ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Mar. 2000, p. 561–567 Vol. 44, No. 3
0066-4804/00/$04.00+0

ampR Gene Mutations That Greatly Increase Class C β-Lactamase Activity in *Enterobacter cloacae*

AKIO KUGA, RYOICHI OKAMOTO, AND MATSUHISA INOUE*

Department of Microbiology, Kitasato University School of Medicine, 1-15-1 Kitasato, Sagamihara, Kanagawa 228-8555, Japan

Received 26 August 1999/Returned for modification 12 November 1999/Accepted 8 December 1999

The *ampC* and *ampR* genes of *Enterobacter cloacae* GN7471 were cloned into pMW218 to yield pKU403. Four mutant plasmids derived from pKU403 (pKU404, pKU405, pKU406, and pKU407) were isolated in an AmpD mutant of *Escherichia coli* ML4953 by selection with ceftazidime or aztreonam. The β-lactamase activities expressed by pKU404, pKU405, pKU406, and pKU407 were about 450, 75, 160, and 160 times higher, respectively, than that expressed by the original plasmid, pKU403. These mutant plasmids all carried point mutations in the *ampR* gene. In pKU404 and pKU405, Asp-135 was changed to Asn and Val, respectively. In both pKU406 and pKU407, Arg-86 was changed to Cys. The ease of selection of AmpR mutations at a frequency of about 10^{-6} in this study strongly suggests that derepressed strains, such as AmpD or AmpR mutants, could frequently emerge in the clinical setting.

Chromosomal class C β-lactamase is an inducible enzyme produced by *Enterobacter cloacae* and many other gram-negative bacilli (4, 14, 17, 29, 31, 38). The AmpD, AmpG, and AmpR proteins are reported to be involved in the induction of class C β-lactamase (32, 33, 35).

AmpD is a novel *N*-acetylmuramyl-L-alanine amidase that participates in the intercellular recycling of peptidoglycan fragments (11, 15). AmpD degrades cytoplasmic 1,6-anhydro-*N*-acetylmuramyl-tripeptide (1,6-anhMurNAc-tripeptide) to release the tripeptide L-Ala-D-Glu-*meso*-diaminopimelic acid (*meso*-DAP) for direct utilization in the construction of new peptidoglycans (15, 16). An *ampD* mutation that results in β-lactamase expression even in the absence of a β-lactamase inducer coincides with the accumulation of 1,6-anhMurNAc-tripeptide (15). Inactivation of AmpD leads to semiconstitutive or hyperinducible overproduction of AmpC in *Citrobacter freundii* and *E. cloacae* (8, 19, 21). On the other hand, AmpD mutants with increased levels of β-lactamase expression show one of three phenotypes (hyperinducible, derepressed, and partially derepressed), which are associated with different mutations or which may depend on environmental regulation of unknown genes (40). AmpG is a transmembrane protein involved in the permease for an *N*-acetylglucosaminyl (GluNAc)-1,6-anhMurNAc-tripeptide (15, 25). Dietz et al. (7) have reported that AmpG primarily affects aD-pentapeptide (disaccharide-pentapeptide; GluNAc-1,6-anhMurNAc-L-Ala-D-Glu-*meso*-DAP-D-Ala-D-Ala), a periplasmic muropeptide that is converted into the cytoplasmic signaling molecule for β-lactamase induction, aM-pentapeptide (monosaccharide-pentapeptide; L1,6-anhMurNAc-L-Ala-D-Glu-*meso*-DAP-D-Ala-D-Ala) (7). Without *ampG*, neither induction nor high-level expression of β-lactamase is possible (20). AmpR acts as a transcriptional activator by binding to a DNA region immediately upstream of the *ampC* promoter (2, 12, 24). In the absence of a β-lactam inducer, AmpR represses the synthesis of β-lactamase by 2.5-fold, whereas expression is induced 10- to 200-fold in the presence of a β-lactam inducer (22, 23). On the other hand, many clinical isolates of the family *Enterobacteriaceae* show high-level production of class C β-lactamase even without induction.

In the present study, we selected mutant strains by culture with an expanded-spectrum cephalosporin and a monobactam and examined the genetic background of *ampC* and *ampR* mutations that conferred high levels of resistance to β-lactam antibiotics, as well as compared the enzyme activity with that of the parental strain. The possible mechanisms by which these mutant strains had a strong response to an expanded-spectrum cephalosporin and a monobactam are discussed.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1. pACYC184 and pMW218 are vector plasmids that confer resistance to tetracycline-chloramphenicol and kanamycin, respectively, and were purchased from Nippon Gene (Tokyo, Japan) (5). pMW218 was derived from pSC101 (3).

Antibiotics. Reference samples of various antibiotics of known potency were kindly supplied in powder form by the respective manufacturers, as follows: ampicillin, Meiji Seika (Tokyo, Japan); cephaloridine, Shionogi (Osaka, Japan); cefotaxime, Nippon Hoechst Marion Roussel (Tokyo, Japan); cefotiam, Takeda Chemical Industries (Osaka, Japan); ceftazidime, Nippon Glaxo (Tokyo, Japan); aztreonam, Eisai (Tokyo, Japan); latamoxef, Shionogi; cefpodoxime, Sankyo (Tokyo, Japan); imipenem, Banyu Pharmaceutical (Tokyo, Japan); cefepime, Bristol-Myers Squibb K. K. (Tokyo, Japan); and kanamycin, Meiji Seika.

Determination of antibiotic sensitivity. The MICs of the antibiotics were determined by the agar dilution method. Briefly, an overnight culture in Muller-Hinton broth (Nissui, Tokyo, Japan) was diluted to about 5×10^7 CFU/ml and was inoculated onto agar plates containing various concentrations of the test antibiotic by using an inoculating device which applied spots of bacterial suspensions containing 5×10^4 CFU.

Transformation of *Escherichia coli*. Plasmid DNAs were isolated and were used to transform *E. coli* ML4947 (AmpD wild type) and ML4953 (AmpD mutant), as well as *E. cloacae* ATCC 13047 and clinical isolates of *E. cloacae*, by electroporation (6, 34, 37).

Cloning of *ampC* and *ampR* genes. Genomic DNA was purified by the procedure of Marmur (28). Plasmid DNA was purified by extracting plasmid DNA by the small-scale alkaline method (37). Restriction enzymes and T4 DNA ligase were purchased from Takara shuzo (Kyoto, Japan) and Nippon Gene, respectively. The plasmid size was calculated from the sizes of the fragments obtained by cleaving the plasmid with restriction enzymes and by using λ phage DNA cleaved with *Hin*dIII as a molecular marker. PCR primers were obtained from Amersham Pharmacia Biotech (Tokyo, Japan). PCR was carried out according to the instructions with the GeneAmp PCR reagent kit (Perkin-Elmer Cetus, Emeryville, Calif.). All PCRs were performed on a Perkin-Elmer Cetus DNA thermal cycler (model 480) (34, 36).

The genomic DNA from *E. cloacae* GN7471 was digested with *Eco*RI. The digested genomic DNAs were shotgun cloned into *Eco*RI-digested plasmid

* Corresponding author. Mailing address: Department of Microbiology, Kitasato University School of Medicine, 1-15-1 Kitasato, Sagamihara, Kanagawa 228-8555, Japan. Phone: 81-427-78-9355. Fax: 81-427-78-9350. E-mail: matsu@kitasato-u.ac.jp.

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.

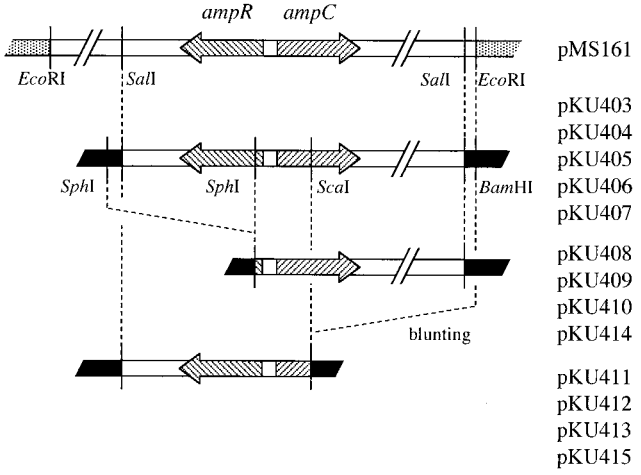

FIG. 1. Cloning strategy for *E. cloacae* GN7471 *ampC* and *ampR*. pMS161 was used to clone an 8-kb *Eco*RI fragment containing *ampC* and *ampR* from *E. cloacae* GN7471 into pACYC184. pKU403 was constructed by cloning a *Sal*I fragment of pMS161 into pMW218. pKU404, pKU405, pKU406, and pKU407 were 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

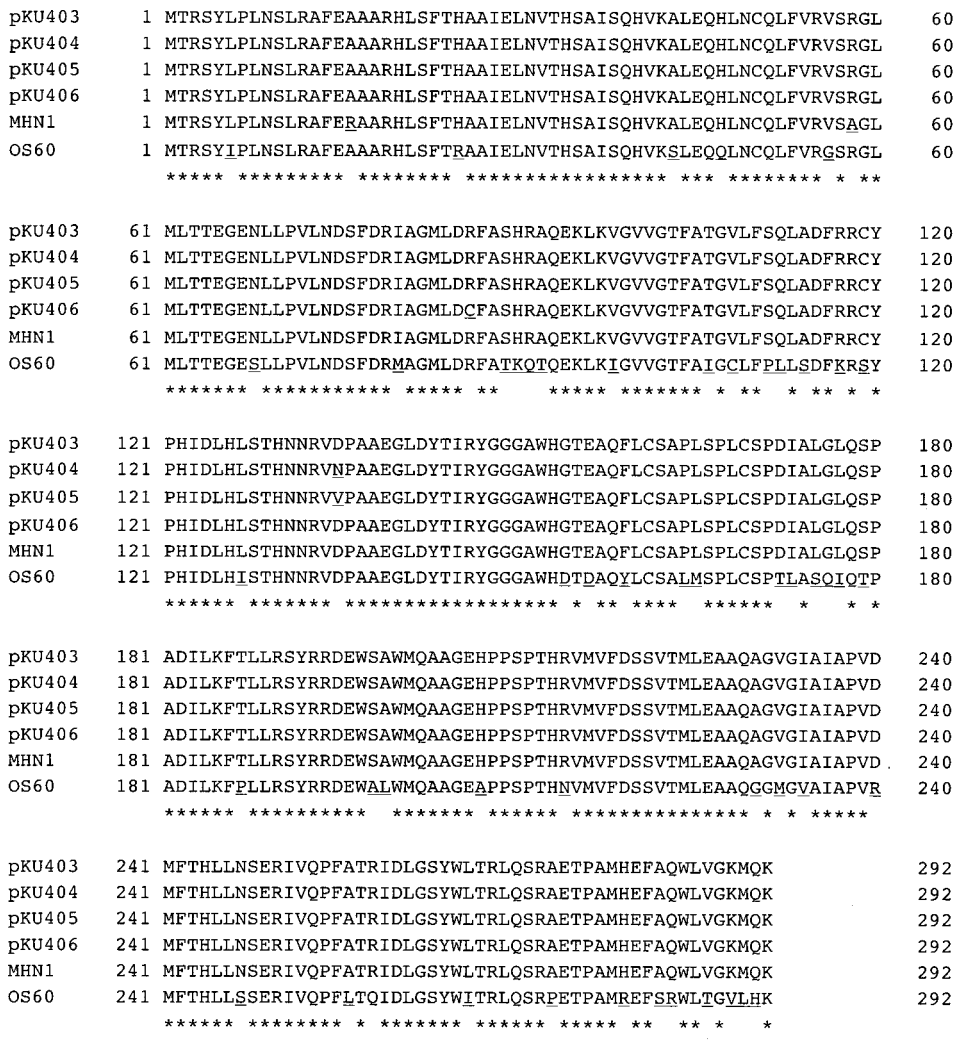

FIG. 2. Comparison of deduced amino acid sequences of AmpR from pKU403, pKU404, pKU405, pKU406, *E. cloacae* MHN1 (12), and *C. freundii* OS60 (24). Identical amino acids in all five sequences are marked with an asterisk. Nonidentical amino acids compared to those in pKU403 are underlined.

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.

ACKNOWLEDGMENTS

We thank Y. Ohya for excellent technical assistance.

This work was supported by grant 09670296 (to M.I.) from the Japanese Ministry of Education and by a grant for diagnosis of antibiotic resistance from the Japanese Ministry of Health and Welfare.

REFERENCES

1. **Bartowsky, E., and S. Normark.** 1991. Purification and mutant analysis of *Citrobacter freundii* AmpR, the regulator for chromosomal AmpC β-lactamase. Mol. Microbiol. **5:**1715–1725.
2. **Bartowsky, E., and S. Normark.** 1993. Interactions of wild-type and mutant AmpR of *Citrobacter freundii* with target DNA. Mol. Microbiol. **10:**555–565.
3. **Bernardi, A., and F. Bernardi.** 1984. Complete sequence of pSC101. Nucleic Acids Res. **12:**9415–9426.
4. **Bush, K., G. A. Jacoby, and A. A. Medeiros.** 1995. A functional classification scheme for β-lactamases and its correlation with molecular structure. Antimicrob. Agents Chemother. **39:**1211–1233.
5. **Chang, A. C. Y., and S. N. Cohen.** 1978. Construction and characterization of

amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. J. Bacteriol. **134:**1141–1156.
6. **Cohen, S. N., A. C. Chang, and L. Hsu.** 1972. Nonchromosomal antibiotic resistance in bacteria: genetic transformation of *Escherichia coli* by R-factor DNA. Proc. Natl. Acad. Sci. USA **69:**2110–2114.
7. **Dietz, H., D. Pfeifle, and B. Wiedemann.** 1997. The signal molecule for β-lactamase induction in *Enterobacter cloacae* is the anhydromuramyl-pentapeptide. Antimicrob. Agents Chemother. **41:**2113–2120.
8. **Ehrhardt, A. F., C. C. Sanders, J. R. Romero, and J. S. Leser.** 1996. Sequencing and analysis of four new *Enterobacter ampD* alleles. Antimicrob. Agents Chemother. **40:**1953–1956.
9. **Fung-Tomc, J. C., E. Gradelski, E. Huczko, T. J. Dougherty, R. E. Kessler, and D. P. Bonner.** 1996. Differences in the resistant variants of *Enterobacter cloacae* selected by extended-spectrum cephalosporins. Antimicrob. Agents Chemother. **40:**1289–1293.
10. **Galleni, M., F. Lindberg, S. Normark, S. Cole, N. Honore, B. Joris, and J.-M. Frere.** 1988. Sequence and comparative analysis of three *Enterobacter cloacae ampC* β-lactamase genes and their products. Biochem. J. **250:**753–760.
11. **Holtje, J. V., U. Kopp, A. Ursinus, and B. Wiedemann.** 1994. The negative regulator of β-lactamase induction AmpD is a *N*-acetyl-anhydromuramyl-L-alanine amidase. FEMS Microbiol. Lett. **122:**159–164.
12. **Honore, N., M. H. Nicolas, and S. T. Cole.** 1986. Inducible cephalosporinase production in clinical isolates of *Enterobacter cloacae* is controlled by a regulatory gene that has been deleted from *Escherichia coli*. EMBO J. **5:**3709–3714.
13. **Inoue, M., J. Itoh, and S. Mitsuhashi.** 1983. pMS76, a plasmid capable of amplification by treatment with chloramphenicol. Plasmid **9:**86–97.
14. **Jacobs, C., J. M. Frere, and S. Normark.** 1997. Cytosolic intermediates for cell wall biosynthesis and degradation control inducible β-lactam resistance in gram-negative bacteria. Cell **88:**823–832.
15. **Jacobs, C., L. Huang, E. Bartowsky, S. Normark, and J. T. Park.** 1994. Bacterial cell wall recycling provides cytosolic muropeptides as effectors for β-lactamase induction. EMBO J. **13:**4684–4694.
16. **Jacobs, C., B. Joris, M. Jamin, K. Klarsov, J. V. Beeumen, D. Mengin-Lecreulx, J. V. Heijenoort, J. T. Park, S. Normark, and J. M. Frere.** 1995. AmpD, essential for both β-lactamase regulation and cell wall recycling, is a novel cytosolic *N*-acetylmuramyl-L-alanine amidase. Mol. Microbiol. **15:**553–559.
17. **Jones, R. N., F. Baquero, G. Privitera, M. Inoue, and B. Wiedemann.** 1997. Inducible β-lactamase-mediated resistance to third-generation cephalosporins. Clin. Microbiol. Infect. **3:**7–20.
18. **Joris, B., J. Dusart, J.-M. Frere, J. V. Beeumen, E. L. Emanuel, S. Petursson, J. Gagnon, and S. G. Waley.** 1984. The active site of the P99 β-lactamase from *Enterobacter cloacae*. Biochem. J. **223:**271–274.
19. **Kopp, U., B. Wiedemann, S. Lindquist, and S. Normark.** 1993. Sequences of wild-type and mutant *ampD* genes of *Citrobacter freundii* and *Enterobacter cloacae*. Antimicrob. Agents Chemother. **37:**224–228.
20. **Korfmann, G., and C. C. Sanders.** 1989. *ampG* is essential for high-level expression of AmpC beta-lactamase in *Enterobacter cloacae*. Antimicrob. Agents Chemother. **33:**1946–1951.
21. **Lindberg, F., S. Lindquist, and S. Normark.** 1987. Inactivation of the *ampD* gene causes semiconstitutive overproduction of the inducible *Citrobacter freundii* β-lactamase. J. Bacteriol. **169:**1923–1928.
22. **Lindberg, F., and S. Normark.** 1987. Common mechanism of *ampC* β-lactamase induction in enterobacteria: regulation of the cloned *Enterobacter cloacae* P99 β-lactamase gene. J. Bacteriol. **169:**758–763.
23. **Lindberg, F., L. Westman, and S. Normark.** 1985. Regulatory components in *Citrobacter freundii ampC* β-lactamase induction. Proc. Natl. Acad. Sci. USA **82:**4620–4624.
24. **Lindquist, S., F. Lindberg, and S. Normark.** 1989. Binding of the *Citrobacter freundii* AmpR regulator to a single DNA site provides both autoregulation and activation of the inducible *ampC* β-lactamase gene. J. Bacteriol. **171:**3746–3753.
25. **Lindquist, S., K. Weston-Hafer, H. Schmidt, C. Pul, G. Korfmann, J. Erickson, C. Sanders, H. H. Martin, and S. Normark.** 1993. AmpG, a signal transducer in chromosomal β-lactamase induction. Mol. Microbiol. **9:**703–715.
26. **Livermore, D. M.** 1995. β-Lactamases in laboratory and clinical resistance. Clin. Microbiol. Rev. **8:**557–584.
27. **Maejima, T., Y. Ohya, S. Mitsuhashi, and M. Inoue.** 1987. Cloning and expression of the gene(s) for chromosome-mediated β-lactamase production of *Proteus vulgaris* in *Escherichia coli*. Plasmid **18:**120–126.
28. **Marmur, J.** 1961. A procedure for the isolation of deoxyribonucleic acid from micro-organisms. J. Mol. Biol. **3:**208–218.
29. **Medeiros, A. A.** 1997. Evolution and dissemination of β-lactamases accelerated by generations of β-lactam antibiotics. Clin. Infect. Dis. **24:**19–45.
30. **Minami, S., M. Inoue, and S. Mitsuhashi.** 1980. Purification and properties of a cephalosporinase from *Enterobacter cloacae*. Antimicrob. Agents Chemother. **18:**853–857.
31. **Minami, S., A. Yotsuji, M. Inoue, and S. Mitsuhashi.** 1980. Induction of β-lactamase by various β-lactam antibiotics in *Enterobacter cloacae*. Antimicrob. Agents Chemother. **18:**382–385.
32. **Normark, S.** 1995. β-Lactamase induction in gram-negative bacteria is intimately linked to peptidoglycan recycling. Microb. Drug Resist. **1:**111–114.
33. **Normark, S., E. Bartowsky, J. Erickson, C. Jacobs, F. Lindberg, S. Lindquist, K. W. Hafer, and M. Wikstrom.** 1994. Mechanisms of chromosomal β-lactamase induction in gram-negative bacteria, p. 485–503. *In* J. M. Ghuysen and R. Hakenbeck (ed.), Bacterial cell walls. Elsevier Science B. V., Amsterdam, The Netherlands.
34. **Okamoto, R., T. Okubo, and M. Inoue.** 1996. Detection of genes regulating β-lactamase production in *Enterococcus faecalis* and *Staphylococcus aureus*. Antimicrob. Agents Chemother. **40:**2550–2554.
35. **Park, J. T.** 1995. Why does *Escherichia coli* recycle its cell wall peptides? Mol. Microbiol. **17:**421–426.
36. **Saiki, R. K., D. H. Gelfand, S. Stoffel, S. J. Scharf, R. Higuchi, G. T. Horn, K. B. Mullis, and H. A. Erlich.** 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science **239:**487–491.
37. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
38. **Sanders, C. C., and W. E. Sanders.** 1979. Emergence of resistance to cefamandole: possible role of cefoxitin-inducible β-lactamases. Antimicrob. Agents Chemother. **15:**792–797.
39. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
40. **Stapleton, P., K. Shannon, and I. Phillips.** 1995. DNA sequence differences of *ampD* mutants of *Citrobacter freundii*. Antimicrob. Agents Chemother. **39:**2494–2498.