactamase is dependent on mutation of the *ampR* gene.

In summary, *E. coli* plasmid pKU601 was characterized as harboring a novel plasmid-encoded AmpC β -lactamase, CFE-1, with an *ampR* gene. The high level of constitutive CFE-1 expression in *E. coli* is presumably caused by the mutation in the *ampR* gene, in which the Asp at position 135 is changed to Ala. These results indicate the dissemination of a resistance gene to different enterobacterial species through mobilization of a plasmid and transposable event-mediated events.

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