TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics[a]	Source or reference
Strains		
E. cloacae		
GN7471	Clinical isolate from Japan, resistance to cephaloridine and cefotiam	30
KU3261	Clinical isolate from Japan	This study
KU3262	Clinical isolate from Japan	This study
KU3263	Clinical isolate from Japan	This study
ATCC 13047	Purchased from ATCC; type strain, RF4738	
E. coli		
ML4947	F⁻ *galK2 galT22 hsdR hsdM lacY1 metB1 relA supE*44 RIF^r	13
ML4953	F⁻ *ampD9 galK2 galT22 hsdR hsdM lacY1 metB1 relA supE*44 RIF^r	*ampD9* is *deb9* (27)
Plasmids		
pMS161	8-kb *Eco*RI fragment containing *ampC* and *ampR* from GN7471 cloned into pACYC184	This study
pKU403	6-kb *Sal*I fragment containing *ampC* and *ampR* from pMS161 cloned into pMW218	This study
pKU404	Mutant from pKU403 selected with aztreonam	This study
pKU405	Mutant from pKU403 selected with ceftazidime	This study
pKU406	Mutant from pKU403 selected with aztreonam	This study
pKU407	Mutant from pKU403 selected with ceftazidime	This study
pKU408	Prepared by deleting 1.7-kb *Sph*I fragment containing *ampR* from pKU403	This study
pKU409	Prepared by deleting 1.7-kb *Sph*I fragment containing *ampR* from pKU404	This study
pKU410	Prepared by deleting 1.7-kb *Sph*I fragment containing *ampR* from pKU405	This study
pKU414	Prepared by deleting 1.7-kb *Sph*I fragment containing *ampR* from pKU406	This study
pKU411	Prepared by deleting 4-kb *Bam*HI-*Sca*I fragment containing *ampC* from pKU403	This study
pKU412	Prepared by deleting 4-kb *Bam*HI-*Sca*I fragment containing *ampC* from pKU404	This study
pKU413	Prepared by deleting 4-kb *Bam*HI-*Sca*I fragment containing *ampC* from pKU405	This study
pKU415	Prepared by deleting 4-kb *Bam*HI-*Sca*I fragment containing *ampC* from pKU406	This study
pACYC184	Cloning vector, purchased from Nippon Gene (Tokyo, Japan); CP^r TC^r	5
pMW218	Cloning vector, purchased from Nippon Gene (Tokyo, Japan); KM^r	3

[a] RIF, rifampin; CP, chloramphenicol; TC, tetracyclin; KM, kanamycin; r, resistance; ATCC, American Type Culture Collection.

pACYC184 and were used to transform *E. coli* ML4947. Transformants with a plasmid carrying the *E. cloacae* genomic 8-kb fragment (containing *ampC* and *ampR*) were selected for increased resistance to cephaloridine. This hybrid plasmid (12 kb) was designated pMS161 (Fig. 1). pMS161 was digested with *Sal*I and was ligated into the *Sal*I site of pMW218. The resulting plasmid was used to transform *E. coli* ML4947. Transformants harboring this plasmid, which had a 6-kb *Sal*I fragment carrying *ampC* and *ampR*, were selected with kanamycin by using the plasmid marker. This hybrid plasmid (9.9 kb) was renamed pKU403.

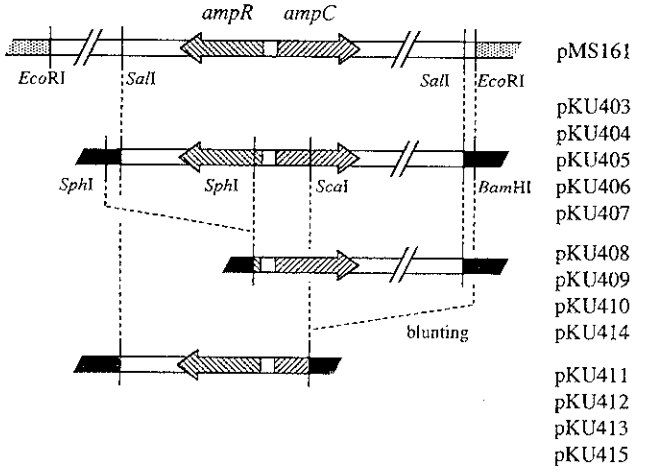

FIG. 1. Cloning strategy for *E. cloacae* GN7471 *ampC* and *ampR*. pMS161 was used to clone an 8-kb *Eco*RI fragment containing *ampC* and *ampR* from *E. cloacae* GN7471 into pACYC184. pKU403 was constructed by cloning a *Sal*I fragment of pMS161 into pMW218. pKU404, pKU405, pKU406, and pKU407 were mutated plasmids derived from pKU403. pKU408, pKU409, pKU410, and pKU414 were self-ligated at the *Sph*I site of pKU403, pKU404, pKU405, and pKU406, respectively. pKU411, pKU412, pKU413, and pKU415 were also self-ligated at the *Bam*HI-*Sca*I site of pKU403, pKU404, pKU405, and pKU406, respectively. Symbols: ▨, pACYC184; ■, pMW218.

To construct plasmids containing only the *ampC* gene, pKU403, pKU404, pKU405, or pKU406 was digested with *Sph*I and was then self-ligated. These plasmids were used to transform *E. coli* ML4947, and plasmids with deletion of the 1.7-kb *Sph*I fragment containing *ampR* were identified from the result of the plasmid DNA size obtained after digestion with *Sal*I (8.7 kb) and by PCR. PCR primers AR3 and AR4, directed against the sequence for an *ampR* gene (GenBank accession no. AB016612), amplified a fragment of 743 bp. Forward primer AR3 (5′-CCGCCAGACACCTCAGTTTT-3′) is located between nucleotides 127 and 146 on the sequence, while reverse primer AR4 (5′-GTAACTCCCCA GGTCAATCC-3′) is located between nucleotides 869 and 850. The plasmids derived from pKU403, pKU404, pKU405, and pKU406 were named pKU408, pKU409, pKU410, and pKU414, respectively (Fig. 1). Similarly, to construct plasmids containing only the *ampR* gene, pKU403, pKU404, pKU405, and pKU406 were digested with *Bam*HI-*Sca*I and were blunt ended at the *Bam*HI site. The blunt end of the *Bam*HI site was obtained with a DNA blunting kit (Takara shuzo) (37). After ligation, these plasmids were used to transform *E. coli* ML4947. Plasmids with deletion of the 4-kb *Bam*HI-*Sca*I fragment containing *ampC*, which therefore carried only *ampR*, were identified from the plasmid DNA size obtained after digestion with *Sal*I (5.9 kb) and by PCR. The PCR primers AC1 and AC2, directed against the sequence for an *ampC* gene (GenBank accession no. AB016611), amplified a fragment of 333 bp. Forward primer AC2 (5′-TTATCAGGGTCAGCCGCACT-3′) is located between nucleotides 262 and 281 on the sequence, while reverse primer AC1 (5′-GGTTTCCACTG CGGTTGCCA-3′) is located between nucleotides 594 and 575. The plasmids derived from pKU403, pKU404, pKU405, and pKU406 were named pKU411, pKU412, pKU413, and pKU415, respectively (Fig. 1).

Assay of β-lactamase activity. β-Lactamase activity was detected as described previously (34). Briefly, imipenem (a carbapenem), a β-lactamase inducer, was added to mid-logarithmic-phase cultures and the cells were incubated for another 2 h. Imipenem was added at several concentrations (1/4× the MIC of imipenem) so that the cell protein concentration was not less than 75% compared with that for the controls. Cell lysis was negligible under these conditions, allowing enzyme activity to be assessed. The 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, the β-lactamase activity and the protein concentration in the extract were measured and were compared between cultures. 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.

Isolation of ceftazidime- or aztreonam-resistant mutants. Mutants with elevated levels of resistance to ceftazidime or aztreonam were obtained by plating about 10^9 CFU/ml of washed late-logarithmic-phase ML4953/pKU403 grown in L broth on agar plates containing ceftazidime or aztreonam at 4× to 16× the MIC.

DNA sequencing. Analysis of the *ampC* and *ampR* sequences of pKU403 was performed as described by Sanger et al. (39). The DNA sequences of the *ampR* genes carried by pKU404, pKU405, pKU406, and pKU407 were determined with an ALFred DNA sequencer (Amersham Pharmacia Biotech) and the Thermo Sequenase fluorescence-labeled primer sequencing kit (Amersham Pharmacia Biotech). Sequencing primers were obtained from Amersham Pharmacia Biotech. The sequencing primers for the *ampR* gene, CY5AP4, CY5AR1 CY5AR2, and CY5AR3, were designed from the sequence of *ampR* (GenBank accession no. AB016612). Forward primers CY5AR1 (5′-CCCAGGAGAAGCTAAAAG TGG-3′) and CY5AR3 (5′-GATGGTCTTTGATTCGTCCGTG-3′) are located at nucleotides 352 to 372 and nucleotides 722 to 743, respectively, on the sequence, while reverse primers CY5AP4 (5′-TGCGTAAAACTGAGGTGTCTG GCG-3′) and CY5AR2 (5′-TAGGAGCGCAGCAGGGTAAACT-3′) are located at nucleotides 151 to 128 and 652 to 631, respectively.

Nucleotide sequence accession numbers. The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL, and GenBank nucleotide sequence databases with the accession nos. AB016611 (*ampC*) and AB016612 (*ampR*).

RESULTS

Base sequences of *ampC* and *ampR* from *E. cloacae* GN7471. The 8-kb DNA fragment from an *Eco*RI digest of *E. cloacae* GN7471 containing the *ampC* and *ampR* genes was introduced into the *Eco*RI site of pACYC184. The resulting plasmid, designated pMS161, was digested with *Sal*I and was ligated into pMW218 at the *Sal*I site, and the plasmid thus obtained was named pKU403. In this case, the DNA fragment was 6 kb in length. Next, the base sequence of pKU403 was determined. The degree of identity of *ampC* between *E. cloacae* GN7471 and *E. cloacae* MHN1 was 81.8%, while that between *E. cloacae* GN7471 and *E. cloacae* P99 was 81.8%. However, the degree of identity of *ampC* between *E. cloacae* P99 and MHN1 was 98.3% (10). In contrast, the degree of identity of *ampR* between DNA derived from *E. cloacae* GN7471 and that derived from *E. cloacae* MHN1 was 99.3% (12).

Isolation of mutants and MICs. For isolation of mutants and determination of MICs, *E. coli* ML4953, which carried an AmpD mutant background, was used in order to avoid selecting only an AmpD mutant. Mutant strains were isolated from ML4953/pKU403 by selection with ceftazidime or aztreonam. When selection was performed with ceftazidime at 4× to 8× the MIC (2 to 4 μg/ml), mutants were obtained at a frequency of 6.2×10^{-6} to 2.0×10^{-6}. With selection at 16× the MIC (8 μg/ml), mutants were obtained at a frequency of 3.4×10^{-8}. When selection was performed with aztreonam at 4× to 8× the MIC (2 to 4 μg/ml), mutants were obtained at a frequency of 6.4×10^{-6} to 1.4×10^{-6}, while the frequency of occurrence of mutants was 3.8×10^{-9} at 16× the MIC (8 μg/ml).

Among the 60 mutants thus obtained, a group of strains for which ceftazidime and aztreonam MICs were similarly high was chosen. Four strains were selected from this group at random. After DNA extraction and transformation of *E. coli* ML4953 (AmpD mutant), all four of these strains were confirmed to carry mutant plasmids, because a high-level cephalosporin resistance phenotype was transferred to ML4953. The mutant plasmids derived from selection with aztreonam at 8× the MIC were named pKU404 and pKU406, and those derived from selection with ceftazidime at 8× the MIC were named pKU405 and pKU407. All four plasmids were used to transform *E. coli* ML4947 (AmpD wild type) and ML4953 (AmpD mutant), and the MICs for each transformant were determined (Table 2).

When *E. coli* ML4947 (AmpD wild type) was used as the host, the MIC of ampicillin for transformants carrying pKU404, pKU405, pKU406, or pKU407 was increased 16-fold or more compared with that for ML4947 carrying pKU403. Also, the MICs of cephems (cefotiam, latamoxef, ceftazidime, and cefotaxime) and aztreonam were increased 16-fold or more compared with those for ML4947 carrying pKU403. In addition, the MIC of cefepime increased twofold or more, and that of imipenem rose two- to fourfold. The MICs obtained when *E. coli* ML4953 (AmpD mutant) was the host were similar to those obtained when *E. coli* ML4947 was the host, and the MICs of ampicillin, cefotiam, latamoxef, and imipenem did not vary greatly.

On the other hand, the MICs for transformants carrying Δ*ampC* or Δ*ampR* plasmids were almost the same as the MICs for the parental strains.

β-Lactamase activities of mutants. The β-lactamase activities encoded by the plasmids are shown in Table 3. When *E. coli* ML4947 (AmpD wild type) was the host cell, the β-lactamase activities encoded by pKU404, pKU405, pKU406, and pKU407 were about 470, 75, 160, and 180 times higher, respectively, than the activity encoded by the original plasmid (pKU403). However, the activity of the β-lactamase encoded by pKU403 increased about 50-fold when it was induced by imipenem, whereas the β-lactamase activity encoded by pKU405, pKU406, and pKU407 rose only three- to fivefold. The β-lactamase activity encoded by pKU404 was not induced by imipenem.

When *E. coli* ML4953 (AmpD mutant) was used as the host, the β-lactamase activities encoded by pKU403, pKU404, pKU405, pKU406, and pKU407 were much higher compared with those when *E. coli* ML4947 was the host. Induction with imipenem resulted in an eightfold increase for pKU403 and a twofold increase for pKU405. However, no increase in activity was observed for pKU404, pKU406, or pKU407. These results indicated that pKU406 and pKU407 encoded similar levels of β-lactamase activity and had activities intermediate between those of pKU404, which encoded a high level of enzyme activity, and pKU405, which encoded a low level of enzyme activity. Hence, pKU404, pKU405, and pKU406 (which encoded different levels of enzyme activity) were used in subsequent experiments.

The specific enzyme activities encoded by Δ*ampR* plasmids (pKU408, pKU409, pKU410, and pKU414) in *E. coli* ML4947 (AmpD wild type) and ML4953 (AmpD mutant) were 0.06 to 0.16 U/mg of protein and were two to four times higher than the β-lactamase activity encoded by ML4953/pKU403 but were markedly lower than the activities encoded by pKU404, pKU405, and pKU406. Similarly, since Δ*ampC* plasmids (pKU411, pKU412, pKU413, and pKU415) lacked the structural gene for β-lactamase, their enzyme activities were always less than 0.02 U/mg of protein and did not differ from the activities of the host cells.

Amino acid sequence of AmpR. Figure 2 shows the AmpR amino acid sequences encoded by pKU403, pKU404, pKU405, and pKU406 derived from *E. cloacae* GN7471, as well as those from the AmpR form of *E. cloacae* MHN1 and *C. freundii* OS60 (13, 25). G-538 in the base sequence of pKU403 was converted to A in pKU404, resulting in the replacement of Asp-135 by Asn. A-539 in the base sequence of pKU403 was converted to T in pKU405, and Asp-135 was replaced by Val. C-256 in the base sequence of pKU403 was converted to T in both pKU406 and pKU407, with Arg-86 being replaced by Cys.

Effect of mutant AmpR on chromosomal β-lactamase. In the experiment described above no difference in enzyme activity was found among Δ*ampR* plasmids, while the β-lactamase activities encoded by pKU404, pKU405, and pKU406 were sig-

TABLE 2. MICs for *E. coli* mutants

Host	Plasmid	Status[a]	MIC (μg/ml)[b]									
			ABPC	CER	CTM	LMOX	CTX	CAZ	CPDX	CFPM	AZT	IPM
ML4947 (AmpD wild type)	pKU403	Wild type	16	128	2	0.125	0.125	0.25	1	0.03	0.06	0.5
	pKU404	H-mut	>128	>128	>128	8	32	64	>128	0.25	32	1
	pKU405	L-mut	>128	>128	>128	4	16	32	>128	0.125	16	2
	pKU406	I-mut	>128	>128	>128	2	8	8	128	0.06	4	2
	pKU407	I-mut	>128	>128	>128	2	8	8	128	0.06	4	2
	pKU408	pKU403 Δ*ampR*	8	16	2	0.125	0.125	0.25	2	0.03	0.125	0.5
	pKU409	pKU404 Δ*ampR*	16	16	4	0.125	0.125	0.25	2	0.03	0.125	0.5
	pKU410	pKU405 Δ*ampR*	8	8	2	0.125	0.125	0.25	2	0.03	0.06	0.25
	pKU414	pKU406 Δ*ampR*	8	16	4	0.125	0.125	0.25	2	0.03	0.125	0.25
	pKU411	pKU403 Δ*ampR*	8	2	0.125	0.125	0.06	0.125	0.5	0.03	0.06	0.25
	pKU412	pKU404 Δ*ampC*	4	2	0.25	0.125	0.06	0.125	0.5	0.03	0.06	0.25
	pKU413	pKU405 Δ*ampC*	8	4	0.5	0.125	0.125	0.125	1	0.03	0.06	0.25
	pKU415	pKU406 Δ*ampC*	8	4	0.25	0.125	0.125	0.125	0.5	0.03	0.06	0.25
	pMW218	Vector	8	4	0.25	0.06	0.06	0.125	0.5	0.03	0.06	0.25
			8	2	0.25	0.125	0.06	0.125	0.5	0.03	0.06	0.25
ML4953 (AmpD mutant)	pKU403	Wild type	>128	>128	128	2	0.5	0.5	32	0.03	0.25	1
	pKU404	H-mut	>128	>128	>128	8	32	32	>128	0.25	16	1
	pKU405	L-mut	>128	>128	>128	4	16	16	>128	0.125	8	1
	pKU406	I-mut	>128	>128	>128	4	8	8	>128	0.06	8	1
	pKU407	I-mut	>128	>128	>128	4	8	8	>128	0.06	8	2
	pKU408	pKU403 Δ*ampR*	8	8	1	0.125	0.125	0.125	2	0.03	0.06	1
	pKU409	pKU404 Δ*ampR*	8	16	1	0.125	0.125	0.125	2	0.03	0.125	1
	pKU410	pKU405 Δ*ampR*	8	8	0.5	0.125	0.125	0.25	2	0.03	0.125	1
	pKU414	pKU406 Δ*ampR*	8	8	1	0.125	0.125	0.25	2	0.03	0.06	1
	pKU411	pKU403 Δ*ampC*	8	4	0.25	0.125	0.06	0.125	1	0.03	0.06	1
	pKU412	pKU404 Δ*ampC*	8	8	0.5	0.125	0.06	0.125	1	0.03	0.06	1
	pKU413	pKU405 Δ*ampC*	8	8	0.25	0.125	0.06	0.125	1	0.03	0.03	1
	pKU415	pKU406 Δ*ampC*	8	4	0.5	0.125	0.06	0.125	1	0.03	0.03	1
	pMW218	Vector	4	4	0.25	0.125	0.06	0.125	1	0.03	0.06	1
			4	4	0.25	0.125	0.06	0.25	1	0.03	0.06	1

[a] L-, I-, and H-mut, mutant plasmids with low, intermediate, and high levels of β-lactamase activity, respectively.
[b] ABPC, ampicillin; CER, cephaloridine; CTM, cefotiam; LMOX, latamoxef; CTX, cefotaxime; CAZ, ceftazidime; CPDX, cefpodoxime; CFPM, cefepime; AZT, aztreonam; IPM, imipenem.

nificantly higher than the activity of the pKU403-encoded β-lactamase. Therefore, the high level of enzyme activity encoded by the plasmids isolated in the present study appeared to be due to a mutation of *ampR*. To confirm this, Δ*ampC* plasmids (pKU411, pKU412, pKU413, and pKU415) were used to transform *E. cloacae* ATCC 13047 as well as clinical isolates of *E. cloacae* (KU3261, KU3262, and KU3263), and the effects of mutations in AmpR were examined. Table 4 shows that the β-lactamase activities of the pKU411 transformants were almost the same as those of the host strains, whereas the activities of the pKU412, pKU413, and pKU415 transformants were 20 to 350 times, 10 to 130 times, and 15 to 250 times higher, respectively.

DISCUSSION

The frequency of occurrence of mutants that stably express derepressed class C β-lactamase in subpopulations of resistant organisms and the widespread use of β-lactams in the hospital environment have resulted in the emergence of clinically important endemic bacterial resistance (9). The differences between individual inducible strains that cause infection remain unclear, but these organisms appear to carry mutations in either AmpD or AmpR.

Many gram-negative bacilli (e.g., *Enterobacter* spp., *C. freundii*, *Pseudomonas aeruginosa*, and *Serratia marcescens*) produce chromosomal class C β-lactamases. The *ampC* gene used in the present study was derived from a clinical isolate, *E. cloacae* GN7471, and showed about 80% identity with those reported in *E. cloacae* P99 and *E. cloacae* MHN1 (10). However, microbiological comparison of *E. cloacae* GN7471 and *E. cloacae* P99 has shown that they belong to the same species (18). That is, among bacterial strains assigned to the same species by microbiological methods, classification into close relatives may be possible when identification is done at the gene level.

The degree of identity of *ampR* between DNA derived from *E. cloacae* GN7471 and that derived from *E. cloacae* MHN1 was 99.3%. In contrast, the degree of identity of AmpR between DNA derived from *E. cloacae* GN7471 and *C. freundii* OS60 was only 73.0%. However, the AmpR amino acid sequences of Arg-86, Gly-102, and Asp-135 were conserved between *E. cloacae* GN7471, *E. cloacae* MHN1, and *C. freundii* OS60 (Fig. 2).

As shown in Table 2, MICs appeared to be inconsistent with β-lactamase activity (Table 3). In *E. coli* ML4947 (AmpD wild type), the β-lactamase activity of pKU404 was sixfold higher than that of pKU405. In the case of *E. coli* ML4953 (AmpD mutant), the enzyme activity of pKU404 was only 1.6-fold higher than that of pKU405. On the contrary, the β-lactamase activities encoded by Δ*ampR* plasmids (pKU408, pKU409, pKU410, and pKU414) in *E. coli* were markedly lower than the activities encoded by pKU404, pKU405, and pKU406. This result maybe indicates that β-lactamase was induced on a plate with drug and that the mutations in pKU404 and pKU405 are located in different sites of AmpR.

Two of the mutant plasmids obtained in the present study,

TABLE 3. β-Lactamase activity

Host	Plasmid	Status[b]	β-Lactamase activity (U/mg of protein)[c]	
			Not induced	Induced[a]
ML4947 (AmpD wild type)	pKU403	Wild type	0.04 ± 0.01	1.94 ± 0.84
	pKU404	H-mut	18.86 ± 3.10	25.94 ± 5.53
	pKU405	L-mut	2.99 ± 1.09	8.26 ± 0.68
	pKU406	I-mut	6.34 ± 1.01	25.95 ± 4.01
	pKU407	I-mut	7.10 ± 0.60	30.95 ± 7.66
	pKU408	pKU403 Δ*ampR*	0.14 ± 0.02	0.12 ± 0.01
	pKU409	pKU404 Δ*ampR*	0.13 ± 0.01	0.12 ± 0.01
	pKU410	pKU405 Δ*ampR*	0.13 ± 0.01	0.10 ± 0.01
	pKU414	pKU406 Δ*ampR*	0.13 ± 0.01	0.12 ± 0.01
	pKU411	pKU403 Δ*ampC*	0.01	0.01
	pKU412	pKU404 Δ*ampC*	0.01	0.01
	pKU413	pKU405 Δ*ampC*	0.01	0.01
	pKU415	pKU406 Δ*ampC*	0.01	0.01
	pMW218	Vector	0.01	0.01
			0.01	0.01
ML4953 (AmpD mutant)	pKU403	Wild type	4.89 ± 1.28	37.68 ± 9.53
	pKU404	H-mut	43.77 ± 5.88	41.54 ± 13.82
	pKU405	L-mut	27.72 ± 5.11	48.48 ± 7.52
	pKU406	I-mut	35.06 ± 4.46	41.39 ± 5.21
	pKU407	I-mut	31.99 ± 2.97	38.28 ± 9.74
	pKU408	pKU403 Δ*ampR*	0.11 ± 0.00	0.09 ± 0.01
	pKU409	pKU404 Δ*ampR*	0.09 ± 0.01	0.06 ± 0.01
	pKU410	pKU405 Δ*ampR*	0.11 ± 0.02	0.08 ± 0.01
	pKU414	pKU406 Δ*ampR*	0.11 ± 0.01	0.07 ± 0.01
	pKU411	pKU403 Δ*ampC*	0.01	0.01
	pKU412	pKU404 Δ*ampC*	0.02	0.02
	pKU413	pKU405 Δ*ampC*	0.02	0.02
	pKU415	pKU406 Δ*ampC*	0.01	0.01
	pMW218	Vector	0.01	0.01
			0.02	0.01

[a] One to 4× the MIC of imipenem was used for induction of β-lactamase.
[b] L-, I-, and H-mut, mutant plasmids with low, intermediate, and high levels of β-lactamase activity, respectively.
[c] Values are means ± standard deviations of three independent experiments. Standard deviations for pKU411, pKU412, pKU413, pKU415, pMW218, and the host were <±0.001. 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.

pKU404 and pKU405, had single-base mutations of *ampR*, resulting in mutation of Asp-135. pKU406 and pKU407 also had only a one-base mutation, with a consequent change in Arg-86. Bartowsky and colleagues (1, 33) have reported on the variability of AmpR from *C. freundii*, and they isolated AmpR with alterations of Ser-35, Tyr-264, Gly-102, and Asp-135 by using nitrosoguanidine mutagenesis and site-directed mutagenesis. In our study, a change of wild-type Asp-135 to Asn (pKU404) or Val (pKU405) resulted in 470-fold and 75-fold increases in basal levels of β-lactamase expression, respectively, while a change of wild-type Arg-86 to Cys (pKU406 and pKU407) resulted in 160-fold and 180-fold increases, respectively (Table 3).

As for the mutations of Asp-135 (pKU404 and pKU405) and Arg-86 (pKU406 and pKU407), these amino acids also appear to be important for *ampC* activation. A change of either the 86th or the 135th amino acid of AmpR affected the *ampC* promoter. In other words, these mutants were considerably more active than wild-type AmpR as transcriptional activators for the *ampC* promoter. These high levels of expression of β-lactamase were shown in the presence or absence of a β-lactam inducer and in the AmpD wild type (ML4947) or AmpD mutant (ML4953).

On the other hand, for about 3% of clinical isolates the cefotaxime and ceftazidime MICs were less than 0.125 μg/ml, and we could not detect any class C β-lactamase in clinical isolates of *E. cloacae* (data not shown). In this study, we selected three isolates of *E. cloacae* (KU3261, KU3262, and KU3263) with β-lactamase activities of 0.02, 0.03, and <0.02 U/mg of protein, respectively. As shown in Table 4, the activities of pKU412, pKU413, and pKU415 transformants (Δ*ampR* plasmids) were 20 to 350 times, 10 to 130 times, and 15 to 250 times higher, respectively.

In the present study, since mutant plasmids were selected by using AmpD mutant strains as the host cells, the resulting mutants may also have had mutations at sites other than *ampD*. On the other hand, the frequency of selection of AmpD mutants was about 10^{-5} in another study (27). The frequency of occurrence of stably derepressed class C β-lactamase mutations in a bacterial population can be as high as 10^{-5} (26). The existence of such mutants has serious clinical implications with regard to the generation of AmpC-producing strains during selective therapy with broad-spectrum β-lactams. These strains appear to carry a mutation of either AmpD or AmpR. The selection of AmpR mutants at a frequency of 10^{-6} or less strongly suggests that frequent generation of derepressed mutant strains, such as AmpD mutants, might occur first in the clinical setting, followed by selection of AmpR mutants. In the real situation both *ampR* and *ampD* are chromosomal single-copy genes. In these experiments, *ampR* is on a multicopy plasmid. This clearly affects the mutation frequency. Hence, it is much more likely that clinical *E. cloacae* isolates resistant to β-lactamase contain *ampD* mutations than *ampR* mutations, since any harmful event to the AmpD basically increases the

```
pKU403      1 MTRSYLPLNSLRAFEAAARHLSFTHAAIELNVTHSAISQHVKALEQHLNCQLFVRVSRGL    60
pKU404      1 MTRSYLPLNSLRAFEAAARHLSFTHAAIELNVTHSAISQHVKALEQHLNCQLFVRVSRGL    60
pKU405      1 MTRSYLPLNSLRAFEAAARHLSFTHAAIELNVTHSAISQHVKALEQHLNCQLFVRVSRGL    60
pKU406      1 MTRSYLPLNSLRAFEAAARHLSFTHAAIELNVTHSAISQHVKALEQHLNCQLFVRVSRGL    60
MHN1        1 MTRSYLPLNSLRAFERAARHLSFTHAAIELNVTHSAISQHVKALEQHLNCQLFVRVSAGL    60
OS60        1 MTRSYIPLNSLRAFEAAARHLSFTRAAIELNVTHSAISQHVKSLEQQLNCQLFVRGSRGL    60
              ***** ********* ******** ***************** *** ******** * **

pKU403     61 MLTTEGENLLPVLNDSFDRIAGMLDRFASHRAQEKLKVGVVGTFATGVLFSQLADFRRCY    120
pKU404     61 MLTTEGENLLPVLNDSFDRIAGMLDRFASHRAQEKLKVGVVGTFATGVLFSQLADFRRCY    120
pKU405     61 MLTTEGENLLPVLNDSFDRIAGMLDRFASHRAQEKLKVGVVGTFATGVLFSQLADFRRCY    120
pKU406     61 MLTTEGENLLPVLNDSFDRIAGMLDCFASHRAQEKLKVGVVGTFATGVLFSQLADFRRCY    120
MHN1       61 MLTTEGENLLPVLNDSFDRIAGMLDRFASHRAQEKLKVGVVGTFATGVLFSQLADFRRCY    120
OS60       61 MLTTEGESLLPVLNDSFDRMAGMLDRFATKQTQEKLKIGVVGTFAIGCLFPLLSDFKRSY    120
              ******* *********** ***** **    ***** ******* * **  * ** * *

pKU403    121 PHIDLHLSTHNNRVDPAAEGLDYTIRYGGGAWHGTEAQFLCSAPLSPLCSPDIALGLQSP    180
pKU404    121 PHIDLHLSTHNNRVNPAAEGLDYTIRYGGGAWHGTEAQFLCSAPLSPLCSPDIALGLQSP    180
pKU405    121 PHIDLHLSTHNNRVVPAAEGLDYTIRYGGGAWHGTEAQFLCSAPLSPLCSPDIALGLQSP    180
pKU406    121 PHIDLHLSTHNNRVDPAAEGLDYTIRYGGGAWHGTEAQFLCSAPLSPLCSPDIALGLQSP    180
MHN1      121 PHIDLHLSTHNNRVDPAAEGLDYTIRYGGGAWHGTEAQFLCSAPLSPLCSPDIALGLQSP    180
OS60      121 PHIDLHISTHNNRVDPAAEGLDYTIRYGGGAWHDTDAQYLCSALMSPLCSPTLASQIQTP    180
              ****** ******* ****************** * ** ****  ******  *   * *

pKU403    181 ADILKFTLLRSYRRDEWSAWMQAAGEHPPSPTHRVMVFDSSVTMLEAAQAGVGIAIAPVD    240
pKU404    181 ADILKFTLLRSYRRDEWSAWMQAAGEHPPSPTHRVMVFDSSVTMLEAAQAGVGIAIAPVD    240
pKU405    181 ADILKFTLLRSYRRDEWSAWMQAAGEHPPSPTHRVMVFDSSVTMLEAAQAGVGIAIAPVD    240
pKU406    181 ADILKFTLLRSYRRDEWSAWMQAAGEHPPSPTHRVMVFDSSVTMLEAAQAGVGIAIAPVD    240
MHN1      181 ADILKFTLLRSYRRDEWSAWMQAAGEHPPSPTHRVMVFDSSVTMLEAAQAGVGIAIAPVD    240
OS60      181 ADILKFPLLRSYRRDEWALWMQAAGEAPPSPTHNVMVFDSSVTMLEAAQGGMGVAIAPVR    240
              ****** **********  ******* ****** *************** * * *****

pKU403    241 MFTHLLNSERIVQPFATRIDLGSYWLTRLQSRAETPAMHEFAQWLVGKMQK             292
pKU404    241 MFTHLLNSERIVQPFATRIDLGSYWLTRLQSRAETPAMHEFAQWLVGKMQK             292
pKU405    241 MFTHLLNSERIVQPFATRIDLGSYWLTRLQSRAETPAMHEFAQWLVGKMQK             292
pKU406    241 MFTHLLNSERIVQPFATRIDLGSYWLTRLQSRAETPAMHEFAQWLVGKMQK             292
MHN1      241 MFTHLLNSERIVQPFATRIDLGSYWLTRLQSRAETPAMHEFAQWLVGKMQK             292
OS60      241 MFTHLLSSERIVQPFLTQIDLGSYWITRLQSRPETPAMREFSRWLTGVLHK             292
              ****** ******** * ******* ****** ***** **  ** *   *
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FIG. 2. Comparison of deduced amino acid sequences of AmpR from pKU403, pKU404, pKU405, pKU406, *E. cloacae* MHN1 (12), and *C. freundii* OS60 (24). Identical amino acids in all five sequences are marked with an asterisk. Nonidentical amino acids compared to those in pKU403 are underlined.

level of resistance, whereas specific *ampR* mutations are needed to create an AmpR that works as an activator even in the absence of a muropeptide inducer. The potentially interesting aspect of this study is that an *ampD* mutation may perhaps be followed by an *ampR* mutation, creating further resistance. The problem is that it is not known if a single-copy version of the *ampR* mutants studied here actually will increase the level of β-lactamase expression in an *ampD* knockout mutant of *E. cloacae*. It is not known whether replacement of a single-copy version of the mutated *ampR* gene by wild-type *ampR* on the chromosome of *E. cloacae* will actually increase the level of resistance in AmpD wild-type strains.

TABLE 4. Effects of AmpR mutants against production of class C β-lactamase in *E. cloacae* ATCC 13047 and three clinical isolates

Plasmid	β-Lactamase activity (U/mg of protein)[a]			
	KU3261	KU3262	KU3263	ATCC 13047
	<0.02	0.06	<0.02	0.07
pKU411	0.02	0.03	<0.02	0.06
pKU412	6.96	6.40	1.72	1.38
pKU413	2.56	2.01	0.34	0.78
pKU415	4.82	4.34	0.93	1.07

[a] 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.

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Presence of Genes for β-Lactamases of Two Different Classes on a Single Plasmid from a Clinical Isolate of *Serratia marcescens*

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NOTES

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The majority of β-lactam antibiotics have become increasingly ineffective therapeutic agents due to the rapid development of resistant strains[1]. Mechanisms responsible for the development of resistance to β-lactams include (i) hydrolysis by β-lactamases, (ii) creation of nonhydrolytic barriers by β-lactamases, (iii) reduction in affinity of target proteins, and (iv) reduction in permeability[2]. For Gram-negative enteric bacteria, the emergence of resistance to broad-spectrum penicillins and β-lactamase-stable cephalosporins occurs frequently[3]. The complexity of β-lactamase epidemiology has greatly increased with the emergence of extended-spectrum β-lactamases (ESBLs) derived from plasmid-encoded TEM and SHV-type enzymes[4]. These β-lactamases show greater specificity for β-lactam antibiotics.

β-Lactamases are divided by their substrates into classes A, B, C and D. When an isolate produces two different classes of β-lactamases, the genes coding for each β-lactamase are located separately on a plasmid and on a chromosome. For example, plasmid-encoded class A enzymes and chromosome-encoded class C enzymes are produced in Gram-negative bacteria. On the other hand, plasmid-encoded class B enzymes have been reported from Japan[5]. However, there are no reports of plasmids carrying the genes of two different classes of β-lactamases, such as class A and class B or class A and class D.

In this report, we describe a novel plasmid carrying the genes for both class A and class B β-lactamases in a clinical isolate of *Serratia marcescens* in Japan.

Materials and Methods

Test Organisms

The *S. marcescens* strain described here was isolated from the urine of a patient with a urinary tract infection at a hospital in northern Japan in 1996. The organism was identified by standard methods. *Escherichia coli* K12 ML4901 and ML4947 strains, plasmid-regulated β-lactamase-producing bacteria, and plasmids of various incompatibility groups (R9-5, F*lac-tet*, R621a, N3, RP4-1, S-a, R27, R446b and R14) were stocked at the Department of Microbiology, Kitasato University School of Medicine.

Culture Medium

The test strains were incubated in sensitivity assay liquid medium (MH broth, Nissui, Tokyo) for 18 hours at 35°C. Minimum inhibitory concentrations (MICs) were determined using sensitivity assay agar medium (MH agar medium, Nissui)[6]. BTB lactose agar medium (Nissui) was used for the identification of incompatibility group plasmids and for pure culture of transconjugants, while L broth was used for assay of β-lactamases[7].

Test Drugs

The following antibacterial agents were used in this study and reference powder of different drugs of known potency were provided by the respective manufacturers. Benzylpenicillin (PCG, Banyu, Tokyo) and piperacillin (PIPC, Toyama Chemical, Tokyo) were used as representative penicillins, while cephalotin (CET, Shionogi, Osaka), cefotiam (CTM, Takeda Chemical Industries, Osaka), cefmetazole (CMZ, Sankyo, Tokyo) and cefotaxime (CTX, Hoechst Japan, Tokyo) were used as representative cephalosporins. Other β-lactams, including imipenem (IPM, Banyu, Tokyo), meropenem (MEPM, Sumitomo, Osaka), panipenem (PAPM, Sankyo, Tokyo) and aztreonam (AZT, Eisai, Tokyo), as well as kanamycin (KM, Meiji Seika, Tokyo), chloramphenicol (CM, Sankyo) and tetracycline (TC, Lederle Japan, Tokyo) were used. Other reagents, including sulfonamide (SA), streptomycin

(SM), nalidixic acid (NA, Daiichi, Tokyo), various restriction enzymes, and marker DNAs were purchased from commercial sources.

Drug Susceptibility Assay

The susceptibility of the test strains to each drug was assayed in accordance with the specified drug sensitivity assay methods of the Japan Society of Chemotherapy except for the antibiotic concentrations used[6]. After overnight incubation at 35°C, medium containing organisms was diluted in buffered saline with gelatin to a concentration of $5\sim6\times10^6$ cfu/ml. Using a microplanter (Sakuma Seisakusho, Tokyo), aliquots of this suspension (about $5\sim6\times10^4$ cfu/spot) were inoculated onto plates containing the test drug at various concentrations, and each plate was incubated for 18 hours at 35°C. The lowest concentration at which no bacterial growth was observed macroscopically was determined to be the minimum inhibitory concentration (MIC).

Characterization of Plasmids

For determination of incompatibility groups, the plasmid under study was introduced into *E. coli* KI2 ML4901 together with a standard plasmid (previously grouped by conjugal transfer or transformation), and the incompatibility group of the test plasmid was determined as follows[7]. Organisms containing both the test plasmid and a standard plasmid of each incompatibility group were again incubated overnight in MH broth at 35°C. This broth was then smeared on MH agar medium and again incubated overnight at 35°C. The resulting colonies were collected and streaked onto plates containing a drug corresponding to the resistance pattern of the two plasmids, followed by further incubation overnight at 35°C. When organisms proliferated on both drug-containing plates, the test plasmid was considered to be of a different group from that of the standard plasmid. When organisms proliferated on only one of the two plates, the test plasmid was considered to belong to the same incompatibility group as the standard plasmid.

Preparation and Assay of β-Lactamase

Test strains were incubated overnight in L broth, which was then diluted 20-fold with fresh L broth. A 10-ml aliquot of this diluted culture broth was incubated at 35°C with shaking. Organisms in late logarithmic growth phase were collected by centrifugation at $18,000\times g$ for 10 minutes, washed with 0.05 M phosphate buffer (pH 7.0) with 10 μM $ZnCl_2$, and suspended in 3 ml of the same buffer. This bacterial suspension was sonicated and then centrifuged at $18,000\times g$ for 20 minutes at 4°C. The supernatant was used as the crude enzyme preparation[8]. Enzyme activity was determined by spectrophotometry (UV2000, Shimadzu Corp., Tokyo) at 30°C in 50 mM phosphate buffer (pH 7.0) with or without 10 μM $ZnCl_2$, using antibiotics as the substrate.

Analytical Isoelectric Focusing

Isoelectric focusing was carried out with a Phast system (Pharmacia Biotech, Uppsala, Sweden) and a Phast Gel IEF 3-9 (Pharmacia Biotech). The enzyme protein on the gel plate was detected by staining with Coomassie brilliant blue G-250, and β-lactamase activity was confirmed with nitrocefin (100 μg/ml).

Isolation of DNA

The procedures described in the manuals of Takara Suzo Co., Otsu and in previous reports were followed for the isolation of plasmid DNA[9].

Gene amplification was performed by a polymerase chain reaction (PCR) method using a commercially available PCR kit (Gene Amp PCR Reaction Kit with Ampli TaqDNA Polymerase, Takara) and a DNA Thermal

Table 1. PCR primers.

Type of β-lactamase	Size of PCR product (bp)	Sequence
TEM	108	5'-AAGCCATACCAAACGACGAG-3'
		5'-ATTGTTGCCGGGAAGCTAGA-3'
Class B	448	5'-CATGGTTTGGTGGTTCTTGT-3'
		5'-ATAATTTGGCGGACTTTGGC-3'
SHV-1	593	5'-TCTCCCTGTAAGCCACCCTG-3'
		5'-CCACTGCAGCAGCTGC(A/C)GTT-3'
Toho-1	164	5'-TGGAAGCCCTGGAGAAAAGT-3'
		5'-CTTATCGCTCTCGCTCTGTT-3'

bp, base pair

Cycler PH2000 (Perkin-Elmer Cetus Instruments, Emeryville, Calif). PCR primers were chosen on the basis of the published TEM-1[11], bla_{IPM}[12], SHV-1[13] and Toho-1[14] sequences and are listed in Table 1. Bacterial strains of KU2013, 1917, 2017 and 3522, which were stocked at the Department of Microbiology at Kitasato University School of Medicine, were used as positive controls for TEM, bla_{IPM}, SHV-1 and Toho-1. The reaction mixture contained 61.5 μl of H_2O, 10 μl of 10×buffer, 2 μl each of 10 mM dATP, dCTP, dGTP, and dTTP, 0.5 μl of Taq polymerase (5 units/μl), 0.1 μl each of primers 1 and 2 (2 μM), and 10 μl of template DNA. The PCR involved 25 cycles of denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute, and elongation at 72°C for 1 minute, followed by heating at 72°C for 7 minutes. Five μl of the PCR product were subjected to electrophoresis on 1.2% agarose gel to identify the amplified DNA fragment.

Results and Discussion

The *S. marcescens* strain KU3838 that carries the plasmid described here was isolated from a patient with a urinary tract infection in November 1996 in Japan. *E. coli* KU3999 was a transconjugant that served to transfer the conjugative plasmid from *S. marcescens* KU3838 to *E. coli* K12 ML4901. The frequency of transfer by conjugation to *E. coli* K12 ML4901 was about 10^{-5} to 10^{-6}, and resistance to PIPC and IPM was transferred simultaneously as a single genetic unit, indicating that the plasmid-encoded class A and B β-lactamases investigated in this study were transferable. This plasmid was designated pKU501. The incompatibility group of this plasmid was identified as previously described[7]. When a donor pKU501 culture was mixed with a recipient carrying plasmid S-a, which belongs to incompatibility group W, the resistance conferred by S-a was eliminated from the transconjugants by the newly introduced pKU501. In this case, resistance to both PIPC and IPM was expressed in the recipient strain but the TC resistance mediated by S-a was lost. Therefore, class A and B β-lactamase genes were confirmed to be located on the same plasmid, which belonged to incompatibility group W.

The plasmid R9-5, which belongs to the incompatibility group FIV, is transferable to *E. coli* K12 ML4947 by conjugation at a frequency of 10^{-1}. When pKU501 coexisted with R9-5, the frequency of transfer by conjugation of pKU501 was 10 times higher than that of pKU501 alone. These results suggested the possibility of clinical spread of the plasmid-encoded class A and B β-lactamases by conjugation.

Drug resistance of *S. marcescens* KU3838 and the transconjugant strain of *E. coli* KU3999 was measured by determining the minimum inhibitory concentration (MIC) of various drugs as previously described[6] (Table 2). *S. marcescens* KU3838 and *E. coli* KU3999 were resistant to PIPC, cephalosporins and carbapenems. The MICs of PIPC and PIPC/CVA (clavulanic acid) against *E. coli* KU3999 were >128 and 32 μg/ml, respectively, indicating that the MIC was slightly decreased in the presence of CVA, which inhibits class A β-lactamase activity. Recently, clinical isolates possessing class A ESBLs that differ by a few point mutations have been described[15]. These enzymes hydrolyze cephalosporins and monobactam, including CAZ, CTX and AZT, in addition to ABPC and PIPC, but not cefmetazole and cefminox, which belongs to the cephamycin group, or IPM, PAPM and MEPM. The MICs of IPM, PAPM and

Table 2. Minimum inhibitory concentrations (mg/ml) of different antibiotics for *S. marcescens* KU3838, *E. coli* KU3999 and *E. coli* ML4901.

Antibiotic	*S. marcecsens* KU3838	*E. coli* KU3999	*E. coli* ML4901
Piperacillin	>128	>128	4
Piperacillin/ CVA	>128	32	0.5
Cephalotin	>128	>128	0.13
Cefmetazole	>128	>128	0.25
Cefotiam	>128	128	0.03
Cefotaxime	>128	>128	0.13
Cefepime	>128	16	0.13
Imipenem	32	8	0.25
Panipenem	>128	64	0.25
Meropenem	>128	64	0.5
Aztreonam	128	0.25	0.06

CVA, clavulanic acid

Table 3. Hydrolyzing activities of β-lactamases mediated by the pKU501 plasmid.

Substrate	β-Lactamase activity (U/ mg of protein)		
	KU3999	KU3999/ CVA	KU3991
Penicillin G	9.7 (3590)	0.17(68)	0.15 (88)
Piperacillin	6.6 (2440)	<0.01	0.02 (12)
Cephalothin	4.7 (1740)	3.09(1240)	0.10 (58)
Cefotaxime	0.65(240)	0.96(384)	0.05 (29)
Aztreonam	<0.01	<0.01	0.09 (52)
Imipenem	*0.19(100)	*0.20(100)	0.17(100)
Panipenem	*0.26(136)	*0.33(173)	0.07 (41)
Meropenem	*0.35(184)	*0.28(147)	0.03 (17)

( ) indicates the relative rate of enzyme activity, normalized to activity against IPM as 100.
* indicates the average of two separate experiments.
CVA; Clavulanic acid,

MEPM for *E. coli* KU3999 were 8, 64 and 64 μg/ml, respectively. *S. marcescens* KU3838 showed an MIC for AZT of 128 μg/ml, while *E. coli* KU3999 was inhibited at an AZT concentration of 0.25 μg/ml as was the recipient *E. coli* strain ML4901. These MICs suggested that pKU501 carries genes encoding class A and B β-lactamases.

PCR was performed to determine whether pKU501 does carry class A and B β-lactamase genes. PCR primers were chosen on the basis of published sequences and were obtained from Takara Suzo. As the results of PCR, 108 bp and 448 bp PCR products, which were consistent with expected length, were obtained (data not shown). In addition, the enzyme isolated from pKU501 yielded two bands in SDS-polyacrylamide gel electrophoresis, simultaneously. We estimated isoelectric points of 5.4 and 9.2, which were identical to that of TEM type and bla_{IMP} type β-lactamase. These results confirmed that pKU501 carries both TEM type and bla_{IMP} type β-lactamase genes.

Enzyme activity was determined by spectrophotometry (UV2000, Shimadzu Corp.) at 30°C in 50 mM phosphate buffer (pH 7.0) with or without 10 μM $ZnCl_2$, using antibiotics as the substrate. As a positive control, the enzyme activity of *E. coli* KU3991, which produces a plasmid-mediated class B β-lactamase, was also determined. The activities of these β-lactamases are shown in Table 3. The enzyme from *E. coli* KU3999 had a broad substrate profile, hydrolyzed PCG, PIPC and CET frequently, and was inhibited by CVA. Moreover, when enzyme activity was assayed in the presence of CVA, hydrolysis of CTX, IPM, PAPM and MEPM but not of AZT was still observed. These results suggested that pKU501 encodes both class A and B β-lactamases. In addition, the enzyme activities against PAPM and MEPM were higher than against IPM, and substrate profiles of the enzyme encoded by pKU501 were quite different from that of the enzyme produced by *E. coli* KU3991. These results reflected the MICs of IPM, PAPM and MEPM. The results showed that the activity of the enzyme encoded by pKU501 differed from that of the class B β-lactamase reported in another study[5]. From these results, the class B β-lactamase encoded by pKU501 appears to be an extended-spectrum β-lactamase. We are now studying the genetic and molecular properties of pKU501. The DNA sequence analysis will be described elsewhere.

Bacteria have developed many ways to counteract antibiotics, but knowledge of the mechanisms involved yields a variety of strategies to counter antimicrobial resistance. Recently, there have been increasing reports of Gram-negative bacteria carrying the transferable carbapenem resistance gene bla_{IMP}, including *Pseudomonas aeruginosa* and *S. marcescens*, with close genetic relationships of strains demonstrated by pulsed-field gel electrophoresis (PFGE)[16]. This suggested that rates of nosocomial spread may differ between hospitals. The incidence of Gram-negative bacteria carrying bla_{IMP} was high in one hospital[17], but low in another[18]. More rational and appropriate use of antibiotics can reduce the selective pressure for resistance mutations and the spread of resistance plasmids. In addition, controlling nosocomial infection is also very effective to limit to spread of resistance genes in these organisms.

In conclusion, we report a transferable plasmid carrying

genes encoding both class A and B β-lactamases found in a *S. marcescens* clinical isolate. It is important to monitor such strains closely and prevent their spread.

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NOTE

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Estimation of outer membrane permeability of carbapenem antibiotics to *Pseudomonas aeruginosa*

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Abstract
The outer membrane permeability of carbapenems (imipenem [IPM], panipenem [PAPM], meropenem [MEPM], and biapenem [BIPM]) and ceftazidime (CAZ) to *Pseudomonas aeruginosa* was determined by the Zimmermann and Rosselet method. The permeability coefficients of β-lactams tested at 50 μM concentration of substrates ranged from $(0.40 \pm 0.10) \times 10^{-6}$ cm/s for CAZ to $(2.33 \pm 0.33) \times 10^{-6}$ cm/s for IPM, indicating that the outer membrane permeability of carbapenems to *P. aeruginosa* was high in comparison with that of CAZ. In particular, IPM and BIPM showed a higher rate of penetration than MEPM and PAPM.

Key words Outer membrane permeability · *Pseudomonas aeruginosa* · Carbapenem

Introduction

Pseudomonas aeruginosa shows intrinsic resistance to many categories of antibiotics, including β-lactams,[1,2] and this resistance has often been attributed to the low permeability of its outer membrane.[3,4] A method to measure the permeability of β-lactams to intact cells has been developed by Zimmermann and Rosselet[5] and Sawai et al.[6] This method is already used for several species of gram-negative bacteria;[7,8] however, the method is useful only for β-lactams which are hydrolyzed by β-lactamases. In a previous study,[9] we reported a plasmid, pMS350, which encoded a novel β-lactamase classified as a metalloenzyme (carbapenemase).[10] In this study, we determined the permeability rate of various β-lactams through the outer membrane of *P. aeruginosa* by the Zimmermann and Rosselet method.

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Materials and methods

The antimicrobial agents IPM, PAPM, MEPM, BIPM, and CAZ were obtained from Banyu Pharmaceuticals, Sankyo, Sumitomo Pharmaceuticals, Lederle (Japan), and Nippon Glaxo, respectively (all in Tokyo, Japan). Antibiotic susceptibility tests were carried out against *P. aeruginosa* PAO 4141 (β-lactamase-deficient mutant of PAO1) and PAO 4141 harboring pMS354,[11] which was used for the assay of outer membrane permeability.

The Michaelis constant (Km) of the β-lactamase was determined spectrophotometrically using purified enzyme, while the rates of hydrolysis of β-lactams were measured spectrophotometrically, using intact cells or sonic extract cells. Enzyme activity was determined at 30°C in 50 mM 3-morpholinopropanesulfonate (MOPS)-NaOH buffer (pH 7.0) containing 5 mM $MgCl_2$. The rate of hydrolysis by the sonic extract was used to calculate the maximum velocity (Vmax) of the β-lactamase, and Vmax was calculated from the modified Michaelis equation as follows:

$$\mathrm{Vmax} = (1 + \mathrm{Km}/\mathrm{Co}) \times \mathrm{Vd} \quad (1)$$

where Vd is the rate of hydrolysis of β-lactams by the sonic extract of cells and Co denotes the β-lactam concentration outside. The β-lactam concentration inside (Ci) was calculated from the formula of Zimmermann and Rosselet[5] as follows:

$$\mathrm{Ci} = (\mathrm{Vi} \times \mathrm{Km})/(\mathrm{Vmax} - \mathrm{Vi}) \quad (2)$$

where Vi is the rate of hydrolysis of β-lactams by the intact cells. The permeability coefficient, Pz, was calculated ac-

cording to the equation of Zimmermann and Rosselet[5] or according to Nikaido et al.:[12]

$$Pz \times A = Ci \times Vmax/(Ci + Km) \times (Co - Ci) \quad (3)$$

where A denotes the area of the membrane. According to the method of Nikaido et al.,[12] the approximate surface/weight ratio, 132 cm^2/mg (dry weight), was used.

Results

The minimum inhibitory concentrations (MICs) of carbapenems and CAZ against PAO 4141 and PAO 4141 harboring pMS354 are shown in Table 1. The plasmid pMS354 conferred resistance to IPM, PAPM, MEPM, and BIPM, and higher resistance to CAZ.

The permeability coefficients of *P. aeruginosa* PAO 4141 outer membrane harboring pMS354 to β-lactam antibiotics are shown in Table 2. The permeability coefficients Pz, was calculated by the formula of Zimmermann and Rosselet (formula 3). The order of penetrability for these β-lactams at 50 μM concentration of substrates was:

IPM = BIPM > MEPM = PAPM > CAZ

that is, IPM and BIPM showed higher rates of penetration than MEPM, PAPM, and CAZ (*t*-test; $P < 0.05$).

Table 1. Antibacterial activity of carbapenems and ceftazidime against *P. aeruginosa* PAO4141 strains

Antibiotic	MIC (μg/ml)	
	PAO4141	pMS354/PAO4141
Imipenem	0.20	3.13
Panipenem	0.20	12.5
Meropenem	0.20	12.5
Biapenem	0.10	6.25
Ceftazidime	0.78	200

MIC, Minimum inhibitory concentration

Table 2. Permeation rates of carbapenem antibiotics and ceftazidime through outer membrane of *P. aeruginosa* PAO4141 harboring pMS354

Antibiotic	Mol wt[a]	Permeability coefficient Pz ($\times 10^{-6}$ cm/s)[b] at 50 μM	No. of experiments[c]
Imipenem	299	2.33 ± 0.33	4
Panipenem	339	0.81 ± 0.20	3
Meropenem	384	0.85 ± 0.21	3
Biapenem	350	2.03 ± 0.45	3
Ceftazidime	547	0.40 ± 0.10	3

[a] Molecular weights of compounds in free form
[b] The permeability coefficients of antibiotics measured at 50 μM of the substrates were calculated from formula 3 (Materials and Methods), and the mean ± SD values of the coefficients of independent experiments were calculated
[c] Number of independent experiments

Discussion

The outer membrane barrier of gram-negative bacteria contributes to the degree of resistance to β-lactams, as the influx of the antibiotics is slow via the barrier and the drugs are hydrolyzed by β-lactamase.[3,4] We determined the permeability coefficients of β-lactams to *P. aeruginosa* by the Zimmermann and Rosselet method. Leakout of β-lactamase was a serious problem in the assay of outer membrane permeability of β-lactams to *P. aeruginosa* . We kept intact cells at 20°C in MOPS-NaOH buffer containing 5 mM $MgCl_2$ buffer during experiments, and cell lysis was reduced to an undetectable order in the assay system. In an earlier report,[13] changes in permeability coefficients due to the concentration of substrate were pointed out in the Zimmermann-Rosselet assay. When pMS354-mediated enzyme was used for the assay, the permeability coefficient, Pz, for IPM was not significantly different between the concentrations of 50 and 100 μM (data not shown). Moreover, the adequacy of this experiment was confirmed by the finding that our results of pilot experiments for cephaloridine, cefepime, and cefpirome were close to the results found with the other assay system, of Nikaido et al.[14] (data not shown). In this study, the permeability coefficients of carbapenems were higher than those of CAZ.

The permeablity coefficient of carbapenems in *P. aeruginosa* is considered to be the value which would be influenced by deficiency of the porin protein, OprD, or overproduction of MexAB-OprM efflux pump proteins.[15]

From our results, it is suggested that both the higher rates of penetration of carbapanems, especially IPM and BIPM, and the high stabilities to chromosomal β-lactamase[11] may contribute to the in potent antibacterial activities against *P. aeruginosa* strains, except for carbapenemase-producing strain. We are convinced that this finding will increasingly spur further research and the development of new carbapenem derivatives.

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Inducible β-lactamase-mediated resistance to third-generation cephalosporins

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The emergence of multiple resistance to β-lactam antimicrobial agents is a major problem in the treatment of patients infected with Enterobacteriaceae that characteristically produce inducible β-lactamases. Inducible and 'derepressed' AmpC β-lactamases are produced by *Enterobacter* spp., *Citrobacter freundii, Serratia marcescens, Morganella morganii* and *Providencia* spp. Resistance to broad-spectrum β-lactams has emerged in 16–44% of these strains from infections treated with one of the newer cephalosporins, even in combination with other antimicrobials. Multiply resistant organisms have spread widely both locally, within hospitals, and nationally. This trend has been shown to correlate closely with the extent of usage of some third-generation cephalosporins. These resistant strains, especially *Enterobacter* spp., are more regularly isolated from seriously ill patients (especially from respiratory sources), or in intensive care units and pose one of the greatest challenges to contemporary chemotherapy of infections in hospitalized patients. Zwitterionic fourth-generation cephalosporins combine the properties of rapid bacterial outer membrane penetration with high stability to AmpC β-lactamase with good affinity for the penicillin-binding proteins to achieve in vitro activity against AmpC-producing organisms, including the majority of strains highly resistant to ceftazidime and other earlier generation cephalosporins. These features have contributed to their clinical success in the therapy of infections caused by *Enterobacter* spp. with and without resistance to third-generation compounds. Other alternative agents for chemotherapy of infections due to AmpC β-lactamase-producing strains (inducible or derepressed expression) should also be considered e.g. carbapenems, aminoglycosides and fluoroquinolones.

Key Words: AmpC, induction, derepression, cefpirome, β-lactamase

INTRODUCTION

The emergence of bacterial resistance to antimicrobial agents continues to represent an important clinical problem. In recent years, many classes of antimicrobials have become less effective as a result of evolving microbial resistance mechanisms. In some cases this has been linked to extensive use of selecting drugs [1,2]. In nosocomial infections, resistance continues to be a threat to contemporary antimicrobial chemotherapy. Current resistance problems among Gram-positive bacteria include multidrug-resistant staphylococci, glycopeptide-resistant enterococci and penicillin- and multidrug-resistant pneumococci. Resistance among Gram-negative bacteria is attributable to ceftazidime-resistant Bush Group 1 producing Enterobacteriaceae, extended-spectrum β-lactamases in *Klebsiella* spp., *Proteus mirabilis* and *Escherichia coli*, and multidrug resistance among *Pseudomonas* spp. The likelihood of encountering *Stenotrophomonas maltophilia* as a nosocomial pathogen is increasing [3]. Fluoroquinolone resistance is also present and increasing in staphylococci, enteric bacilli and *Pseudomonas* spp. [3].

Although reduced outer membrane permeability and modification of the penicillin binding proteins (PBPs) are among the most important mechanisms of bacterial resistance to β-lactam antimicrobial agents, β-lactamase production accounts for a major source of resistance [4]. Virtually all bacteria produce chromosomally-mediated β-lactamases and plasmid-mediated β-lactamases are

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widespread in Gram-negative bacteria. More recently, the plasmid-mediated, extended-spectrum β-lactamases have emerged as clinically important resistance determinants in the Enterobacteriaceae [4–6]. Metallo β-lactamases confer resistance to carbapenems and, although still uncommon at present, may pose a threat in the future [4].

The introduction of third-generation cephalosporins improved the effectiveness of therapy for the vast majority of infections caused by Gram-negative bacteria; however, the use of these highly β-lactamase stable compounds has led to the emergence of resistant species [2]. Bacteria that possess chromosomally-mediated Bush Group 1 β-lactamase have been implicated in the development of resistance and multiply-resistant, stably derepressed mutants have emerged during therapy [7,8].

Beta-lactamases are present in virtually all Gram-negative bacilli. However, in some bacterial strains, such as *E. coli* and *Klebsiella* spp., the β-lactamase is produced at a low level and cannot be induced to greater production by the presence of β-lactams. In other species, β-lactamase production occurs at low levels, but is inducible when exposed to certain β-lactams, commonly resulting in resistance to these agents. These inducible β-lactamases are frequently found in *Enterobacter* spp., *Citrobacter freundii, Providencia* spp., *Morganella* spp. and *Serratia* spp. (Table 1) [7,8]. These organisms also routinely undergo spontaneous mutation to become constitutive β-lactamase producers. This, in turn, confers resistance to most β-lactams, including third-generation cephalosporins. However, β-lactam antimicrobial agents differ, not only in their sensitivity to these enzymes, but also in their ability to induce synthesis of the enzyme and selection of resistant, derepressed mutants [4,9].

Beta-lactamases are an enormously varied class of enzymes, classified until recently both on the basis of their substrate hydrolytic spectrum and whether encoded by plasmid- or chromosomally-located genes [10–12]. However, such phenotypic classification schemes were found to be compromized in satisfactorily recognizing the point mutations which could dramatically alter substrate specificity and inhibitor susceptibilities. Therefore, β-lactamases are increasingly classified at a molecular level on the basis of amino acid sequence [13], as originally proposed by Ambler [14]. Four classes are recognized under this scheme: classes A, C and D are serine active site enzymes, whereas class B metallo-enzymes require zinc for activity. Expression of β-lactamase can be constitutive or inducible. Constitutive and non-induced enzyme levels are normally quite low; however, induction can lead to several hundred-fold increases in activity. Mutations in genetic control mechanisms can also result in derepression of the enzyme, whereby β-lactamase production is maintained at a very high level.

The genetics of induction are discussed below; however, it is important at this stage to define the terms 'induction' and 'derepression'. 'Induction' is defined as the synthesis of enzyme-protein in direct response to induction by the substrate (inducer), a phenotypic, temporary response to an environmental change. 'Derepression', in contrast, is a constitutive, permanent feature of the mutant (stable genetic change) whereby large amounts of enzyme-protein are produced consistently.

Ambler Class A β-lactamases, including the common plasmid-mediated TEM enzymes, are produced constitutively by *K. pneumoniae, Bacteroides fragilis* and inducibly by *K. oxytoca* and *Staphylococcus aureus*. Class B metallo-enzymes are relatively uncommon and mainly produced by *S. maltophilia, Bacillus cereus* and some strains of *Bacteroides* spp. However, the Class B enzymes have the important ability to rapidly hydrolyze those drugs generally stable to the other enzyme classes, such as the carbapenems and the cephamycins.

AmpC β-lactamases are produced by many bacterial species. Production of inducible AmpC β-lactamases is limited to a group of organisms including *Enterobacter* spp., *C. freundii, S. marcescens, M. morganii, Providencia* spp. and *P. aeruginosa*. These bacterial species are regularly isolated from hospitalized patients, including the seriously ill, and pose one of the greatest challenges to contemporary nosocomial or hospitalized patient infection chemotherapy [2,4,7,8,15].

Table 1 Enterobacteriaceae species often possessing inducible Bush Group 1 β-lactamases and associated with strains having resistance to so-called 'third-generation' cephalosporins

Genus	Species
Enterobacter	*aerogenes, cloacae*
Citrobacter	*freundii*
Serratia	*marcescens*
Morganella	*morganii*
Providencia	*rettgeri, stuartii*

Enterobacter spp. are increasing in clinical practice [3,16,17]. In a survey conducted in the USA in 1994, of > 8,500 organisms isolated from patients residing in 43 medical centers, *Enterobacter* spp. were responsible for 6.3% of all infections [3]. *Enterobacter* was found to be the fourth most prevalent genus in respiratory tract infections, accounting for 9.2% of infections (with *S. marcescens* accounting for a further 4.6% of infections). Of the 3,224 organisms isolated from urinary tract infections, 4.7% were *Enterobacter* spp. and 1.8% were *C. freundii*. *Enterobacter* was also a significant pathogen in skin and soft tissue infections, accounting for 6.8% of the total number of isolates (with *S. marcescens* responsible for another 2.4%). In blood stream infections, *Enterobacter* spp. accounted for 3.9% of the total. Similar data were obtained in the 1995/96 SCOPE study, where *Enterobacter* spp. and *S. marcescens* accounted for 5% and 2%, respectively, of nosocomial blood stream infections [16].

Enterobacter spp. was also found to be a significant pathogen isolated in Intensive Care Units (ICUs). In the National Nosocomial Infections Surveillance system (NNIS) of ICU infections conducted in 1990, *Enterobacter* spp. was among the top five pathogens [17]. In this study, the incidence per site of infection was: respiratory tract (5.3%), surgical wound (10.3%) and urinary tract (6.1%). These findings were confirmed by the results of a European study, where *Enterobacter* spp. accounted for 8% of pathogens isolated from infections in medical ICUs, surgical ICUs and hematology/oncology units [18].

GENETICS OF INDUCIBLE AmpC EXPRESSION IN ENTEROBACTERIACEAE

Translation of the *ampC* gene is regulated by the *ampR* gene product, AmpR [19]. AmpR is a bifunctional protein, being a transcriptional activator in the presence of some β-lactams and a repressor in their absence. Deletion mutations of *ampR* generate a non-inducible phenotype, with AmpC being expressed at a level two- to three-fold higher than the normal, uninduced basal level [20,21].

At least two other genes, *ampD* and *ampG*, are involved in AmpC induction. A third gene, *ampE*, was initially thought to be involved in β-lactamase expression, but recent work has shown that it is not required [22]. *AmpD* and *ampG* are present in all Enterobacteriaceae tested to date, even those lacking an inducible AmpC β-lactamase, suggesting other primary functions for AmpD and AmpG [23]. AmpD is, in fact, a cytosolic *N*-acetyl muramyl-L-alanine amidase which participates in the intracellular recycling of peptidoglycan fragments [23,24]. DNA protection studies have failed to show binding to the regulatory region upstream from *ampC*; hence, it is unlikely that AmpD directly influences the expression of *ampC*. Null mutations in *ampD* cause derepression, while other mutations generate a hyper-inducible phenotype, whereby lower levels of inducer are required to promote *ampC* expression.

AmpG is believed to be a permease for a large muropeptide which might be a hypothetical activating ligand for β-lactamase induction [25]. In the absence of this protein no induction occurs, nor does constitutive activation of *ampC* take place in *ampG*, *ampD* double mutants [26,27].

Several models have been proposed to show the interaction of the various genes and gene products involved in AmpC induction. New insights into the relationship between β-lactamase induction and peptidoglycan recycling have given rise to an alternative view of the Bennett and Chopra model [28]. This suggests that AmpR controls β-lactamase production by sensing the cytoplasmic level of muropeptides, which is influenced by the activities of AmpD and AmpG in peptidoglycan recycling and indicative of the presence or absence of β-lactam antimicrobials (Figure 1) [25,29,30]. Peptidoglycan recycling has a signalling role in β-lactamase induction and derepression and is part of a communication link between the dynamic state of the cell wall, essential for growth and cell division, and the transcription mechanism of *ampC*.

RESISTANCE AMONG ENTERIC BACTERIAL SPECIES

The clinical and epidemiological importance of inducible β-lactamases and their stably derepressed mutants in Gram-negative bacteria has increased dramatically since the introduction of the third-generation cephalosporins [31]. These stably derepressed mutants were present in significant numbers among clinical isolates even before the clinical introduction of the third-generation cephalosporins. Occurrence rates of more than 10% for high β-lactamase-producing strains (derepressed AmpC) among Enterobacteriaceae were not uncommon between 1976 and 1981, although the incidence of such strains varied according to site of infection, geographical location and selective pressures [32]. In 1982, before the introduction of third-generation cephalosporins, *E. cloacae*, *C. freundii* and *S. marcescens* isolated from medical centers in the USA were all relatively susceptible to cefotaxime, with MIC_{90} values ≤ 5 mg/L [32]. In contrast, data reported from Europe and the Far East showed that strains of *C. freundii* and *E. cloacae* were more resistant, with

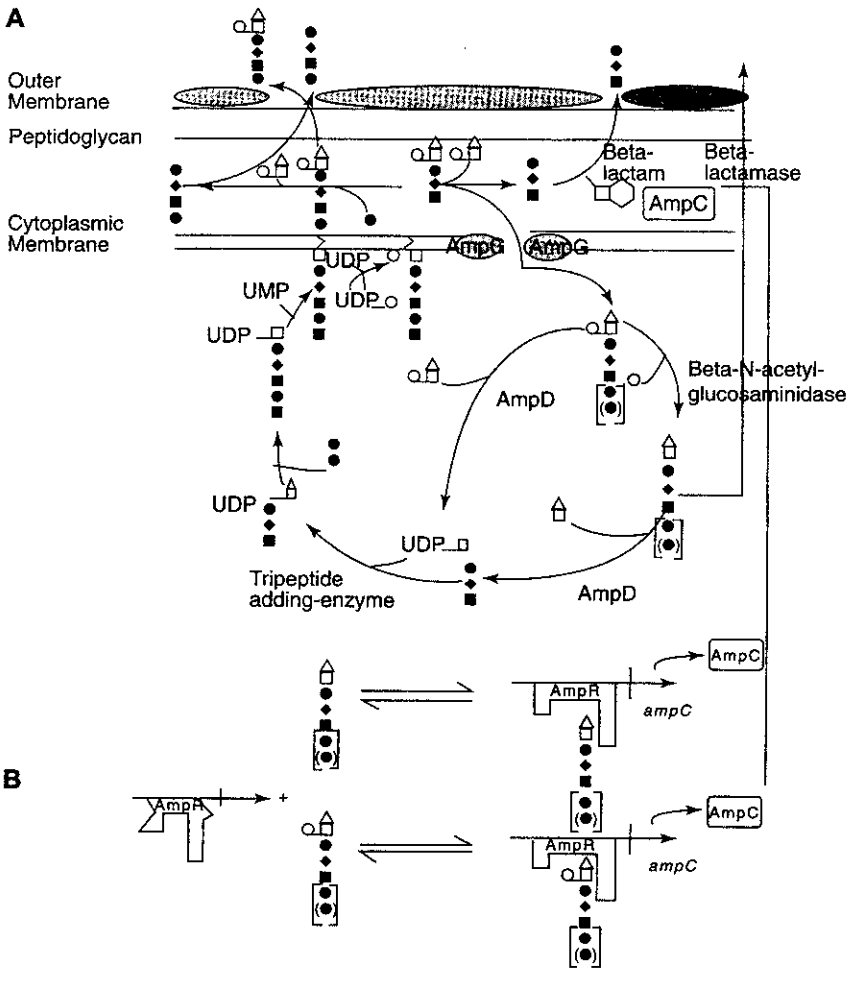

Figure 1 Hypothetical model for control of the expression of inducible *ampC* genes in Gram-negative bacteria [24–26,29,30]. The proposed interconnected pathway for recycling muropeptides and for their involvement in β–lactamase induction is illustrated. ○ GlcNac; ⌂ anhMurNac; □ MurNac; ● Ala; ◆ Glun; ■ DAP.

(A) The recycling pathway. As shown, murein is degraded by known enzymes in the periplasm to muramyl peptides. The muropeptide GlcNac-anhMurNac-tripeptide, tetrapeptide and pentapeptide are transported into the cytoplasm through AmpG. Disaccharides are cleaved by β-*N*-acetyl-glucosaminidase into monosaccharides. The muropeptides are then degraded into GlcNac-anhMurNac and free tripeptide, tetrapeptide or pentapeptide by AmpD. Free tripeptide can then be added directly to UDP-*N*-acetylmuramic acid by an as yet unidentified enzyme, thereby reintroducing it into the biosynthetic pathway for murein synthesis.

Derepression can occur because of mutations in the *ampD* or *ampR* gene. The *ampR* mutations change the AmpR protein into an activator. The *ampD* mutations alter the AmpD protein to an inactive enzyme, which results in an accumulation of the muramyl peptides in the cytoplasm.

(B) Muropeptides as inducers of β-lactamase. Intracellular accumulation of GlcNac-anhMurNac-tripeptide as a result of the presence of the β-lactam antibiotics or of anhMurNac-tripeptide as the result of inactivation of *ampD* triggers production of *C. freundii* AmpC β-lactamase. The muropeptides presumably bind to the transcriptional regulator AmpR and convert it into an activator for *ampC* expression. (*C. freundii ampR* and *ampC* are expressed from a plasmid.)

MIC_{90} values three- and 30-fold higher, respectively, clearly attributable to derepressed AmpC production (Table 2). Further reports [33–35] have also indicated that up to 40% of isolates (1987–91) had stably derepressed β-lactamases.

Over the following decade, with increased use of broad-spectrum β-lactams, resistance levels rose markedly throughout the world in general such as *Enterobacter* and in *C. freundii*, although there continued to be regional and national differences. International variations in resistance to third-generation cephalosporins have been documented in a review of surveys between selected hospitals in five nations (USA, France, Germany, Italy and Japan) [36]. Cefotaxime, used as an index third-generation cephalosporin, had relatively high susceptibilities in Germany, where 80% and 100% of *E. cloacae* and *S. marcescens* were inhibited by ≤ 8mg/L. In contrast, high levels of resistance were observed in Japan and Italy, where only 57.7% of *E. cloacae* (Japan) and 63.3% of *S. marcescens* (Italy) were susceptible.

High levels of resistance to the third-generation cephalosporins have also been reported from studies in the USA. In one report in 1993, using reference NCCLS tests and breakpoint criteria, only 66–82% of some Enterobacteriaceae remained susceptible to cefotaxime [37]. In another US survey involving >30,000 enteric bacilli isolated during 1994, 18% of *S. marcescens*, 23% of *C. freundii* and 34% of *E. cloacae* were resistant to cefotaxime. These resistance levels have been confirmed by more recent surveillance data [36–38]. Data from 1994–1995, using two standardized methods, indicated that 21–40% of *E. cloacae* isolated from blood, lower respiratory tract, urinary tract and skin and soft tissue infections were resistant to ceftazidime [3,38] (Table 3). In 1995, in a five-hospital study (>1,000 strains/site), 20–30% of strains (depending on the species tested) were resistant to the third-generation cephalosporins [39].

Resistance levels have also increased in other areas of the world, although the incidence varies according to geographic location, the testing method and interpretation criteria used. In a Belgian study conducted in 1993, the susceptibility of 8,625 ICU and hematology patient isolates was examined. Of these, 30% of *E. cloacae* and 41% of *C. freundii* strains were found to be resistant

Table 2 Activity of cefotaxime against Bush Group 1 (Ambler class A) β-lactamase-producing Enterobacteriaceae isolated prior to its widespread clinical use[a]

Organism	Collection source (no. tested)	MIC (mg/L)[b]		No. of refs. cited
		MIC_{50}	MIC_{90}	
Citrobacter freundii	USA (48)	0.11	5.0	4
	World (88)	0.35	18.2	
Enterobacter aerogenes	USA (152)	0.12	1.0	8
	World (42)	0.20	6.3	
Enterobacter cloacae	USA (153)	0.12	1.3	10
	World (245)	6.2	37.0	
Serratia marcescens	USA (597)	0.47	5.2	25
	World (449)	0.82	5.2	

[a]Modified from report of 15,672 enteric bacilli by Jones and Thornsberry [32].
[b]The MIC_{50} and MIC_{90} are the lowest concentration inhibiting growth of 50% and 90% of tested strains, respectively.

Table 3 Rates of resistance to third-generation cephalosporins (ceftazidime) among *E. cloacae* isolates reported in the USA in 1994–1995 [3,38][a]

	Monitored centers (No.)	% susceptible by infection site[b,c]			
		Blood	LRTI	UTI	SSTI
Jones et al. (1995)	43	60	66	74	71
Baron and Jones (1995)	236	75	75	ND	79[c]

[a]Data derived from NCCLS standardized test (disk diffusion and broth microdilution).
[b]Blood = bacteremias; LRTI = lower respiratory tract infections; UTI = urinary tract infections; SSTI = skin and soft tissue infections; ND = Not determined.
[c]Isolates from intra-abdominal and gynaecology wound infections exhibited 74–76% susceptibility to ceftazidime.