ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Jan. 1989, p. 63–70
0066-4804/89/010063-08$02.00/0

Vol. 33, No. 1

Chromosomal β-Lactamase of *Klebsiella oxytoca*, a New Class A Enzyme That Hydrolyzes Broad-Spectrum β-Lactam Antibiotics

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Received 6 April 1988/Accepted 12 October 1988

The chromosomally encoded β-lactamase gene of *Klebsiella oxytoca* E23004, a strain resistant to cefoperazone and aztreonam, was cloned and expressed in *Escherichia coli* HB101. The molecular mass and pI of this enzyme were 28 kilodaltons and 7.4, respectively. Although the β-lactamase of *K. oxytoca* hydrolyzed many cephalosporins, including broad-spectrum drugs, the nucleotide sequence and deduced amino acid sequence lacked homology with chromosomal class C β-lactamase genes (*ampC*) of *E. coli* or *Citrobacter freundii*. Rather, about 45% nucleotide sequence homology and 40% deduced amino acid sequence homology were observed between the *K. oxytoca* β-lactamase and TEM-1, a class A β-lactamase which does not efficiently hydrolyze cephalosporins. Values of K_m, relative V_{max}, and relative V_{max}/K_m for the *K. oxytoca* β-lactamase indicated that the enzyme is a penicillinase but that it can hydrolyze cefoperazone effectively and other broad-spectrum cephems weakly. Hence, the chromosomal β-lactamase of *K. oxytoca* E23004 belongs to class A but differences in its amino acid sequence provide a broader spectrum of activity.

Most β-lactamases encountered clinically have been classified into two major groups, class A and class C, based on substrate specificities and amino acid sequence. Class A includes TEM-type β-lactamases (2, 38) and some chromosomally encoded β-lactamases, such as those of *Klebsiella pneumoniae* (3, 26, 31) and *Bacillus cereus* (35). These enzymes have penicillinase activity and inactivate ampicillin and penicillin G preferentially. In contrast, chromosomally encoded β-lactamases of *Escherichia coli* K-12 (11), *Citrobacter freundii* (22), and *Enterobacter cloacae* (14) belong to class C and inactivate many cephalosporins (32), as well as penicillins. Newly developed broad-spectrum β-lactam antibiotics are generally refractory not only to penicillinases but also to the cephalosporinases mentioned above. However, the increasing use of these new broad-spectrum β-lactam antibiotics has caused a profound change in epidemiological patterns of bacterial isolates in hospitals. Recently, there has been an increase in the number of indole-positive *Klebsiella* strains identified as *Klebsiella oxytoca* which have been isolated from patients with pneumonia, bacteremia, urinary tract infection, and enterocolitis. Some of these isolates are resistant to the new broad-spectrum β-lactam antibiotics (20, 39). In this paper, we report the sequence of a chromosomally encoded β-lactamase gene of *K. oxytoca*, together with the enzymological characterization of its product. The deduced amino acid sequence at the active site of the β-lactamase from *K. oxytoca* E23004 was compared with that of other class A β-lactamases.

MATERIALS AND METHODS

Bacterial strains, plasmids, and bacteriophages. Strains used for the cloning and analysis of the β-lactamase gene are listed in Table 1. Reidentification of *K. pneumoniae* SC10436, kindly provided by K. Bush, The Squibb Institute for Medical Research, was done in our laboratory by biochemical tests including indole production, pectate degradation, and gentisate utilization (28). For the sources and characteristics of *Klebsiella* and other strains of the family *Enterobacteriaceae* used for Southern hybridization analysis, see the legend to Fig. 4 and Table 7. Bacteria were grown in L broth or M9 medium (25) supplemented with appropriate antibiotics.

Media and reagents. Restriction endonuclease, T4 DNA ligase, and the nucleotide sequencing kit were purchased from Takara Syuzo Co., Kyoto, Japan. The multiprime DNA labeling kit and [α-^{32}P]dCTP were from Amersham, Buckinghamshire, United Kingdom. The antibiotics were purchased as follows: ampicillin and kanamycin, Meiji-seika Co., Tokyo, Japan; cephaloridine, Glaxo Group Research, Greenford, United Kingdom; cefoperazone, Toyama Chemical Co., Tokyo, Japan; cefotaxime, Farbwerke Hoechst AG, Frankfurt, Federal Republic of Germany; ceftizoxime, Fujisawa Pharmaceutical Co., Osaka, Japan; cefmenoxime, Takeda Pharmaceutical Co., Osaka, Japan; and aztreonam, Eizai Co., Ltd., Tokyo, Japan.

Susceptibility testing. Bacteria were cultured in Mueller-Hinton broth (Becton Dickinson Microbiology Systems, Cockeysville, Md.) at 37°C for 18 h. A 5-μl portion of a 10^6-CFU/ml dilution of each culture was inoculated on a Mueller-Hinton II agar (Becton Dickinson) plate containing serially diluted antibiotics. MICs were determined after incubation at 37°C for 18 h.

Purification of β-lactamase. *E. coli* HB101(pKOB5-3) was cultured to late log phase (approximately 5×10^9 CFU/ml) at 37°C in 3 liters of L broth containing 25 μg of kanamycin per ml. Cells were harvested by centrifugation and washed twice with 50 mM phosphate buffer (pH 7.0) and suspended in 3 ml of the same buffer. For preparation of cell extracts, the cells were ruptured by ultrasonic treatment at 4°C. Cell debris was removed by centrifugation at 40,000 × *g* for 1 h. The supernatant was used for most of the β-lactamase assays. Further purification was as follows. The supernatant was chromatographed through a Sephadex G-75 (Pharmacia, Uppsala, Sweden) column preequilibrated with 50 mM phosphate buffer (pH 7.0). Elution was performed with the same buffer. Fractions with activity were pooled and concentrated

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TABLE 1. Bacterial strains, phages, and plasmids used

Bacterial strain, phage, or plasmid	Characteristics	Source or reference
Strains		
Klebsiella oxytoca E23004	Clinical isolate, a cefoperazone-resistant strain isolated in Tokyo, Japan (1980)	K. Katu, Tsukuba Research Laboratories, Eizai Co. Ltd.
K. pneumoniae LEN-1	Laboratory strain (*leu*, O3:K1−)	3
K. pneumoniae SC10436	Laboratory strain (reidentified as *K. oxytoca*)	K. Bush, The Squibb Institute for Medical Research (12)
Escherichia coli D21	*his-51 trp-30 proA23 lac-28 ampA1 tsx-81 strA173*	B. Bachmann, *E. coli* Genetic Stock Center, Yale University
E. coli HB101	F⁻ *hsdS20* (r⁻, m⁻) *recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1 supE44* λ⁻	A. Ohta, Department of Biochemistry, Saitama University
E. coli JM109	*recA1* Δ(*lac pro*) *endA1 gyrA96 thi-1 hsdR⁻ supE44 relA1* F'(*traD36 proA⁺ B⁺ lacI^q ZΔM15*)	Takara Syuzo Co.
Plasmids and phages		
pMK16	Cloning vector; Km^r Tc^r	A. Ohta
M13 mp10 and mp11	Vectors for DNA sequencing	Takara Syuzo Co.
Clones		
pKOB5-3	Recombinant plasmid encoding β-lactamase gene of *K. oxytoca* E23004	This study
pMKampl-6	Recombinant plasmid encoding β-lactamase gene of *K. pneumoniae* LEN-1	3
pDAB22	Recombinant plasmid encoding *ampC* gene of *E. coli* D21	This study

by using Centricut U-10 (Kurabo Co., Osaka, Japan). To remove the few remaining proteins, the β-lactamase was injected into a TSK3000 (Toyo Soda Co., Tokyo, Japan) column and high-performance liquid chromatography gel filtration was performed in buffer (50 mM sodium phosphate, 100 mM NaCl [pH 7.0]). Centricut was used for the final concentration of enzyme solution. The molecular mass and pI of the purified enzyme were determined by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and isoelectric focusing (Multiphor II; LKB, Bromma, Sweden), respectively.

Enzyme assays. β-Lactamase activities were determined with a spectrophotometer (model 557; Hitachi, Tokyo, Japan) at 30°C in 50 mM phosphate buffer (pH 7.0). The molar extinction coefficients used were as follows: for ampicillin (235 nm), $\Delta\varepsilon = 1.2\ mM^{-1}\ cm^{-1}$; for cephaloridine (295 nm), $\Delta\varepsilon = 1.0\ mM^{-1}\ cm^{-1}$; for cefoperazone (276 nm), $\Delta\varepsilon = 9.0\ mM^{-1}\ cm^{-1}$; for cefotaxime (264 nm), $\Delta\varepsilon = 7.25\ mM^{-1}\ cm^{-1}$; for ceftizoxime (250 nm), $\Delta\varepsilon = 7.0\ mM^{-1}\ cm^{-1}$; for cefmenoxime (280 nm), $\Delta\varepsilon = 3.12\ mM^{-1}\ cm^{-1}$; and for aztreonam (315 nm), $\Delta\varepsilon = 0.68\ mM^{-1}\ cm^{-1}$. These values were calculated by the method of Seeberg et al. (32). K_m and V_{max} values were obtained by a least-squares fit to plots of the initial steady-state velocities at different substrate concentrations. Relative V_{max} and relative V_{max}/K_m values were calculated for comparison of enzyme activities, as recommended by Bush and Sykes (5). Purified β-lactamase was used for studies with cefoperazone, cefotaxime, ceftizoxime, cefmenoxime, and aztreonam.

Preparation of DNA. Plasmid DNA was prepared by the rapid alkaline extraction method and was purified by NA-agarose (Pharmacia) gel electrophoresis followed by electroelution (25). Bacterial chromosomal DNA was extracted by the method of Stauffer et al. (37).

Cloning of β-lactamase genes. Fragments of completely restriction enzyme-digested genomic DNA were ligated into the same restriction enzyme cloning site of pMK16. *E. coli* HB101 was transformed with the ligated DNA, and ampicillin-resistant colonies were selected on an L agar plate (25) supplemented with 50 μg of ampicillin per ml.

Sequencing. The dideoxynucleotide chain-terminating method of Sanger et al. (30) was used for DNA sequencing of M13 recombinant subclones. The approximately 4-kilobase *Eco*RI-*Sal*I DNA fragment of pKOB5-3 containing the *K. oxytoca* β-lactamase gene was isolated by NA-agarose gel electrophoresis followed by electroelution. To obtain M13 subclones, this fragment was digested partially with a 4-base-recognizing restriction enzyme, *Sau*3AI or *Taq*I, and the resulting fragment was ligated into the *Bam*HI or *Acc*I (= *Sal*I) cloning site of M13 bacteriophage mp10, respectively. Subclones via appropriate endonucleases were also used for sequencing (see Fig. 1). Sequencing was performed in both directions by using the DEAZA sequencing kit (Takara).

DNA hybridization. Transfer of DNA onto nylon membrane (Hybond-N; Amersham) was achieved by the method of Southern (25). As a hybridization probe, the 960-base-pair *Eco*RI-*Sma*I DNA fragment of pKOB5-3 was excised from low-melting-temperature agarose gel (IBI, New Haven, Conn.) after electrophoresis. The gel slice containing the DNA fragment was melted, diluted, and labeled with [α-^{32}P]dCTP by using the multiprime labeling kit. Before the hybridization reaction, the nylon membrane was preincubated in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1 M sodium phosphate (pH 7.0)–2.5× Denhardt solution (26) for 3 h at 65°C. Hybridization was done in 6× SSC–0.1 M sodium phosphate (pH 7.0)–2.5× Denhardt solution–0.1 mM EDTA–5 μg of sheared denatured salmon sperm DNA per ml of hybridization fluid at 65°C for 24 h. Washes were performed in 2× SSC–0.1% SDS two times for 5 min at room temperature and in 0.2× SSC–0.1% SDS at 65°C for 15 min.

Analysis of DNA sequences. DNA sequences were analyzed on a personal computer (PC9801Vm2; NEC, Tokyo, Japan) by using the SDC-GENETYX system (Software Development Co., Tokyo, Japan).

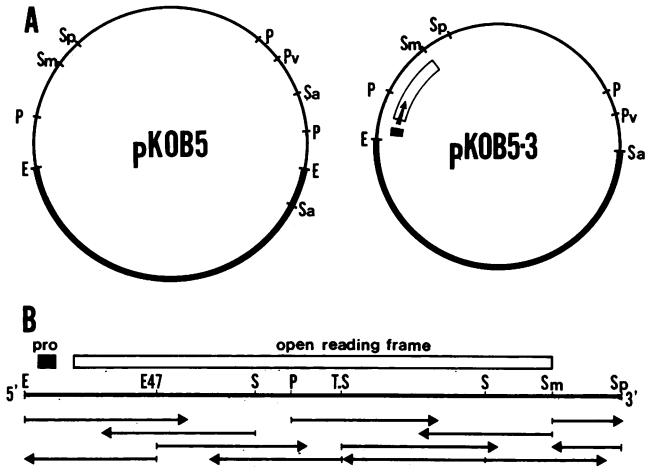

FIG. 1. Map of restriction enzyme cleavage sites of pKOB5 and pKOB5-3 and sequencing strategy. The cleavage sites for restriction endonucleases were as follows: E, *Eco*RI; E47, *Eco*47III; P, *Pst*I; Sa, *Sal*I; S, *Sau*3AI; Sm, *Sma*I; Sp, *Sph*I; and T, *Taq*I. (A) Chromosomal DNA fragment of *K. oxytoca* is represented by the thin line, with the arrow indicating the position and direction of transcription of the β-lactamase structural gene. The thick line represents plasmid pMK16. The small arrow indicates the direction of transcription. (B) The black box (pro) indicates the potential promoter region, and the open box represents the structural gene of β-lactamase. The arrows indicate the direction and extent of each sequence determination.

RESULTS

Cloning. Genomic DNA from *K. oxytoca* E23004, a strain resistant to cefoperazone and aztreonam, was digested completely with *Eco*RI and ligated into pMK16 at its *Eco*RI site. Transformation of *E. coli* HB101 with these recombinants gave six ampicillin-resistant colonies. These clones harbored plasmids containing about 5.5 kb of added DNA. A representative plasmid, pKOB5, was cut with *Sal*I and religated for the deletion of an approximately 1.5-kb *Sal*I DNA fragment. The newly created recombinant plasmid contained an approximately 4-kilobase-pair *Eco*RI-*Sal*I genomic DNA fragment of *K. oxytoca* and carried the intact β-lactamase gene. A subclone termed pKOB5-3 was mapped with restriction enzymes (Fig. 1). The *E. coli ampC* gene (mapped at 94 min on the chromosome) was also cloned in the same way from the genomic DNA of *E. coli* D21, a K-12 strain that produces β-lactamase constitutively. This clone was designated pDAB22 and carried about 9 kb of *E. coli* genomic DNA. Plasmid pMKamp1-6, containing about 3 kb of the chromosomal β-lactamase gene from *K. pneumoniae* LEN-1, was described previously (3).

MICs. Parent strain *K. oxytoca* E23004 showed higher resistance to all broad-spectrum cephems tested than *E. coli* D21, which produces a cephalosporinase constitutively, except perhaps for ceftizoxime. *E. coli* HB101 harboring pKOB5-3 showed a higher level of resistance than the parent or host strains (Table 2).

β-Lactamase activity. Sonic extracts prepared from *E. coli* HB101 harboring pKOB5-3, pMKamp1-6, or pDAB22 were used as crude preparations of β-lactamase. These three preparations had similar values of K_m and V_{max} for ampicillin, but the hydrolytic efficiency (relative V_{max}/K_m) of the *K. oxytoca* β-lactamase for cephaloridine was lower (37.6/100) than that for ampicillin, although it was much higher than the hydrolytic efficiency of the *K. pneumoniae* LEN-1 β-lactamase for cephaloridine. In contrast, the hydrolytic efficiency of the *E. coli* β-lactamase for cephaloridine was about two times higher than that for ampicillin (Table 3). These results indicate that the *K. oxytoca* β-lactamase is primarily a penicillinase rather than a cephalosporinase. However, the purified preparation of *K. oxytoca* β-lactamase showed a very low K_m for cefoperazone compared with other broad-spectrum drugs, so that the relative V_{max}/K_m became high (Table 4). β-Lactamases of *K. pneumoniae* LEN-1 and *E. coli* D21 did not detectably hydrolyze these new β-lactam antibiotics after 2 h of incubation at 30°C.

TABLE 2. Resistance profiles of donor, transconjugant, and recipient strains

Antibiotic	MIC (μg/ml) for:				
	K. oxytoca E23004	*E. coli* HB101 (pKOB5-3)	*E. coli* HB101	*E. coli* D21	*E. coli* HB101 (pDAB22)
Ampicillin	>100	>100	3.2	25	>100
Cephaloridine	>100	>100	1.6	6.25	50
Cefoperazone	>100	>100	0.4	0.2	0.8
Cefmenoxime	0.2	0.8	<0.025	0.1	0.4
Cefotaxime	0.2	0.8	<0.025	0.1	0.8
Ceftizoxime	0.05	0.1	<0.025	0.1	3.13
Aztreonam	6.25	50	0.025	0.8	12.5

Purification of β-lactamase. About 100 mg of purified enzyme was obtained from 3 liters of culture medium. The purified enzyme gave a clear single band on SDS-polyacrylamide gel electrophoresis and isoelectric focusing, and no other bands were detected by Coomassie blue staining. Therefore, we supposed the purity of the enzyme to be above 95%. Its apparent molecular mass was estimated to be 28 kilodaltons (Fig. 2A). The pI of the enzyme was 7.4 (Fig. 2B).

DNA sequencing. The 4-kb *Eco*RI-*Sal*I fragment of pKOB5-3 was sequenced. The strategy and the fragments used for dideoxy sequencing of the β-lactamase gene are shown in Fig. 1. The complete nucleotide sequence of the *K.*

TABLE 3. Hydrolysis of β-lactam antibiotics by *K. oxytoca* β-lactamase and class A and class C β-lactamases

Antibiotic and characteristic	β-Lactamase from:		
	E. coli HB101 (pKOB5-3)	*E. coli* HB101 (pMKamp1-6)	*E. coli* HB101 (pDAB22)
Ampicillin			
K_m (μM)	2.87×10^1	9.47×10^1	4.06×10^1
V_{max} (μM/min)	6.98	1.98	4.68
Relative V_{max}	100[a]	100	100
Relative V_{max}/K_m	100	100	100
Cephaloridine			
K_m (μM)	9.60×10^1	1.35×10^3	7.48×10^2
Relative V_{max}	126	26.8	4,130
Relative V_{max}/K_m	37.6	1.88	225
Cefotaxime			
K_m (μM)	3.10×10^2	ND[b]	ND
Relative V_{max}	1.98		
Relative V_{max}/K_m	0.183		

[a] The relative V_{max} and relative V_{max}/K_m for each antibiotic were normalized with respect to ampicillin.

[b] ND, Not detected. Hydrolytic activities of both *K. pneumoniae* and *E. coli* β-lactamases for cefotaxime were not detected after 2 h of incubation at 30°C.

TABLE 4. Hydrolysis of β-lactam antibiotics by purified β-lactamase of *K. oxytoca*

Antibiotic	K_m (μM)	Relative V_{max}	Relative V_{max}/K_m
Cefoperazone	1.70	85	1,570
Cefotaxime	3.15×10^2	100[a]	100
Ceftizoxime	1.18×10^2	3.84	10.2
Cefmenoxime	1.60×10^2	955	18.7
Aztreonam	4.36×10^3	1,550	111

[a] Hydrolysis rates for each antibiotic were normalized with respect to cefotaxime.

oxytoca β-lactamase gene, its 5′- and 3′-flanking regions, and the deduced amino acid sequence are shown in Fig. 3. There was an 840-nucleotide open reading frame specifying a protein of 280 amino acid residues, which is somewhat longer than that of the mature enzyme. The initiation codon (ATG) was preceded by a possible −10 region (CATAAT) and −35 region (TTGTCA) of a putative promoter. The termination codon (TAA) was followed by several potential inverted repeats. The nucleotide homology of the coding regions between the *K. oxytoca* β-lactamase gene and the *K. pneumoniae* β-lactamase gene (3) was only 45%. No homology of nucleotide sequence was found in the sequence at the promoter region or in the adjacent sequences.

Comparison of amino acid sequences. About 40% homology of amino acid sequence was observed between the β-lactamase of *K. oxytoca* and class A β-lactamases, such as that of *K. pneumoniae* LEN-1 (3) and the TEM-1 β-lactamase mediated by Tn*3* (8) (Table 5). On the other hand, the overall amino acid sequence of *K. oxytoca* β-lactamase had no similarity to that of the *E. coli ampC* cephalosporinase (11). The amino acid sequence of the active site of the enzyme was compared with those of other β-lactamases (Table 6). The consensus sequence at the active site (18) of β-lactamases (-Phe-aa-aa-aa-Ser-aa-aa-Lys-) was found at positions 58 to 65 of the *K. oxytoca* β-lactamase. However, a conspicuous difference from others was a cysteine residue just before the active-site serine residue. It is the sole

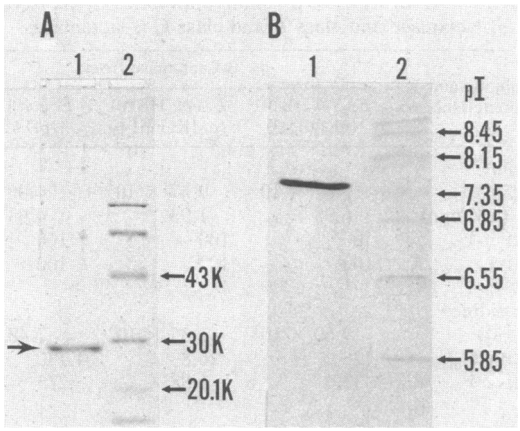

FIG. 2. SDS-polyacrylamide gel electrophoresis and analytical isoelectric focusing of purified β-lactamase. (A) Protein (100 μg) was subjected to SDS-polyacrylamide gel electrophoresis and stained with Coomassie blue. Lanes: 1, purified β-lactamase of *K. oxytoca* (indicated by the arrow); 2, protein molecular weight standards. (B) Purified protein (200 μg) was subjected to isoelectric focusing on a PAG plate (LKB) and stained with Coomassie blue. Lanes: 1, purified β-lactamase; 2, standard proteins as pI markers.

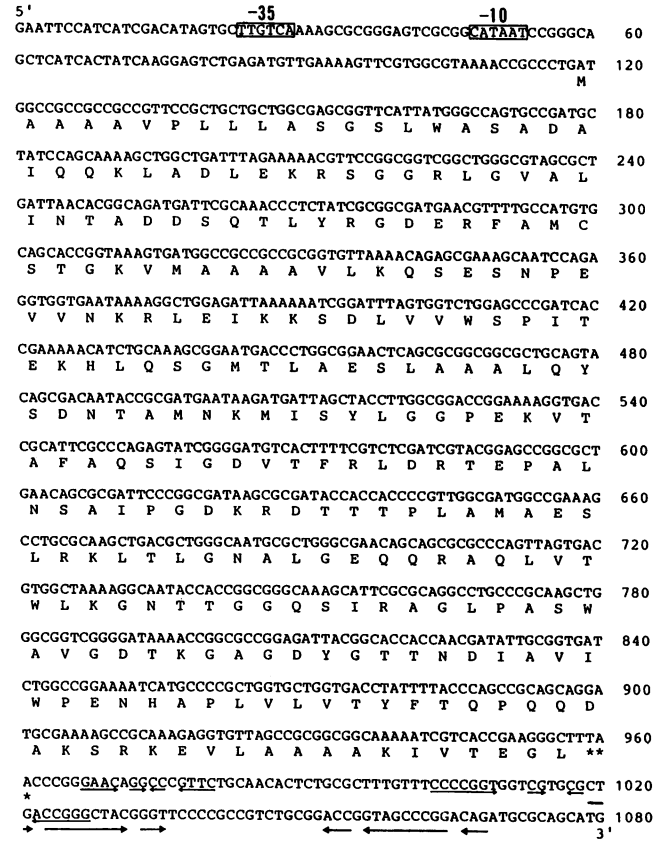

FIG. 3. Nucleotide sequence and deduced amino acid sequence of *K. oxytoca* β-lactamase gene. Nucleotide and deduced amino acid sequences of the complete β-lactamase gene from *K. oxytoca* E23004 are shown, together with the nucleotide sequence of its flanking region. The nucleotide sequence of the antisense strand is numbered from the 5′-terminal *Eco*RI cleavage site. An 840-nucleotide open reading flame is preceded by the presumptive −35 and −10 region (boxed), and the stop codon (TAA) is marked with ***. The arrows indicate regions of possible dyad symmetries. The deduced amino acid residues are indicated by a one-letter code below the corresponding codons.

cysteine residue found in the entire sequence of the *K. oxytoca* β-lactamase, whereas there are two cysteine residues outside the active site in the β-lactamase of *K. pneumoniae* LEN-1 (3) or TEM-1 (2, 38) β-lactamase. It was reported by Joris et al. (15) that the cysteine residue was found just before the active-site serine residue in the K1 β-lactamase of *K. pneumoniae* SC10436 (Table 6). We

TABLE 5. Matrix of amino acid sequence homologies among class A β-lactamases

Source of β-lactamase	Homology (%) of β-lactamase[a]			
	K. oxytoca E23004	*K. pneumoniae* LEN-1	TEM-1	*S. aureus* PC1
K. oxytoca E23004	100	39	40	33
K. pneumoniae LEN-1		100	64	28
TEM-1			100	32
S. aureus PC1				100

[a] These values were calculated based on previously published data for *K. pneumoniae* LEN-1 (3), TEM-1 (2, 38), and *S. aureus* PC1 (1). Calculations were performed with the SDC-GENETYX system by the technique of Lipman and Pearson (24).

TABLE 6. Amino acid sequence at active site of β-lactamases

Bacterial strain	Amino acid sequence of β-lactamase at active site[a]	Reference(s)
Class A		
K. oxytoca E23004	-Phe-Ala-Met-Cys-Ser-Thr-Gly-Lys-	This study
K. pneumoniae SC10436[b]	-Phe-Ala-Met-Cys-Ser-Thr-Ser-Lys-	12
K. aerogenes K1082E[c]	-Phe-Ala-Met-Asn-Ser-Thr-Ser-Lys-	7
K. pneumoniae LEN-1	-Phe-Pro-Met-Val-Ser-Thr-Phe-Lys-	3
TEM-1	-Phe-Pro-Met-Met-Ser-Thr-Phe-Lys-	2, 38
B. cereus 596H	-Phe-Ala-Phe-Ala-Ser-Thr-Thr-Lys-	35
S. aureus PC1	-Phe-Ala-Tyr-Ala-Ser-Thr-Ser-Lys-	1
Class C		
E. coli K-12	-Phe-Glu-Leu-Gly-Ser-Val-Ser-Lys-	11
C. freundii OS60	-Phe-Glu-Leu-Gly-Ser-Val-Ser-Lys-	22
E. cloacae P99	-Phe-Glu-Leu-Gly-Ser-Ile-Ser-Lys-	14
P. aeruginosa 18S	-Phe-Glu-Ile-Gly-Ser-Val-Ser-Lys-	18

[a] The amino acid residues at the active site are shown by the three-letter code. The sequences of amino acid residues of *K. pneumoniae* SC10436, *K. aerogenes* K1082E, *S. aureus* PC1, and *P. aeruginosa* 18S were obtained by peptide sequencing; the others were deduced from DNA sequences.
[b] This strain was confirmed in our laboratory to belong to *K. oxytoca*.
[c] The designation of *K. aerogenes* in the earlier classification was a synonym of *K. pneumoniae* (10).

found, however, that strain SC10436 was indole production positive, pectate degradation positive, and gentisate utilization positive. Therefore, strain SC10436 must be reidentified as a strain of *K. oxytoca*.

Southern hybridization analysis. The 960-base-pair *Eco*RI-*Sma*I DNA fragment of pKOB5-3, which includes the promoter and entire coding region, hybridized strongly with the bands of chromosomal DNA of all *K. oxytoca* strains tested (Fig. 4), whether these strains were resistant or susceptible to β-lactam antibiotics (Table 7). In contrast, the probe hybridized only faintly with the bands of chromosomal DNA extracted from *E. cloacae*. No bands of chromosomal DNA from *K. pneumoniae*, *K. oxytoca*, *E. aerogenes*, *Proteus mirabilis*, *Proteus vulgaris*, and *Serratia marcescens* hybridized detectably with the probe.

DISCUSSION

Resistance to β-lactam antibiotics can be acquired mainly by three mechanisms. Firstly, β-lactamase, produced and secreted inducibly (21, 23) or constitutively, may hydrolyze β-lactam antibiotics in the periplasmic space or outside of the cell wall. Secondly, porins of the outer membranes of many gram-negative bacteria can function as a barrier to the penetration of β-lactam antibiotics into the cell (6). Thirdly, changes may occur in the affinity to β-lactams of the penicillin-binding proteins, which are the target proteins of β-lactam antibiotics and play a role in the synthesis of the peptidoglycan layer (40). This study showed that newly developed broad-spectrum cephalosporins, such as cefoperazone, cefotaxime, cefmenoxime, and ceftizoxime, and a monobactam, aztreonam, were hydrolyzed by the class A β-lactamase of *K. oxytoca*. *E. coli* HB101, which harbors a plasmid encoding the cloned *K. oxytoca* β-lactamase gene, became somewhat more resistant than the parent strain to these β-lactams as the result of gene amplification (Table 2). It is therefore likely that the resistance of *K. oxytoca* E23004 to cefoperazone was the result essentially of the strong hydrolyzing action of its own β-lactamase. This is in contrast to the resistance of *E. cloacae* to cefotaxime that is achieved mostly through the barrier function of outer membrane porin proteins in combination with the weak hydrolyzing activity of its β-lactamase to broad-spectrum β-lactams (6, 41).

The genus *Klebsiella* is classified into four species, *K. pneumoniae*, *K. oxytoca*, *K. planticola*, and *K. terrigena* (28). Indole-positive *Klebsiella* strains are either *K. oxytoca* or *K. planticola*. *K. planticola* strains are not easily identified in clinical laboratories because of the lack of tests for identification. However, we found that some 20% of indole-positive *Klebsiella* strains isolated clinically were identified as *K. planticola* (manuscript in preparation). The results of DNA hybridization shown in Fig. 4 revealed that either *K. pneumoniae* strains or indole-positive *K. planticola* strains possessed specific chromosomal DNA sequences which cannot hybridize with the β-lactamase gene of *K. oxytoca*. The substrate profile of the indole-positive *K. planticola* β-lactamase and the observed patterns of resistance to β-lactams of clinical isolates of indole-positive *K. planticola* are similar to those of *K. pneumoniae* rather than *K. oxytoca* (unpublished observation). On this basis, *K. oxytoca* isolates should be discriminated from indole-positive *K. planticola* strains in clinical laboratories for accurate chemotherapy.

The *Eco*RI-*Sma*I fragment of the *K. oxytoca* β-lactamase gene hybridized strongly with DNAs from all *K. oxytoca* strains tested. Otherwise, no hybridization was observed with other *Klebsiella* strains, such as *K. pneumoniae* or *K. planticola*. These results suggest that DNA sequences homologous with the cloned β-lactamase gene from *K. oxytoca* E23004 exist generally on the chromosomes of *K. oxytoca* strains. On the other hand, the same probe hybridized slightly with some chromosomal DNAs from *E. cloacae*. However, the presence of DNA sequences with weak homology does not necessarily mean that their chromosomally encoded β-lactamases have a certain level of similarity to the *K. oxytoca* β-lactamase. Rather, the DNA probe derived from the *K. oxytoca* β-lactamase gene might hybridize partially with other genes, such as those for penicillin-binding proteins (4, 27) or carboxypeptidases (42). Indeed, partial similarities have been found among their DNA sequences (data not shown).

Strong hybridization of a cloned gene probe with all DNAs from various strains of *K. oxytoca* tested was observed, but only two of seven strains show high resistance to cefoperazone and aztreonam (Table 7). Although more studies are required to establish the reason for this finding, it may be speculated that, overall, nucleotide sequences between probe and chromosomal β-lactamase genes of tested strains are highly homologous but that there may be some changes in nucleotide sequences which relate closely to the diversity

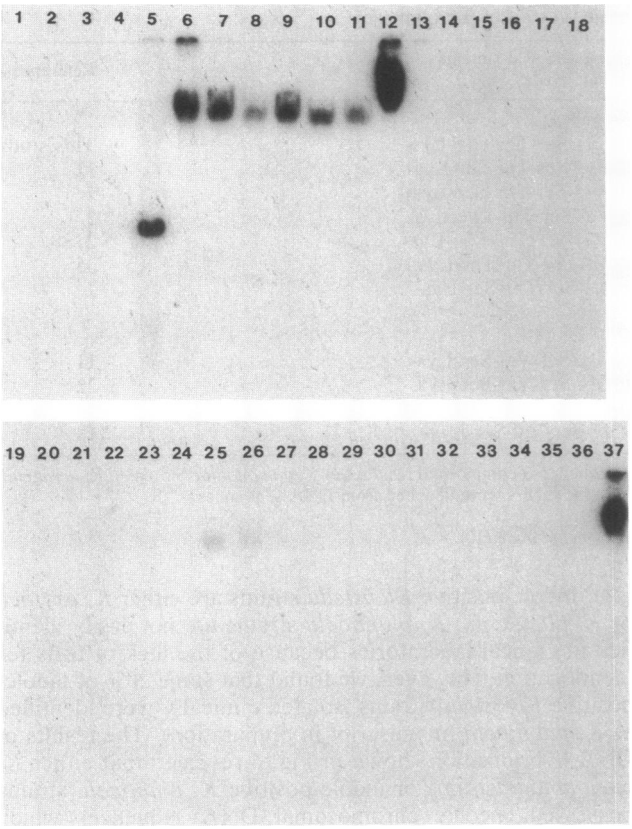

FIG. 4. Southern blot and hybridization analysis of chromosomal DNA. Chromosomal DNA from various strains of the family *Enterobacteriaceae* were hybridized with the *Eco*RI-*Sma*I DNA fragment of the cloned β-lactamase gene of *K. oxytoca*. About 10 μg of DNA was placed in each lane. The DNA probe hybridized strongly with the chromosomal DNAs of all *K. oxytoca* strains tested (lanes 6 to 12). On the other hand, the same probe hybridized only faintly with chromosomal DNA from some strains of *E. cloacae* (lanes 25 to 26). No other DNAs hybridized with the probe. Lanes: 1, *K. pneumoniae* LEN-1 (serotype O3:K1−); 2, *K. pneumoniae* Chedid (O1:K2); 3, *K. pneumoniae* E5051 (O2:K5); 4, *K. pneumoniae* Klebs. 919 (O1:K10); 5, chromosomal DNA of *K. oxytoca* E23004 digested with *Eco*RI; 6, *K. oxytoca* E23004; 7, *K. oxytoca* G3 (K43); 8, *K. oxytoca* A5 (K55); 9, *K. oxytoca* M5 (K60); 10, *K. oxytoca* Y13 (K65); 11, *K. oxytoca* N18; 12, *K. oxytoca* E1 (K3); 13, *K. planticola* S5; 14, *K. planticola* Y4 (K58); 15, *K. planticola* D2; 16, *K. planticola* J1; 17, *K. planticola* H20 (K2); 18, *K. planticola* 132 (K3); 19, *E. aerogenes* 1; 20, *E. aerogenes* 16; 21, *E. aerogenes* 45; 22, *E. aerogenes* 50; 23, *E. cloacae* 3S; 24, *E. cloacae* 12H; 25, *E. cloacae* 35; 26, *E. cloacae* 46; 27, *E. cloacae* 56; 28, *P. mirabilis* PM2; 29, *P. mirabilis* 3045; 30, *P. vulgaris* 3849; 31, *P. vulgaris* PV2; 32, *P. vulgaris* PV1; 33, *S. marcescens* 7; 34, *S. marcescens* 8; 35, *S. marcescens* 23; 36, *S. marcescens* 21; 37, *K. oxytoca* E23004. The strains shown in lanes 1 and 2 were laboratory strains. The strains shown in lanes 3 and 4 were *Klebsiella* K-antigen reference strains provided by I. Ørskov, International Escherichia and Klebsiella Center, Copenhagen, Denmark. All the other strains were clinically isolated from various general hospitals in Japan during the past several years.

in substrate specificities of these enzymes. This speculation is supported by the fact that only two amino acid differences were observed between TEM-2 and CTX-1 (S. Goussard, W. Sougakoff, G. Gerbaud, and P. Courvalin, Program Abstr. 27th Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 517, 1987).

The group of β-lactamases that hydrolyze broad-spectrum

TABLE 7. MICs for bacterial strains used for Southern hybridization analysis

Lane[a]	Strain[b] (serotype)	MIC (μg/ml)[c]		
		CPZ	CZX	AZT
6	*K. oxytoca* E23004[d]	>100	0.05	6.25
7	*K. oxytoca* G3 (K43)	0.2	<0.025	<0.025
8	*K. oxytoca* A5 (K55)	>100	0.1	25
9	*K. oxytoca* M5 (K60)	0.8	<0.025	0.05
10	*K. oxytoca* Y13 (K65)	0.4	<0.025	0.1
11	*K. oxytoca* N18[d]	1.6	<0.025	0.4
12	*K. oxytoca* E1 (K3)	1.6	<0.025	0.1
13	*K. planticola* S5[d]	0.2	<0.025	0.05
14	*K. planticola* Y4 (K58)	0.1	<0.025	<0.025
15	*K. planticola* D2[d]	0.8	<0.025	0.1
16	*K. planticola* J1[d]	0.2	<0.025	0.05
17	*K. planticola* H20 (K2)	0.4	0.05	<0.025
18	*K. planticola* 132 (K3)	0.1	<0.025	0.05

[a] Figure 4.
[b] All strains were clinically isolated from various general hospitals in Japan during the past several years.
[c] CPZ, Cefoperazone; CZX, ceftizoxime; AZT, aztreonam.
[d] These strains have unclassifiable K-antigen types.

β-lactams includes β-lactamases of *K. oxytoca*, SHV-2 (a mutant that originated from plasmid-encoded SHV-1 β-lactamase) (17, 19), CTX-1 (16, 34), CAZ-1 (29), and RHH-1 (36). Most of these new enzymes have been found in *Klebsiella* spp., and a feature of these plasmid-mediated β-lactamases is that they all belong to class A. It is puzzling why so many of the extended-spectrum β-lactamases have turned up in *Klebsiella* spp., which have class A β-lactamase genes on their chromosomes. We have reported the close evolutionary relationship between the TEM β-lactamase gene and the chromosomal β-lactamase gene of *K. pneumoniae* (3). Furthermore, only two amino acid differences between TEM-2 and CTX-1 were reported, and it has also been suggested by Southern hybridization analysis (29) that the CAZ-1 β-lactamase derives from a TEM-type β-lactamase. Therefore, it is possible to speculate that these plasmid-mediated new β-lactamases have something to do with the chromosomal β-lactamase of *Klebsiella* spp.

Because the entire amino acid sequences of the extended-spectrum β-lactamases other than that of *K. oxytoca* are not available, it is impossible to identify precisely the consensus structure of the class A enzymes which hydrolyze broad-spectrum β-lactamas. It has been reported that thiol-β-lactamase can hydrolyze β-lactams (33). *K. oxytoca* β-lactamase has a thiol group and a hydroxyl group at the active site. The presence of a cysteine residue at the active site is not unique to the *K. oxytoca* β-lactamase. A cysteine just before the active-site serine was found in the β-lactamases of *Streptomyces albus* G (15) and *K. pneumoniae* SC10436 (12). However, the latter strain was reidentified as a strain of *K. oxytoca* in our laboratory. The hydrolysis of new β-lactam antibiotics by the *K. pneumoniae* SC10436 β-lactamase has not been reported. In preliminary experiment, the sulfhydryl inhibitor (*p*-chloromercuribenzoic acid) did not decrease the hydrolytic activity of the *K. oxytoca* β-lactamase. These findings suggest that the cysteine just before the active-site serine may not be essential for the hydrolysis of broad-spectrum β-lactams and that other portions of the amino acid sequence participate in the geometry of the active site (9).

We have reported that the gene probes derived from the *K. pneumoniae* LEN-1 β-lactamase gene hybridized strongly with all the chromosomal DNAs extracted from

strains of *K. pneumoniae* which had been isolated in Japan, the United States, and Europe (3). In this study, we also investigated hybridization of the *K. oxytoca* E23004 β-lactamase gene with many DNAs from *K. oxytoca* strains isolated recently from clinical specimens. These findings may be interpreted as reflecting the diversity among *Klebsiella* species. It is one of the most characteristic points that there are considerable differences in nucleotide and amino acid sequences within the β-lactamase genes of *Klebsiella* species (3, 7, 12), whereas the *ampC* genes of *E. coli*, *E. cloacae*, and *C. freundii* show highly conserved nucleotide or amino acid sequences (11, 13, 22).

ACKNOWLEDGMENTS

We thank Kanemasa Katu, Tsukuba Research Laboratories, Eizai Co., Ltd., Ibaraki, Japan, for the gift of *K. pneumoniae* E23004; Karen Bush, The Squibb Institute for Medical Research, Princeton, N.J., for providing *K. pneumoniae* SC10436; A. Ohta, Department of Biochemistry, Saitama University, Saitama, Japan, for providing *E. coli* HB101 and plasmid pMK16; and B. Bachmann, *E. coli* Genetic Stock Center, Yale University, New Haven, Conn., for providing *E. coli* D21.

This work was supported by the Radio-isotope Center Medical Division, Nagoya University.

ADDENDUM

After the submission of our manuscript, we received a letter from K. Bush which told us of the reidentification of *K. pneumoniae* SC10436 as a strain of *K. oxytoca*.

LITERATURE CITED

1. **Ambler, R. P.** 1975. The amino acid sequence of *Staphylococcus aureus* penicillinase. Biochem. J. **151**:197–218.
2. **Ambler, R. P., and G. K. Scott.** 1978. Partial amino acid sequence of penicillinase coded by *Escherichia coli* plasmid R6K. Proc. Natl. Acad. Sci. USA **75**:3732–3736.
3. **Arakawa, Y., M. Ohta, N. Kido, Y. Fujii, T. Komatsu, and N. Kato.** 1986. Close evolutionary relationship between the chromosomally encoded β-lactamase gene of *Klebsiella pneumoniae* and the TEM β-lactamase gene mediated by R plasmids. FEBS Lett. **207**:69–74.
4. **Broome-Smith, J. K., A. Edelman, S. Yousif, and B. G. Spratt.** 1985. The nucleotide sequences of the *ponA* and *ponB* genes encoding penicillin-binding proteins 1A and 1B of *Escherichia coli* K12. Eur. J. Biochem. **147**:437–446.
5. **Bush, K., and R. B. Sykes.** 1986. Methodology for the study of β-lactamases. Antimicrob. Agents Chemother. **30**:6–10.
6. **Bush, K., S. K. Tanaka, D. P. Bonner, and R. B. Sykes.** 1985. Resistance caused by decreased penetration of β-lactam antibiotics into *Enterobacter cloacae*. Antimicrob. Agents Chemother. **27**:555–560.
7. **Emanuel, E. L., J. Gagnon, and S. G. Waley.** 1986. Structural and kinetic studies on β-lactamase K1 from *Klebsiella aerogenes*. Biochem. J. **234**:343–347.
8. **Heffron, F., and B. J. McCarthy.** 1979. DNA sequence analysis of the transposon Tn*3*: three genes and three sites involved in transposition of Tn*3*. Cell **18**:1153–1163.
9. **Herzberg, O., and J. Moult.** 1987. Bacterial resistance to β-lactam antibiotics: crystal structure of β-lactamase from *Staphylococcus aureus* PC1 at 2.5 Å resolution. Science **236**:694–701.
10. **Hormes, B., and R. J. Gross.** 1983. Coliform bacteria; various other members of the *Enterobacteriaceae*, *Klebsiella*, p. 292–296. *In* M. T. Parker (ed.), Topley and Wilson's principles of bacteriology, virology and immunity, 7th ed. vol. 2. Edward Arnold Ltd., London.
11. **Jaurin, B., and T. Grundström.** 1981. ampC cephalosporinase of *Escherichia coli* K-12 has a different evolutionary origin from that of β-lactamases of the penicillinase type. Proc. Natl. Acad. Sci. USA **78**:4897–4901.
12. **Joris, B., F. De Meester, M. Galleni, J. M. Frère, and J. Van Beeumen.** 1987. The K1 β-lactamase of *Klebsiella pneumoniae*. Biochem. J. **243**:561–567.
13. **Joris, B., F. De Meester, M. Galleni, G. Reckinger, J. Coyette, and J. M. Frere.** 1985. The β-lactamase of *Enterobacter cloacae* P99. Biochem. J. **228**:241–248.
14. **Joris, B., J. Dusart, J. M. Frere, J. Van 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.
15. **Joris, B., J.-M. Ghuysen, G. Dive, A. Renard, O. Dideberg, P. Charlier, J.-M. Frère, J. A. Kelly, J. C. Boyington, P. C. Moews, and J. R. Knox.** 1988. The active-site serine penicillin-recognizing enzymes as members of the *Streptomyces* R61 DD-peptidase family. Biochem. J. **250**:313–324.
16. **Kitzis, M. D., D. Billot-Klein, F. W. Goldstein, R. Williamson, G. Tran Van Nhieu, J. Carlet, J. F. Acar, and L. Gutmann.** 1988. Dissemination of the novel plasmid-mediated β-lactamase CTX-1, which confers resistance to broad-spectrum cephalosporins, and its inhibition by β-lactamase inhibitors. Antimicrob. Agents Chemother. **32**:9–14.
17. **Kliebe, C., B. A. Nies, J. F. Meyer, R. M. Tolxdorff-Neutzling, and B. Wiedemann.** 1985. Evolution of plasmid-coded resistance to broad-spectrum cephalosporins. Antimicrob. Agents Chemother. **28**:302–307.
18. **Knott-Hunziker, V., S. Petursson, G. S. Jayatilake, S. G. Waley, B. Jaurin, and T. Grundström.** 1982. Active sites of β-lactamases. Biochem. J. **201**:621–627.
19. **Korfmann, G., C. Kliebe, and B. Wiedemann.** 1986. β-Lactam antibiotics and selection of resistance: speculation on the evolution of R-plasmids. J. Antimicrob. Chemother. **18**:113–121.
20. **Labia, R., A. Morand, M. Guionie, M. Heitz, and J. S. Pitton.** 1986. Bêtalactamases de *Klebsiella oxytoca*: etude de leur action sur les céphalosporines de troisième génération. Pathol. Biol. **34**:611–615.
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.** 1986. Sequence of the *Citrobacter freundii* OS60 chromosomal *ampC* β-lactamase gene. Eur. J. Biochem. **156**:441–445.
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. **Lipman, D. J., and W. R. Pearson.** 1985. Rapid and sensitive protein similarity searches. Science **227**:1435–1441.
25. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
26. **Matsumoto, H., T. Sawai, T. Tazaki, S. Yamagishi, and S. Mitsuhashi.** 1972. Characterization of the chromosomally mediated penicillinase in *Klebsiella pneumoniae*. Jpn. J. Microbiol. **16**:169–176.
27. **Nakamura, M., I. Maruyama, M. Soma, J. Kato, H. Suzuki, and Y. Hirota.** 1983. On the process of cellular division in *Escherichia coli*: nucleotide sequence of the gene for penicillin-binding protein 3. Mol. Gen. Genet. **191**:1–9.
28. **Ørskov, I.** 1984. Genus V. *Klebsiella* Trevisan 1885, 105^{AL}, p. 461–465. *In* N. R. Krieg (ed.), Bergey's manual of systematic bacteriology, vol. 1. The Williams & Wilkins Co., Baltimore.
29. **Petit, A., D. L. Sirot, C. M. Chanal, J. L. Sirot, R. Labia, G. Gerbaud, and R. A. Cluzel.** 1988. Novel plasmid-mediated β-lactamase in clinical isolates of *Klebsiella pneumoniae* more resistant to ceftazidime than to other broad-spectrum cephalosporins. Antimicrob. Agents Chemother. **32**:626–630.
30. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
31. **Sawai, T., S. Yamagishi, and S. Mitsuhashi.** 1973. Penicillinases of *Klebsiella pneumoniae* and their phylogenetic relationship to penicillinases mediated by R factors. J. Bacteriol. **115**:1045–1054.

32. **Seeberg, A. H., R. M. Tolxdorff-Neutzling, and B. Wiedemann.** 1983. Chromosomal β-lactamases of *Enterobacter cloacae* are responsible for resistance to third-generation cephalosporins. Antimicrob. Agents Chemother. **23**:918–925.
33. **Sigal, I. S., B. G. Harwood, and R. Arentzen.** 1982. Thiol-β-lactamase: replacement of the active-site serine of RTEM β-lactamase by a cysteine residue. Proc. Natl. Acad. Sci. USA **79**:7157–7160.
34. **Sirot, D., J. Sirot, R. Labia, A. Morand, P. Courvalin, A. Darfeuille-Michaud, R. Perroux, and R. Cluzel.** 1987. Transferable resistance to third-generation cephalosporins in clinical isolates of *Klebsiella pneumoniae*: identification of CTX-1, a novel β-lactamase. J. Antimicrob. Chemother. **20**:323–334.
35. **Sloma, A., and M. Gross.** 1983. Molecular cloning and nucleotide sequence of the type I β-lactamase gene from *Bacillus cereus*. Nucleic Acids Res. **11**:4997–5004.
36. **Spencer, R. C., P. F. Wheat, T. G. Winstanley, D. M. Cox, and S. J. Plested.** 1987. Novel β-lactamase in a clinical isolate of *Klebsiella pneumoniae* conferring unusual resistance to β-lactam antibiotics. J. Antimicrob. Chemother. **20**:919–921.
37. **Stauffer, G. V., M. D. Plamann, and L. T. Stauffer.** 1981. Construction and expression of hybrid plasmids containing the *Escherichia coli glyA* gene. Gene **14**:63–72.
38. **Sutcliffe, J. G.** 1978. Nucleotide sequence of the ampicillin resistance gene of *Escherichia coli* plasmid pBR322. Proc. Natl. Acad. Sci. USA **75**:3737–3741.
39. **Then, R. L., M. P. Glauser, P. Angehrn, and M. Arisawa.** 1983. Cephalosporin resistance in strains of *Klebsiella oxytoca* isolated during antibiotic therapy. Zentralbl. Bakteriol. Mikrobiol. Hyg. **254**:469–479.
40. **Utsui, Y., and T. Yokota.** 1985. Role of an altered penicillin-binding protein in methicillin- and cephem-resistant *Staphylococcus aureus*. Antimicrob. Agents Chemother. **28**:397–403.
41. **Vu, H., and H. Nikaido.** 1985. Role of β-lactam hydrolysis in the mechanism of resistance of a β-lactamase-constitutive *Enterobacter cloacae* strain to expanded-spectrum β-lactams. Antimicrob. Agents Chemother. **27**:393–398.
42. **Waxman, D. J., and J. L. Strominger.** 1980. Sequence of active site peptides from the penicillin-sensitive D-alanine carboxypeptidase of *Bacillus subtilis*. J. Biol. Chem. **255**:3964–3976.