ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Apr. 1994, p. 761–766
0066-4804/94/$04.00+0

Vol. 38, No. 4

Multiply Resistant *Klebsiella pneumoniae* Strains from Two Chicago Hospitals: Identification of the Extended-Spectrum TEM-12 and TEM-10 Ceftazidime-Hydrolyzing β-Lactamases in a Single Isolate

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Received 26 August 1993/Returned for modification 15 December 1993/Accepted 26 January 1994

Ceftazidime-resistant *Klebsiella pneumoniae* strains began to appear when ceftazidime usage was increased in two unrelated Chicago hospitals. These strains produced a β-lactamase with an isoelectric point of 5.6 (RP-5.6) and strong hydrolyzing activity against ceftazidime. Two different restriction digest profiles were associated with the ceftazidime resistance plasmids. A second β-lactamase with a pI of 5.2 (RP-5.2) was coproduced in two representative strains. The second β-lactamase hydrolyzed ceftazidime, cefotaxime, and aztreonam with relative hydrolysis rates of <8% of that observed for benzylpenicillin. Both enzymes were inhibited by clavulanic acid and tazobactam. Nucleotide sequencing of the genes coding for RP-5.2 and RP-5.6 revealed sequences identical to those of the TEM-12 and TEM-10 β-lactamase genes, respectively. Both genes were derived from a TEM-1 sequence related to that of the gene encoded on the Tn*2* transposon. Single point mutations are required to progress from TEM-1 to TEM-12 and from TEM-12 to TEM-10. Extracts from broths grown from single cell isolates of the strain producing TEM-12 and TEM-10 were shown to contain both enzymes. Transconjugants producing either the TEM-12 or the TEM-10 β-lactamase were obtained. A significant finding was that both enzymes were encoded by plasmids with identical restriction digest patterns. These studies show that mutations leading to extended-spectrum β-lactamases can occur sequentially in the same organism, with the genes encoding both enzymes maintained stably.

Extended-spectrum β-lactamases in gram-negative pathogens have been implicated as enzymes responsible for resistance to β-lactam antibiotics such as ceftazidime and aztreonam (12, 23). Initially these enzymes were identified in isolates in western Europe, where major outbreaks of ceftazidime-resistant members of the family *Enterobacteriaceae* have been described, particularly involving *Klebsiella pneumoniae* (7, 14, 24). The first ceftazidime-hydrolyzing extended-spectrum enzymes in the United States were identified almost simultaneously in Boston and Chicago (13, 25). These enzymes have now been fully characterized as TEM-10 β-lactamases with amino acid sequences that differ from the TEM-1 enzyme by two amino acid substitutions (26, 28). The TEM-12 β-lactamase has also been identified in the United States (28, 34) as an enzyme with one amino acid substitution from both the TEM-1 and TEM-10 β-lactamases (18) and may represent the intermediate form of β-lactamase between the two enzymes.

The original *K. pneumoniae* strains that produced the TEM-10 enzyme in Chicago appeared in only two patients in a single hospital within a 2-week period in January 1988 (25). No further identification of the enzyme was made in Chicago until late 1990, when additional ceftazidime-resistant isolates began to be recognized in at least three hospitals. Some of these isolates were also associated with nursing home patients who were referred to the various hospitals (26). In the present study, we showed that at least three different antibiotic-resistant plasmid profiles have been identified in Chicago city hospitals, with six different β-lactamases present among the samples. Most importantly, a single *K. pneumoniae* isolate has been shown to produce two extended-spectrum β-lactamases: TEM-10 and TEM-12.

(This report was presented in part at the 33rd Interscience Conference on Antimicrobial Agents and Chemotherapy, 12–20 October 1993 [abstract 1514].)

MATERIALS AND METHODS

Bacterial strains and epidemiology. *K. pneumoniae* strains with increased resistance to ceftazidime were isolated in 1990 and 1991 from the blood, urine, and sputum of patients at two Chicago area hospitals, Mercy Hospital and Medical Center, a 450-bed primary care facility (strains MH2 through MH6), and Rush-Presbyterian-St. Lukes Hospital, an 800-bed tertiary care institution (strains RP1 through RP24). The two hospitals were separated by several miles and did not share medical staff or training programs. They did, however, draw from the same patient population of several area nursing homes. Representative strains to be examined in this study were chosen by the clinical microbiologists at the two institutions (5, 15).

Streptomycin-resistant *Escherichia coli* MC1061 was used as the recipient for mating experiments. *K. pneumoniae* KC2(pJPQ100) expressing TEM-10; *E. coli* KC2X, a transconjugant containing TEM-10-expressing plasmid pJPQ100; and *E. coli* DH5α(pDAW403) expressing TEM-12 cloned from *E.*

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TABLE 1. Antimicrobial susceptibilities, IEF of β-lactamases, and plasmid profile patterns of *K. pneumoniae* clinical isolates

Strain	MIC (μg/ml)[a]											Isoelectric point(s) of β-lactamase(s)				Plasmid profile pattern
	CAZ	CTX	ATM	AMC	SAM	TIC	TIM	PIP	P/T	IPM	BIA					
KC2	128	0.25	64	16	ND[b]	>128	>128	>128	2	0.25	≤0.06		5.6			A
RP1	>128	4	>128	32	32	>128	>128	>128	32	0.125	≤0.06	5.2	5.6			C
RP3	>128	2	>128	32	32	>128	>128	>128	8	0.125	0.125		5.6			B
RP10	>128	4	128	32	32	>128	>128	>128	4	0.125	≤0.06		5.6	7.6		B
RP16	>128	4	64	16	16	>128	>128	>128	4	0.125	≤0.06		5.6			B
RP23	>128	16	>128	16	32	>128	>128	>128	16	0.125	≤0.06		5.6			B
RP24	>128	4	>128	32	32	>128	>128	>128	16	0.125	≤0.06	5.2	5.6			C
MH2	>128	1	32	32	32	>128	>128	>128	2	0.125	0.125		5.6	7.6		B
MH3	32	0.25	8	16	16	>128	>128	>128	2	0.125	≤0.06		5.6	7.6		B
MH4	>128	2	>128	32	32	>128	>128	>128	4	0.125	0.25		5.6			B
MH5	>128	2	>128	16	32	>128	>128	>128	4	≤0.06	≤0.06		5.6	6.2	7.6	8.3	B
MH6	>128	2	>128	32	32	>128	>128	>128	8	0.125	≤0.06	5.4	5.6				B

[a] MICs were determined in agar dilution tests. Abbreviations: CAZ, ceftazidime; CTX, cefotaxime; ATM, aztreonam; AMC, amoxicillin-clavulanate; SAM, ampicillin-sulbactam; TIC, ticarcillin; TIM, ticarcillin-clavulanate; PIP, piperacillin; P/T, piperacillin-tazobactam; IPM, imipenem; BIA, biapenem.
[b] ND, not done.

coli MG32 were used as reference strains for β-lactamase studies (25, 34).

Identification and susceptibility tests. Initial identification and susceptibility tests were performed with an automated microdilution system (Microscan Walkaway; Baxter). MICs of various β-lactam antibiotics were determined by agar dilution with standard methods (20). MICs of the following antibiotics were determined: piperacillin, tazobactam, and biapenem (Lederle Laboratories, Pearl River, N.Y.); ticarcillin and potassium clavulanate (Beecham Laboratories, Bristol, Tenn.); ceftazidime (Glaxo Group Research Ltd., Greenford, England); cefotaxime (Hoechst-Roussel Pharmaceuticals Inc., Somerville, N.J.); ampicillin, cefoperazone, and sulbactam (Pfizer Inc., New York, N.Y.); imipenem (Merck Sharp & Dohme, Rahway, N.J.); aztreonam (Bristol-Myers-Squibb, Princeton, N.J.); and amoxicillin (Sigma Chemical Company, St. Louis, Mo.). Conditions used to test the β-lactam–β-lactamase inhibitor combinations were as follows: amoxicillin-clavulanate and ampicillin-sulbactam, 2:1 ratio of drug to inhibitor; ticarcillin-clavulanate, constant concentration of 2 μg/ml of inhibitor; piperacillin-tazobactam, constant concentration of 4 μg/ml of inhibitor. Resistance markers of transconjugants to non-β-lactam antibiotics were determined by disk diffusion tests (21).

IEF. Crude preparations of β-lactamases from clinical isolates were made from extracts obtained after four freeze-thaw cycles in 0.2 M sodium acetate (pH 5.5) to release the periplasmic β-lactamase (3). Isoelectric focusing (IEF) was performed by the method of Matthew et al. (19), by using an LKB Multiphor apparatus with prepared PAGplates (pH 3.5 to 9.5; Pharmacia LKB). The isoelectric point of each enzyme was confirmed by activity staining with nitrocefin (Becton Dickinson Microbiology Systems) following IEF.

β-Lactamase assays. β-Lactamases were partially purified from transconjugants producing a single enzyme by using Sephadex G75 chromatography in 0.05 M phosphate buffer (pH 7.0) (32). Initial hydrolysis rates were monitored spectrophotometrically at 25°C in 0.05 M phosphate buffer (pH 7.0) (25). The computer program ENZPACK (Biosoft, Cambridge, England) was used to calculate kinetic parameters by using five methods of calculation (Direct Linear Plot, Lineweaver-Burk, Hanes-Woolf, Eadie-Hofstee, and the method of Wilkinson). Each substrate was analyzed on at least two different days, with either cephaloridine or benzylpenicillin included as a reference each day. Mean coefficients of variation were 29% for relative V_{max} values and 20% for K_m values.

Nucleic acid techniques. Plasmids conferring ceftazidime resistance were transferred from the clinical isolates to a susceptible *E. coli* host by filter mating (8) with selection for resistance to ceftazidime and streptomycin. Plasmid DNA was isolated from the resulting transconjugants by alkaline lysis (2). Restriction enzyme digestions, recombinant DNA techniques, and transformations of plasmid DNA were performed as described by Sambrook et al. (30).

To facilitate DNA sequence analysis, a 15-kb *Bam*HI fragment containing the β-lactamase with a pI of 5.2 from strain RP 1(pCLL3401) was cloned into pCLL2300, a kanamycin resistance-conferring cloning vector (27), and the resulting plasmid was designated pCLL3402. Likewise, a 15-kb *Bam*HI fragment containing the β-lactamase with a pI of 5.6 from plasmid pCLL3403 of the same *K. pneumoniae* strain was cloned into pCLL2300 and designated pCLL3404. One strand of the entire coding sequence of the TEM gene was sequenced by using a nested set of oligonucleotide primers complementary to the TEM coding sequence (26). DNA sequencing was performed on double-stranded plasmid DNA with a Sequenase kit (United States Biochemical) in accordance with the manufacturer's instructions.

RESULTS

Epidemiology. During 1988 and 1989, from 1 to 3% of the *K. pneumoniae* strains isolated at Mercy Hospital were resistant to ceftazidime and aztreonam but not to cefotaxime or ceftriaxone. In 1990, the frequency of resistance to ceftazidime and aztreonam at both hospitals had risen to 15%; however, there was no increase in the frequency of isolates resistant to cefotaxime or ceftriaxone. Concurrently, the amount of ceftazidime used in one of the hospitals (Mercy) rose from 1,000 g in 1987 to 5,888 g in 1991. The levels of cefotaxime and ceftriaxone use remained constant at 12,000 and 3,000 g/year, respectively. Greater than 75% of the patients from whom the resistant strains of *K. pneumoniae* were isolated had received extended-spectrum cephalosporin therapy. In general, these patients were medically unstable, had multiple long-term admissions, and were often in the intensive care unit.

Susceptibility tests. MICs of various β-lactam antibiotics were determined in agar dilution tests with the clinical isolates

TABLE 2. Interactions of β-lactam antibiotics with TEM-10 and TEM-12 β-lactamases

Antibiotic[a]	Value for original strain [enzyme (plasmid)]							
	K. pneumoniae KC2 [TEM-10(pJPQ100)][b]		*K. pneumoniae* RP1 [RP-5.6(pCLL3404)]		*E. coli* MG32 [TEM-12(pDAW403)]		*K. pneumoniae* RP1 [RP-5.2(pCLL3401)]	
	Relative V_{max}[c]	K_m (μM)	Relative V_{max}	K_m (μM)	Relative V_{max}	K_m (μM)	Relative V_{max}	K_m (μM)
Benzylpenicillin	100	6.0	100	7.6	100	20	100	29
Cephaloridine	59	62	45	44	57	100	47	93
Cefotaxime	5.5	46	2.3	16	2.4	94	2.3	56
Ceftazidime	120	150	75	150	3.8	130	3.2	92
Aztreonam	19	28	14	34	6.1	870	8.0	700

[a] The 50% inhibitory concentrations of clavulanic acid, tazobactam, and sulbactam were 4.4 (reference 25), 87 (reference 22), and 940 (reference 25) nM for *K. pneumoniae*(pJPQ100) and 12, 13, and 85 nM for *K. pneumoniae*(pCLL3401), respectively.
[b] These data are from reference 25.
[c] V_{max}, maximum velocity.

(Table 1). All of the *K. pneumoniae* clinical isolates were resistant to ticarcillin, ticarcillin-clavulanate, and piperacillin, with MICs of >128 μg/ml. They were also resistant to ceftazidime, with MICs ranging from 32 to >128 μg/ml. However, these strains had various levels of susceptibility to cefotaxime (MIC range, 0.25 to 16 μg/ml) and aztreonam (MIC range, 8 to >128 μg/ml). Piperacillin-tazobactam had the best activity of the β-lactam–β-lactamase inhibitor combinations, with MICs of 2 to 32 μg/ml for the *K. pneumoniae* strains. The carbapenems imipenem and biapenem were highly active against all of the strains (MIC range, ≤0.06 to 0.25 μg/ml).

IEF. IEF was performed with extracts from the clinical isolates of *K. pneumoniae*. As seen in Table 1, all of the isolates expressed a β-lactamase with a pI of 5.6, consistent with that of a TEM-10 β-lactamase. The original *K. pneumoniae* isolate from Chicago (KC2) possessing TEM-10 expressed only the β-lactamase with a pI of 5.6. However, seven of the strains from the latest outbreak possessed multiple β-lactamases. Four of the strains produced a β-lactamase with a pI of 7.6, consistent with an SHV-type enzyme; two strains produced a β-lactamase with a pI of 5.2; and one strain produced four different β-lactamases (Table 1).

β-Lactamase assays. Strain RP1, possessing two β-lactamases with pIs of 5.2 (β-lactamase RP-5.2) and 5.6 (β-lactamase RP-5.6), was chosen for in-depth analysis. Partially purified RP-5.2 and RP-5.6 enzymes extracted from transconjugants of strain RP1 were used for the hydrolysis studies. For comparison, the TEM-12 β-lactamase from a reference *E. coli* (pDAW403) strain (34) was also purified. Biochemical characteristics of the RP-5.6 β-lactamase were similar to those of the original TEM-10 enzyme (Table 2), in that ceftazidime hydrolysis was faster than hydrolysis of either aztreonam or cefotaxime. In addition, kinetic parameters for hydrolysis by the RP-5.2 β-lactamase were almost identical to those of the original TEM-12 enzyme (Table 2), in that cefotaxime and ceftazidime hydrolysis rates were similar and were lower than the rate of hydrolysis of aztreonam. In contrast to the high rate of ceftazidime hydrolysis by the TEM-10 β-lactamase, all three extended-spectrum β-lactam antibiotics were hydrolyzed by the TEM-12 enzyme at a rate of ≤8% of that of benzylpenicillin. Of note is the high K_m for aztreonam with the TEM-12 enzyme compared with the value observed with the TEM-10 β-lactamase. The 50% inhibitory concentrations of tazobactam and sulbactam for the RP-5.2 β-lactamase were considerably lower than those observed for the TEM-10 β-lactamase (22, 25). Although the 50% inhibitory concentrations of tazobactam for both enzymes were low, piperacillin MICs were reduced to only 16 and 32 μg/ml when tazobactam was added in tests with clinical isolates containing both the RP-5.2 and RP-5.6 β-lactamases, compared with MICs of 2 to 8 μg/ml for almost all of the other strains.

Plasmid characterization. To isolate the plasmids responsible for conferring ceftazidime resistance, the clinical isolates of *K. pneumoniae* were mated to a susceptible *E. coli* host. Transconjugants were selected on the basis of resistance to ceftazidime and streptomycin. The resultant transconjugants of each strain expressed a single β-lactamase with a pI of 5.6. In addition, transconjugants expressing a single β-lactamase with a pI of 5.2 were also obtained from strains RP1 and RP24.

Restriction enzyme digests of plasmids transferring the β-lactamase with a pI of 5.6 were obtained by using *Eco*RI and *Pst*I. Two sets of restriction enzyme digest patterns were detected among these isolates, neither of which was identical to the restriction enzyme digest profile, pattern A, seen with pJPQ100 from strain KC2, the original TEM-10 strain (Table 1). The most common profile, pattern B, was seen in nine of the isolates. This pattern was identical to that seen in a previously reported outbreak from a third Chicago hospital (26). Plasmids with restriction enzyme digest pattern B cotransferred resistance to gentamicin, tobramycin, sulfisoxazole, and trimethoprim-sulfamethoxizole in addition to ceftazidime resistance. Strains RP1 and RP24, expressing resistance markers of gentamicin, sulfamethoxizole, and trimethoprim-sulfamethoxizole, carried identical plasmids with a third restriction enzyme digest profile, pattern C. Plasmids giving the same restriction digest patterns consistently had the same resistance markers.

Strains RP1 and RP24 both expressed β-lactamases with pIs of 5.2 (RP-5.2) and 5.6 (RP-5.6). RP1 was then examined further. β-Lactamase extracts were prepared from culture broths which had been grown from six individual colonies of clinical isolate RP1. Each extract, originating from a single colony, produced both the RP-5.2 and RP-5.6 β-lactamases. However, transconjugants of RP1 produced either the RP-5.2 or RP-5.6 β-lactamase but not both. The transconjugant expressing RP-5.2 and the transconjugant expressing RP-5.6 both acquired resistance to gentamicin, sulfisoxazole, and trimethoprim-sulfamethoxizole in addition to ceftazidime.

Restriction digests of plasmids encoding the RP-5.2 β-lactamase gene from strain RP1 with *Bam*HI, *Bgl*II, *Eco*RI, *Pst*I, and *Sal*I yielded profiles that were identical to those of digests of the plasmid bearing the RP-5.6 β-lactamase gene from the same strain (Fig. 1). In addition, the plasmids expressing either RP-5.2 or RP-5.6 were digested by using *Hha*I, *Hae*III, *Hin*fI,

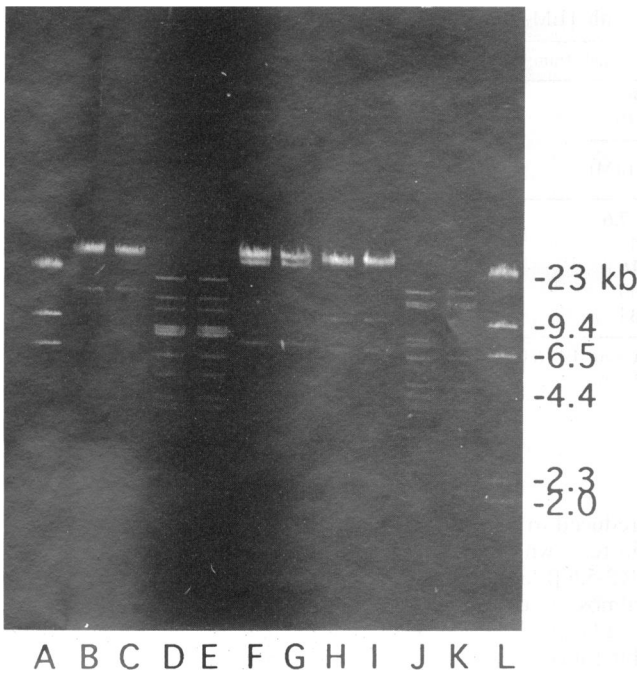

FIG. 1. Restriction digest patterns of plasmids expressing TEM-10 and TEM-12 from *K. pneumoniae* RP1. Lanes: A and L, molecular size marker (λ *Hin*dIII digest); B, pCLL3401 *Bam*HI digest; C, pCLL3403 *Bam*HI digest; D, pCLL3401 *Bgl*II digest; E, pCLL3403 *Bgl*II digest; F, pCLL3401 *Eco*RI digest; G, pCLL3403 *Eco*RI digest; H, pCLL3401 *Pst*I digest; I, pCLL3403 *Pst*I digest; J, pCLL3401 *Sal*I digest; K, pCLL3403 *Sal*I digest.

*Hpa*I, *Sau*3AI, and *Taq*I, restriction enzymes all of which have a four-base recognition sequence. The two plasmids again exhibited identical restriction enzyme digest patterns (data not shown).

Nucleotide sequencing. Nucleotide sequences have been reported previously (26) for two TEM-10 β-lactamase genes, one from pCLL3405 cloned from the original TEM-10 strain, *K. pneumoniae* KC2, and one from pCLL2303, previously cloned from *K. pneumoniae* 2351, a Chicago strain which also had plasmid restriction digest pattern B, common to nine of the current isolates (Table 3). The DNA sequence of the original TEM-10 gene (restriction digest pattern A) corresponds to that of the TEM-1 gene encoded on transposon Tn*2*, whereas the DNA sequence of the TEM-10 gene representing restriction digest pattern B corresponds to the TEM-1 gene encoded on transposon Tn*3*. The determination of TEM-1 gene origin is based upon the three silent nucleotide substitutions between the TEM-1 gene of Tn*2* and the TEM-1 gene of Tn*3* (4, 9, 31). Nucleotide sequences for the β-lactamase genes encoded by plasmids pCLL3402 (cloned from pCLL3401), containing the RP-5.2 β-lactamase gene, and pCLL3404 (cloned from pCLL3403), containing the RP-5.6 β-lactamase gene from strain RP1 (restriction digest pattern C), were determined in the present study and are shown in Table 3. Both β-lactamases appeared to be derived from a TEM-1 gene corresponding to that harbored by transposon Tn*2*. The nucleotide change for pCLL3402, resulting in an amino acid substitution of Ser for Arg-164, was identical to those reported for TEM-12 (18), while the changes in pCLL3404, resulting in amino acid substitutions of Ser for Arg-164 and Lys for Glu-240, were identical to those reported for TEM-10 (26).

DISCUSSION

There has been circumstantial evidence of a link between ceftazidime usage and the appearance of extended-spectrum β-lactamases in previous reports (22, 25, 29). This also appears to be the case with the ceftazidime-resistant strains of *K. pneumoniae* isolated at these two hospitals in Chicago, although not all of the patients from whom ceftazidime-resistant strains were isolated received ceftazidime as therapy. The total use of cefotaxime was far greater at Mercy Hospital than was the use of ceftazidime, suggesting that ceftazidime exerts a greater selective pressure. It is interesting that nine of the strains in this study and one strain in a previous study (26) which carried an identical plasmid were isolated at three different hospitals. Because the three hospitals did not share medical personnel, it was not likely that the resistant strains were carried from one hospital to the next by staff members. A common link among these three facilities has been suggested to be a pool of patients from area nursing homes who were frequently in and out of several of the various hospitals and who may have served as the reservoir for these strains (26).

Production of the TEM-10 β-lactamase was identified as the mechanism by which these *K. pneumoniae* isolates were resistant to ceftazidime. Two different TEM-10-encoding plasmids were identified in the isolates reported here, neither of which was identical to that of the TEM-10-encoding plasmid isolated from the original producing isolate. The finding of multiple plasmids all encoding a TEM-10 enzyme strongly suggests that

TABLE 3. Nucleotide sequences of ceftazidime-hydrolyzing β-lactamase-encoding genes

Enzyme (encoding entity)	Reference(s) or original *K. pneumoniae* strain	Original plasmid	Nucleotide (amino acid)[a] at position:				
			226	436	604	692	917
TEM-1 (Tn*2*)	Ref. 4, 9	R111	T (Phe-8)[b]	T (Gly-78)	T (Ala-134)	C (Arg-164)	G (Glu-240)
TEM-1 (Tn*3*)	Ref. 31	R1	C (Phe-8)[c]	C (Gly-78)	G (Ala-134)	C (Arg-164)	G (Glu-240)
TEM-10(pCLL3405)	KC2	pJPQ100	T (Phe-8)[d]	T (Gly-78)	T (Ala-134)	A (Ser-164)	A (Lys-240)
TEM-10(pCLL2303)	2351	pCLL2301	C (Phe-8)[d]	C (Gly-78)	G (Ala-134)	A (Ser-164)	A (Lys-240)
TEM-10(pCLL3403)	RP1	pCLL3404	T (Phe-8)	T (Gly-78)	T (Ala-134)	A (Ser-164)	A (Lys-240)
TEM-12(pDAW403)	Ref. 34		NA[e]	NA	NA	A (Ser-164)[f]	G (Glu-240)
TEM-12(pCLL3401)	RP1	pCLL3402	T (Phe-8)	T (Gly-78)	T (Ala-134)	A (Ser-164)	G (Glu-240)

[a] Nucleotides and amino acids are numbered according to references 31 and 1, respectively.
[b] References 4 and 9.
[c] Reference 31.
[d] Reference 26.
[e] NA, data not available.
[f] Reference 18.

the TEM-10 genes in these isolates arose independently. It is curious that the TEM-10 enzyme has not been commonly identified in European centers where other extended-spectrum β-lactamases have been associated with outbreaks of antibiotic-resistant members of the family *Enterobacteriaceae* (6, 7, 14, 16, 24). However, the TEM-10 β-lactamase has been reported in one isolate from a patient in France (33) and a TEM-10-like β-lactamase was identified in several isolates of *K. pneumoniae* at a hospital in England (17).

Noteworthy in this study was the identification of two different extended-spectrum β-lactamases in *K. pneumoniae* RP1. It appears that this represents a step-wise progression of mutations from TEM-12 to TEM-10 and that TEM-12 serves as an intermediate form for the TEM-10 β-lactamase. This finding supports the premise that conversion of a TEM-1-encoding gene to a TEM-10 gene proceeds through a TEM-12 gene intermediate. It appears that the two point mutations converting from TEM-1 to TEM-10 need not occur concurrently but are more likely to accrue sequentially.

While many previously identified strains possessing an extended-spectrum β-lactamase also produced one or more additional β-lactamases (14, 16, 24, 34), it is rare to find two extended-spectrum enzymes produced in one strain (6, 18). Recently, TEM-12 and TEM-10 (TEM-23) β-lactamases were identified in sequential isolates of *E. coli* from a single patient; however, the two β-lactamases were produced only one at a time (33). In another report, TEM-12 was identified in an isolate of *K. oxytoca* which also produced an uncharacterized β-lactamase with a pI of 5.6 (11). The TEM-12-encoding gene from this isolate was demonstrated to be harbored on a transposon by means of transposition experiments. This finding is significant in that the epidemiologic spread of extended-spectrum β-lactamase genes may be due, in part, to transposition of the gene from one plasmid to another.

The TEM-12 and TEM-10 β-lactamases produced by strain RP1 of this study were found to be carried on plasmids that were identical by restriction enzyme digest analysis. This suggests that two plasmids, identical except for a single point mutation, were present in this strain. It is a well-established phenomenon that two plasmids of the same origin or "incompatibility group" are not maintained in a single strain (10). However, the two plasmids in strain RP1, differing by a single nucleotide substitution, are likely to be seen as identical by the host strain and therefore both plasmids have been replicated and maintained as though they were one plasmid species.

Biochemically it would make sense for an organism under antibiotic stress to progress from a TEM-1 to a TEM-12 to a TEM-10 β-lactamase. The TEM-1 enzyme hydrolyzes ceftazidime at a rate <0.01% that of benzylpenicillin (34), whereas TEM-12, created by a single nucleotide change from TEM-1, is capable of hydrolyzing ceftazidime at a modest rate. Under heavy stress, however, a second point mutation could yield the TEM-10 β-lactamase, with a high efficiency of ceftazidime hydrolysis, a situation of great advantage to the organism.

From these results it is apparent that the TEM-10 β-lactamase has become a commonly identified extended-spectrum enzyme in Chicago. The epidemiological significance of finding both TEM-10 and TEM-12 in a single organism suggests that mutation of the TEM-1-encoding gene proceeds in sequential order. It is likely that the TEM-12 intermediate of the more potent ceftazidime-hydrolyzing enzyme, TEM-10, may be present before an outbreak of highly resistant organisms is noticed in a clinical setting.

ACKNOWLEDGMENT

We gratefully acknowledge the assistance of Alex Kuritza for providing clinical isolates.

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