ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, June 1997, p. 1322–1325 Vol. 41, No. 6
0066-4804/97/$04.00+0
Copyright © 1997, American Society for Microbiology

A Complex Mutant of TEM-1 β-Lactamase with Mutations Encountered in Both IRT-4 and Extended-Spectrum TEM-15, Produced by an *Escherichia coli* Clinical Isolate

D. SIROT,[1]* C. RECULE,[2] E. B. CHAIBI,[3] L. BRET,[1] J. CROIZE,[2] C. CHANAL-CLARIS,[1]
R. LABIA,[3] AND J. SIROT[1]

Laboratoire de Bactériologie-Virologie, Faculté de Médecine, 63001 Clermont-Ferrand Cedex,[1] Service de Bactériologie et Virologie, Centre Hospitalier Universitaire de Grenoble, 38043 Grenoble Cedex 9,[2] and UMR 175 Centre National de la Recherche Scientifique-MNHN, 29000 Quimper,[3] France

Received 28 October 1996/Returned for modification 3 February 1997/Accepted 6 April 1997

Escherichia coli* GR102 was isolated from feces of a leukemic patient. It expressed different levels of resistance to amoxicillin or ticarcillin plus clavulanate and to the various cephalosporins tested. The double-disk synergy test was weakly positive. Production of a β-lactamase with a pI of 5.6 was transferred to *E. coli* HB101 by conjugation. The nucleotide sequence was determined by direct sequencing of the amplification products obtained by PCR performed with TEM gene primers. This enzyme differed from TEM-1 (*blaT-1B* gene) by four amino acid substitutions: Met→Leu-69, Glu→Lys-104, Gly→Ser-238 and Asn→Asp-276. The amino acid susbstitutions Leu-69 and Asp-276 are known to be responsible for inhibitor resistance of the IRT-4 mutant, as are Lys-104 and Ser-238 substitutions for hydrolytic activity of the extended-spectrum β-lactamases TEM-15, TEM-4, and TEM-3. These combined mutations led to a mutant enzyme which conferred a level of resistance to coamoxiclav (MIC, 64 μg/ml) much lower than that conferred by IRT-4 (MIC, 2,048 μg/ml) but higher than that conferred by TEM-15 or TEM-1 (MIC, 16 μg/ml). In addition, the MIC of ceftazidime for *E. coli* transconjugant GR202 (1 μg/ml) was lower than that for *E. coli* TEM-15 (16 μg/ml) and higher than that for *E. coli* IRT-4 or TEM-1 (0.06 μg/ml). The MICs observed for this TEM-type enzyme were related to the kinetic constants K_m and k_{cat} and the 50% inhibitory concentration, which were intermediate between those observed for IRT-4 and TEM-15. In conclusion, this new type of complex mutant derived from TEM-1 (CMT-1) is able to confer resistance at a very low level to inhibitors and at a low level to extended-spectrum cephalosporins. CMT-1 received the designation TEM-50.

Overproduction of *Escherichia coli* chromosomal β-lactamase is one cause of resistance to β-lactam–β-lactamase inhibitor combinations such as amoxicillin (AMX)-clavulanate (CA) and also results in reduced susceptibility to all β-lactams except carbapenems (15).

In *E. coli*, resistance to all β-lactams except cephamycins and carbapenems may be caused by extended-spectrum β-lactamases. These enzymes are susceptible to β-lactamase inhibitors such as CA (10, 14, 15) and are therefore detected by synergy tests (10), and strains producing such mutants are often susceptible to β-lactam–β-lactamase inhibitor combinations.

In *E. coli* isolates, the most recently discovered mechanism of resistance to AMX-CA is production of inhibitor-resistant TEM β-lactamases (IRT) (8).

E. coli GR102, isolated from feces of a leukemic patient in the hematology unit of the teaching hospital of Grenoble, France, harbored an unusual β-lactam resistance phenotype with resistance to AMX and ticarcillin (TIC) alone and combined with CA and resistance to all cephalosporins, including cephamycins, at various levels. In addition, the double-disk synergy test used for extended-spectrum β-lactamase detection was weakly positive.

This complex phenotype suggested that the β-lactam resistance of the strain was due to the presence of several β-lactamases or a combination of different mechanisms of resistance to β-lactams.

* Corresponding author. Mailing address: Laboratoire de Bactériologie-Virologie, Faculté de Médecine, 28 Place Henri-Dunant, 63001 Clermont-Ferrand Cedex, France. Phone: 33 4 73 60 80 18. Fax: 33 4 73 27 74 94.

MATERIALS AND METHODS

Strains. The strains used included *E. coli* GR102, a clinical isolate producing a novel β-lactamase; *E. coli* HB101, used as a recipient strain for transfer; *E. coli* HB101/p111 (TEM-1 producing); *E. coli* CF0042 (IRT-4–TEM-35 producing) (8); and *E. coli* transformant DH5α (CF244), obtained by electroporation from *Klebsiella pneumoniae* Kp240 (TEM-15 producing) (16).

Susceptibility to β-lactams. The MICs of AMX, TIC, cephalothin (CF), cefotaxime (CTX), ceftazidime (CAZ), aztreonam (ATM), cefepime (FEP), and cefpirome (CPO) alone and combined with CA at a fixed concentration of 2 μg/ml were determined. A method of dilution with Mueller-Hinton agar (Sanofi Diagnostics Pasteur, Marnes-la-Coquette, France) and an inoculum of 10^4 CFU per spot were used. Antibiotics were provided as powders by SmithKline Beecham Pharmaceuticals (AMX, TIC, and CA), Roussel-Uclaf (CTX and CPO), Glaxo Wellcome Research and Development (CAZ), and Bristol-Myers-Squibb (ATM and FEP).

Detection of extended-spectrum β-lactamase was performed with the double-disk synergy test as described by Jarlier et al. (10).

Isoelectric focusing. Isoelectric focusing was performed with polyacrylamide gels containing ampholines with a pH range of 3.5 to 10.0 as previously described (19), and β-lactamases with known pIs (TEM-1 [pI 5.4], TEM-2 [pI 5.6], TEM-15 [pI 6.0], and IRT-4 [pI 5.2]) were used as standards.

Transfer experiment. A transfer experiment was performed with *E. coli* GR102 and the recipient *E. coli* HB101. Transconjugants were selected on agar containing rifampin (300 μg/ml) and gentamicin (8 μg/ml) or CAZ (0.5 μg/ml).

Sequencing of DNA amplified by PCR. On the assumption that the transconjugant strain contained bla_{TEM}, a single-stranded DNA template was generated for sequencing by PCR performed with an asymmetric ratio of amplification primers A and B, and the nucleotide sequence was determined as previously described (3), by direct sequencing of the amplified product obtained from the transconjugant *E. coli* GR202.

Determination of β-lactamase kinetic parameters k_{cat}, K_m, and k_{cat}/K_m. Affinity (K_m) and catalytic activity (k_{cat}) were determined with highly purified extracts (≥97% pure) by using a computerized microacidimetric method (13).

TABLE 1. Nucleotide and amino acid substitutions in bla_{TEM} genes

Nucleotide no.[a]	Nucleotide (amino acid)[b] in:			
	$bla_{TEM-1(Tn2)}$	bla_{TEM-15}	bla_{IRT-4}	bla_{CMT-1}
226	T (Phe)	C	T	T
317	C (Gln-39)	C	C	C
346	A (Glu)	A	G	A
407	A (Met-69)	A	C (Leu)	C (Leu)
436	T (Gly)	C	T	T
512	G (Glu-104)	A (Lys)	G	A (Lys)
604	T (Ala)	G	G	T
682	T (Thr)	T	T	T
914	G (Gly-238)	A (Ser)	G	A (Ser)
925	G (Gly)	G	G	G
1022	A (Asn-276)	A	G (Asp)	G (Asp)

[a] Nucleotide numbering is according to Sutcliffe (21).
[b] The amino acid is indicated when a point mutation leads to an amino acid substitution compared with the sequences of TEM-1(Tn2). Numbering is according to Ambler et al. (1).

All β-lactamases were purified from crude extracts by size exclusion chromatography on Sephadex G-100 (Pharmacia), preparative isoelectric focusing, and reverse-phase high-performance liquid chromatography on a C_{18} Nucleosil 500A column (Interchim) as described by Brun et al. (2). The homogeneity of the preparations was determined by analytical sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The k_{cat}, K_m, and k_{cat}/K_m values of enzyme CMT-1 were compared with those of the β-lactamases TEM-1, TEM-15, and IRT-4. The kinetics of TEM-1 and its mutants toward penicillins and cephalosporins were compared. Inhibition studies of TEM-1 and the mutant enzymes with CA, sulbactam, and tazobactam were performed. The affinity of the enzyme for the inhibitor, expressed as the inhibition constant (K_i), was measured by using competition procedures with benzylpenicillin. It is determined from the extrapolated rate at the time when the inhibitor is added. The 50% inhibitory concentration (IC_{50}) was determined after incubation of the inhibitor and the enzyme for 10 min (completed inactivation) at 37°C before measurement of the remaining enzymatic activity. The IC_{50} is defined as the inhibitor concentration causing 50% inhibition of benzylpenicillin hydrolysis by the enzyme.

RESULTS

Resistance phenotype of *E. coli* GR102. *E. coli* GR102 expressed a complex β-lactam resistance phenotype with resistance to AMX and TIC alone and combined with CA and resistance to cephalosporins at various levels: high-level resistance to narrow-spectrum cephalosporins and low-level resistance to extended-spectrum cephalosporins (MICs, 1 to 32 μg/ml). A positive synergy test with CA suggested the presence of a mutant extended-spectrum β-lactamase of class A origin. In addition, this strain had reduced susceptibility to cefoxitin (MIC, 128 μg/ml) and, to a lesser extent, cefotetan and moxalactam (data not shown). This reduced susceptibility to cephamycins was probably related to decreased permeability of the strain for β-lactams, since *E. coli* GR102 had lost an outer membrane protein with a molecular mass of 40 kDa (data not shown). Overproduction of the chromosomal cephalosporinase was not detected.

This strain was also resistant to aminoglycosides (tobramycin, gentamicin, and netilmicin), probably owing to production of an AAC (3)-II enzyme, and to chloramphenicol, tetracyclines, and sulfonamides.

Conjugative transfer and isoelectric focusing. The gene encoding resistance to β-lactams, except cephamycins, was tranferred by conjugation from *E. coli* GR102 to rifampin-resistant *E. coli* HB101 (GR202). Selection for CAZ or gentamicin resistance revealed the transfer of an 85-kb plasmid conferring resistance to β-lactams, aminoglycosides (tobramycin, gentamicin, and netilmicin), tetracyclines, and sulfonamides.

By isoelectric focusing, two bands at pIs 5.4 and 5.6 were observed in *E. coli* GR102 and one band at pI 5.6 was observed in *E. coli* transconjugant GR202.

Nucleotide sequencing. As shown in Table 1, from the transconjugant GR202 producing a β-lactamase with a pI of 5.6, nucleotide sequencing revealed a bla_{TEM} gene identical to the *blaT-1B* gene (Tn-2) at positions 226, 317, 346, 436, 604, 682, and 925, which discriminate the bla_{TEM} genes (4, 7).

The bla_{TEM} gene from the *E. coli* transconjugant differed from the *blaT-1B* gene by four point mutations. These mutations consisted of the nucleotide change A→C at position 407, which leads to the amino acid substitution Met→Leu at position 69 (1); the nucleotide change G→A at positions 512 and 914, leading to the amino acid substitutions Glu→Lys at position 104 and Gly→Ser at position 238; and the nucleotide change A→G at position 1022, leading to the amino acid substitution Asn→Asp at position 276. The two amino acid substitutions at positions 69 and 276 are observed in the IRT-4–TEM-35 enzyme (2, 8, 22), and the two amino acid substitutions at positions 104 and 238 are observed in the extended-spectrum β-lactamase TEM-15 (16).

β-Lactam MICs for TEM mutants. Consequently, we compared the MICs of β-lactams for *E. coli* GR102 and its transconjugant GR202, producing a complex mutant form of TEM (CMT-1), with MICs for IRT-4-producing *E. coli* CF0042, TEM-15-producing *E. coli* CF244, and TEM-1-producing *E. coli* HB101 (Table 2).

For *E. coli* GR202 producing CMT-1, the MICs of AMX-CA (64 μg/ml) and TIC-CA (64 μg/ml) were much lower than

TABLE 2. MICs of β-lactams for *E. coli* CMT-1 (GR102 and its transconjugant, GR202), *E. coli* TEM-15 (CF244), *E. coli* IRT-4 (CF0042), and *E. coli* TEM-1 (HB101)

E. coli strain (enzyme)	MIC (μg/ml) of:															
	AMX		TIC		CF		CTX		CAZ		ATM		FEP		CPO	
	Alone	With CA[a]	Alone	With CA	Alone	With CA	Alone	With CA	Alone	With CA	Alone	With CA	Alone	With CA	Alone	With CA
GR102 (CMT-1)	4,096	256	>4,096	512	256	128	4	0.5	4	1	1	0.25	8	1	32	4
GR202[b] (CMT-1)	2,048	64	4,096	64	8	4	1	0.03	1	0.12	0.12	0.06	1	0.03	2	0.12
CF244[c] (TEM-15)	>4,096	16	>4,096	32	128	4	8	0.06	16	0.25	4	0.12	1	0.03	2	0.03
CF0042 (IRT-4)	4,096	2,048	1,024	512	8	4	≤0.06	≤0.06	≤0.06	≤0.06	≤0.06	≤0.06	≤0.06	≤0.06	≤0.06	≤0.06
HB101 (TEM-1)	4,096	16	4,096	32	8	2	≤0.06	≤0.06	≤0.06	≤0.06	≤0.06	≤0.06	≤0.06	≤0.06	≤0.06	≤0.06
HB101[d]	4	4	1	1	4	4	≤0.06	≤0.06	≤0.06	≤0.06	≤0.06	≤0.06	≤0.06	≤0.06	≤0.06	≤0.06

[a] CA was used at 2 μg/ml.
[b] *E. coli* HB101 transconjugant.
[c] *E. coli* DH5α transformant.
[d] *E. coli* recipient strain.

TABLE 3. Production of TEM-type β-lactamases in *E. coli*

Enzyme	pI	Producing organism	Activity in crude extract[a]	Sp act[b] of purified protein
TEM-1	5.4	HB101	2.2	2.48
TEM-35–IRT-4	5.2	CF0042	2.1	2.17
TEM-15	6.0	CF244	0.4	0.083
TEM-50–CMT-1	5.6	GR202	0.4	0.23

[a] Micromoles of benzylpenicillin per minute per milligram of protein.
[b] Micromoles of benzylpenicillin per minute per microgram of protein. Determined with highly purified preparations (≥97% pure).

TABLE 5. Inhibition of TEM-1 and its mutant forms by CA, sulbactam, and tazobactam

Enzyme	IC_{50} (μM), K_i (μM)		
	CA	Sulbactam	Tazobactam
TEM-1	0.08, 0.1	6.1, 0.9	0.1, 0.01
TEM-35–IRT-4	28, 27	304, 49	1.8, 0.6
TEM-15	0.01, 0.02	0.03, 0.02	0.01, 0.008
TEM-50–CMT-1	0.25, 0.7	0.5, 0.4	0.04, 0.06

those observed for IRT-4-producing strain CF0042 (2,048 and 512 μg/ml, respectively). Similarly, for strain GR202, MICs of CTX (1 μg/ml) and CAZ (1 μg/ml) were lower than those observed for TEM-15-producing strain CF244 (8 and 16 μg/ml, respectively) and higher than those for IRT-4 producing strain CF0042 (≤0.06 μg/ml). The same 1:8 ratio of MICs was observed for aztreonam (0.12 μg/ml for the CMT-1 producer and 4 μg/ml for the TEM-15 producer). The MICs of cefepime and cefpirome (1 and 2 μg/ml) were identical for the CMT-1 and TEM-15 producers.

E. coli GR202 (CMT-1) was 2 to 4 times less susceptible to AMX or TIC plus CA (64 μg/ml) and 16 times less susceptible to CTX, CAZ, FEP, and CPO (1 to 2 μg/ml) than was *E. coli* HB101 ($TEM-1^+$). MICs of β-lactam substrates in the presence of 2 and 4 μg of sulbactam or tazobactam per ml were about fourfold lower for the CMT-1-producing strain than for the TEM-1-producing strain (data not shown).

Enzymatic and kinetic parameters of β-lactamases. Enzymatic and kinetic parameters of the new complex mutant enzyme CMT-1 with regard to penicillins and cephalosporins were compared with those of the TEM-1, IRT-4, and TEM-15 β-lactamases (Tables 3 to 5). The specific activity of the highly purified CMT-1 protein was 10-fold lower than that of TEM-1 (Table 3).

For all penicillins, the k_{cat} values of CMT-1 were about 10-fold lower than those of TEM-1 and IRT-4 and about twice as high as those of TEM-15. The catalytic efficiencies (k_{cat}/K_m) of the three mutant enzymes were lower than those of the TEM-1 enzyme, and the values observed for CMT-1 and IRT-4 with carboxy- and ureidopenicillins were similar.

For cephalosporins, k_{cat} values of CMT-1 were slightly lower than or similar to those of TEM-15 for ceftriaxone, CTX, CAZ, and ATM; however, the catalytic efficiencies of the CMT-1 enzyme were only 5 to 11% of those of TEM-15 with ceftriaxone and CTX. No activity of TEM-1 or IRT-4 against expanded-spectrum cephalosporins and ATM (k_{cat}s of $<1\ s^{-1}$ associated with K_is of ≥500 μM) was detected.

The IC_{50} of CA for CMT-1 (Table 5) was higher (0.25 μM) than that for TEM-1 (0.08 μM) and TEM-15 (0.01 μM) but 100-fold lower than that for IRT-4 (28 μM). Sulbactam was the least efficient inhibitor of IRT-4 (IC_{50}, 304 μM), while its inhibitor efficiency was similar to that of CA for CMT-1 (IC_{50}, 0.5 μM). Tazobactam was the most efficient inhibitor of all of these β-lactamases. Moreover, CMT-1 (IC_{50}, 0.04 μM) and TEM-15 (IC_{50}, 0.01 μM) were more susceptible to inhibition by tazobactam than was TEM-1 (IC_{50}, 0.1 μM).

TABLE 4. Comparison of the kinetics[a] of TEM-1 and its mutant forms

Drug	k_{cat} (s^{-1}), K_m (μM), k_{cat}/K_m ($\mu M^{-1}S^{-1}$)			
	TEM-1	TEM-35I–IRT-4	TEM-15	TEM-50–CMT-1
Benzylpenicillin	1,200, 25, 48.0	1,050, 140, 7.5	40, 6, 6.7	110, 17, 6.5
AMX	920, 26, 35.4	900, 245, 8.5	26, 5, 5.2	70, 33, 2.1
TIC	115, 10, 11.5	125, 320, 0.4	8, 2, 4.0	15, 30, 0.5
Carbenicillin	132, 13, 10.2	120, 360, 0.3	7, 3, 2.3	13, 60, 0.2
Piperacillin	987, 45, 21.9	945, 320, 2.9	64, 12, 5.3	111, 31, 3.6
CF	122, 250, 0.5	52, 1,200, 0.04	43, 23, 1.7	62, 324, 0.2
Cephaloridine	2,045, 800, 2.5	340, 1,420, 0.2	37, 30, 1.2	320, 310, 1.03
Cefoperazone	470, 260, 1.8	305, 1,325, 0.2	25, 22, 1.1	150, 118, 1.3
Cefuroxime	ND[b]	ND,	24, 91, 0.3	20, 260, 0.08
Ceftriaxone	ND	ND,	92, 50, 1.8	35, 385, 0.09
CTX	1.2, ND	ND,	180, 100, 1.8	150, 873, 0.2
CAZ	ND	ND,	7, 80, 0.1	3, ND
ATM	ND	ND,	0.2, ND	1, ND

[a] The standard deviation for analysis was ≤10%.
[b] ND, not detected; the rate was too small to determine k_{cat} and K_m reliably.

DISCUSSION

The TEM-1 derivative described in this report constitutes a new type of complex mutant, CMT-1, combining mutations responsible for inhibitor resistance (Leu-69 and Asp-276) and those responsible for extended-spectrum activity (Lys-104 and Ser-238). It is the first example of such a β-lactamase produced by a clinical isolate of *E. coli.*

Mutations conferring resistance to β-lactam inhibitors. Replacement of methionine 69, just adjacent to serine 70, by aliphatic amino acids such as leucine influences the positioning of residues (5, 17) because the buried side chain at position 69 lies behind β-strand B3, forming the back wall of the oxyanion pocket in which the β-lactam's carbonyl group is polarized (12). Moreover, crystallographic data indicate that residues in the C-terminal α helix, such as Asn-276, restrict the mobility of the Arg-244 side chain and so play a role in maintaining the integrity of the active site (11). Because small β-lactams such as CA must rely primarily on attractive interactions with the oxyanion hole and Arg-244, inhibitor resistance exists in the natural variant IRT-4, containing changes at residues 69 and 276 (12). This IRT-4 mutant enzyme is one of the most resistant to inhibition by CA among the IRT-type enzymes (2, 22), with a CA IC_{50} 350-fold higher than that for TEM-1. The CA resistance of this mutant is confirmed by high-level resistance to combinations of AMX and TIC with CA (MIC, 2,048 and 512 μg/ml, respectively). Inhibition studies showed that the CMT-1 enzyme was 100-fold less resistant than the IRT-4 mutant but only 3 times as resistant to inhibition by CA as the wild-type TEM-1 β-lactamase. These kinetic results were closely related to the moderate resistance level (64 μg/ml) of the CMT-1-producing strain to AMX-CA or TIC-CA. The inhibitor resistance usually caused by the Leu-69 mutation may be decreased by the close proximity of the Ser-238 mutation (see below).

Mutations conferring extended-spectrum activity. The Glu→Lys change at position 104 contributes to the precise positioning of residues 130 to 132 (SDN loop), which are involved in substrate binding, but seems insufficient alone to confer true resistance to expanded-spectrum cephalosporins (18, 20).

It is generally recognized that the substitution Gly→Ser-238 enlarges the active site, thereby creating an enzyme with increased affinity for the 7-oxyimino cephalosporins (6). All of the TEM variants reported to have Ser-238 contain methionine at position 69, and mutant enzyme CMT-1 is the first harboring both mutations Ser-238 and Leu-69. The affinity may be affected by a change at position 69, since the side chain at position 238, on the inner side of the B3 β-strand, lies very close to the side chain of residue 69 (12).

The TEM-1 variant, with the associated changes Glu→Lys-104 and Gly→Ser-238, is TEM-15 (16). Complex mutant CMT-1, with these last mutations, conferred a lower level of resistance to CTX and CAZ than did TEM-15, and this difference correlated with the kinetic constants. The kinetic comparison of substrate hydrolysis in extended-spectrum β-lactamases TEM-15 and CMT-1 revealed that the catalytic efficiency (k_{cat}/K_m) of CMT-1 was lower than that of TEM-15 for CTX (10-fold) and ceftriaxone (20-fold).

Overall, the hydrolytic properties of this complex TEM mutant enzyme were found to be closer to those of an extended-spectrum enzyme than to those of an inhibitor-resistant enzyme. However, the predominant effect of the mutations Lys-104 and Ser-238, which are responsible for extended-spectrum activity and inhibitor hypersusceptibility, was clearly attenuated by the mutations Leu-69 and Asp-276. In the strain producing CMT-1, complete reversal of CA resistance by mutations enhancing activity against 7-oxyimino cephalosporins was not observed as reported in a Ser-164–Ser-244 mutant obtained by site-specific mutagenesis (9).

E. coli GR102 was isolated from a patient treated with CPO (4 g/day) and amikacin (1 g/day) for 12 days. An *E. coli* strain with a typical β-lactam inhibitor resistance phenotype and the same resistances to other antibiotics had been isolated from the same sample (feces) from this patient a week before. This suggests that an inhibitor-resistant TEM mutant enzyme with the Leu-69 and Asp-276 mutations emerged first, and then, under antibiotic (CPO) and mutagenic agent (cytarabin and daunorubicin) pressure, this mutant underwent the two additional mutations, Lys-104 and Ser-238, responsible for extended-spectrum activity. Unfortunately, this hypothesis could not be confirmed since the initial *E. coli* IRT-producing strain was not kept.

In conclusion, the production of this complex TEM mutant cannot alone account for the high-level multiresistance to β-lactams of the *E. coli* GR102 isolate, in which several resistance mechanisms were involved (TEM-1 and, probably, decreased permeability). It would probably be more beneficial for an *E. coli* strain to produce two different TEM mutants (an extended-spectrum mutant and an inhibitor-resistant mutant) simultaneously than to produce one double mutant. If each mutant conferred its own resistance phenotype, high-level resistance to both CA combinations and extended-spectrum cephalosporins could then be expected.

ACKNOWLEDGMENTS

We thank J. M. Frère for the gift of highly purified TEM-15 extract and P. Courvalin for the gift of *K. pneumoniae* Kp240 producing the TEM-15 enzyme. We thank Rolande Perroux, Marlène Jan, and Dominique Rubio for technical assistance.

This work was supported in part by a grant from the Direction de la Recherche et des Etudes doctorales, Ministère de l'Education Nationale, Paris, France.

REFERENCES

1. **Ambler, R. P., A. F. N. Coulson, J. M. Frère, J. M. Ghuysen, B. Joris, M. Forsman, R. C. Levesque, G. Tiraby, and S. G. Waley.** 1991. A standard numbering scheme for the class A β-lactamases. Biochem. J. **276:**269–272.
2. **Brun, T., J. Péduzzi, M. M. Caniça, G. Paul, P. Névot, M. Barthélémy, and R. Labia.** 1994. Characterization and amino acid sequence of IRT-4, a novel TEM-type enzyme with a decreased susceptibility to β-lactamase inhibitors. FEMS Microbiol. Lett. **120:**111–118.
3. **Chanal, C., M. C. Poupart, D. Sirot, R. Labia, J. Sirot, and R. Cluzel.** 1992. Nucleotide sequences of CAZ-2, CAZ-6, and CAZ-7 β-lactamase genes. Antimicrob. Agents Chemother. **36:**1817–1820.
4. **Chen, S. T., and R. C. Clowes.** 1987. Variations between the nucleotide sequences of Tn*1*, Tn*2*, and Tn*3* and expression of β-lactamase in *Pseudomonas aeruginosa* and *Escherichia coli*. J. Bacteriol. **169:**913–916.
5. **Delaire, M., R. Labia, J. P. Samama, and J. M. Masson.** 1992. Site-directed mutagenesis at the active site of *Escherichia coli* TEM-1 β-lactamase. J. Biol. Chem. **267:**20600–20606.
6. **Du Bois, S. K., M. S. Marriott, and S. G. B. Amyes.** 1995. TEM- and SHV-derived extended-spectrum β-lactamases: relationship between selection, structure and function. J. Antimicrob. Chemother. **35:**7–22.
7. **Goussard, S., and P. Courvalin.** 1991. Sequence of the genes *blaT-1B* and *blaT-2*. Gene **102:**71–73.
8. **Henquell, C., C. Chanal, D. Sirot, R. Labia, and J. Sirot.** 1995. Molecular characterization of nine different types of mutants among 107 inhibitor-resistant TEM β-lactamases from clinical isolates of *Escherichia coli*. Antimicrob. Agents Chemother. **39:**427–430.
9. **Imtiaz, U., E. K. Manavathu, S. Mobashery, and S. A. Lerner.** 1994. Reversal of clavulanate resistance conferred by a Ser-244 mutant of TEM-1 β-lactamase as a result of a second mutation (Arg to Ser at position 164) that enhances activity against ceftazidime. Antimicrob. Agents Chemother. **38:**1134–1139.
10. **Jarlier, V., M. H. Nicolas, G. Fournier, and A. Philippon.** 1988. Extended broad-spectrum β-lactamases conferring transferable resistance to newer β-lactam agents in *Enterobacteriaceae*: hospital prevalence and susceptibility patterns. Rev. Infect. Dis. **10:**867–878.
11. **Jelsch, C., F. Lenfant, J. M. Masson, and J. P. Samama.** 1992. β-Lactamase TEM-1 of *E. coli*. Crystal structure determination at 2.5 A resolution. FEBS Lett. **299:**135–142.
12. **Knox, J. R.** 1995. Extended-spectrum and inhibitor-resistant TEM-type β-lactamases: mutations, specificity, and three-dimensional structure. Antimicrob. Agents Chemother. **39:**2593–2601.
13. **Labia, R., J. Andrillon, and F. Le Goffic.** 1973. Computerized microacidimetric determination of β-lactamase Michaelis-Menten constants. FEBS Lett. **33:**42–44.
14. **Labia, R., A. Morand, K. Tiwari, J. S. Pitton, D. Sirot, and J. Sirot.** 1988. Kinetic properties of two plasmid-mediated β-lactamases from *Klebsiella pneumoniae* with strong activity against third-generation cephalosporins. J. Antimicrob. Chemother. **21:**301–307.
15. **Livermore, D. M.** 1995. β-Lactamases in laboratory and clinical resistance. Clin. Microbiol. Rev. **8:**557–584.
16. **Mabilat, C., and P. Courvalin.** 1990. Development of "oligotyping" for characterization and molecular epidemiology of TEM β-lactamases in members of the family *Enterobacteriaceae*. Antimicrob. Agents Chemother. **34:**2210–2216.
17. **Oliphant, A. R., and K. Struhl.** 1989. An efficient method for generating proteins with enzymatic properties: application to β-lactamase. Proc. Natl. Acad. Sci. USA **86:**9094–9098.
18. **Petit, A., L. Maveyraud, F. Lenfant, J. P. Samama, R. Labia, and J. M. Masson.** 1995. Multiple substitutions at position 104 of β-lactamase TEM-1: assessing the role of this residue in substrate specificity. Biochem. J. **305:**33–40.
19. **Sirot, D., C. Chanal, C. Henquell, R. Labia, J. Sirot, and R. Cluzel.** 1994. Clinical isolates of *Escherichia coli* producing multiple TEM-mutants resistant to β-lactamase inhibitors. J. Antimicrob. Chemother. **33:**1117–1126.
20. **Sowek, J. A., B. Singer, S. Ohringer, M. F. Mally, T. J. Dougherty, J. Z. Gougoutas, and K. Bush.** 1991. Substitution of lysine at position 104 or 240 of TEM β-lactamase enhances the effect of serine-164 substitution on hydrolysis or affinity for cephalosporins and the monobactam aztreonam. Biochemistry **30:**3179–3188.
21. **Sutcliffe, G.** 1978. Nucleotide sequence of the ampicillin resistance gene of *Escherichia coli* plasmid pBR322. Proc. Natl. Acad. Sci. USA **75:**3737–3741.
22. **Zhou, X. Y., F. Bordon, D. Sirot, M. D. Kitzis, and L. Gutmann.** 1994. Emergence of clinical isolates of *Escherichia coli* producing TEM-1 derivatives or an OXA-1 β-lactamase conferring resistance to β-lactamase inhibitors. Antimicrob. Agents Chemother. **38:**1085–1089.