Emergence of Clinical Isolates of *Escherichia coli* Producing TEM-1 Derivatives or an OXA-1 β -Lactamase Conferring Resistance to β -Lactamase Inhibitors

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Sixteen *Escherichia coli* clinical isolates which were resistant to ampicillin and amoxicillin-clavulanate but susceptible to cephalothin were studied. Eight strains showed the presence of a β -lactamase which comigrates with reference OXA-1 enzyme. The eight other strains produced different TEM-1 derivatives which had in common a higher K_m for penicillins and a higher 50% inhibitory concentration for the β -lactamase inhibitors. By oligotyping and sequencing of PCR products, it was shown that Ser (AGC) (TEM-30; also called TRI-1) in three strains and Cys (TGC) (TEM-31; also called TRI-2) in one strain were substituted for Arg-241 (CGC), that Leu (CTG) (TEM-33) and Val (GTG) (TEM-34) in one strain each were substituted for Met-67 (ATG), and that in other mutants the two latter substitutions occurred together with the substitution of Asp (GAT) (TEM-35 and TEM-36) for Asn-272 (AAT). Therefore, different sets of amino acid substitutions of TEM-1 can be found in clinical isolates and lead to resistance to β -lactamase inhibitors.

Resistance to aminopenicillins has become very frequent in *Escherichia coli* and is observed in France in up to 30 to 50% of the *E. coli* strains isolated in the hospital as well as in the community (25). This resistance is generally due to the presence of plasmid-mediated β -lactamases, among which TEM β -lactamases are the most frequently found (22, 25). This is one reason that combinations of β -lactam antibiotics with inhibitors of β -lactamases are now extensively used. Therefore, it is not surprising that many investigators have reported the emergence of clinical isolates of *E. coli* resistant to some of these combinations (3, 4, 26, 28, 31, 32, 34–36). At least two major mechanisms are evoked to explain this resistance: either overproduction of the TEM β -lactamase or a modification of the kinetic properties of the TEM β -lactamase due to some amino acid substitution (4, 20, 32, 36). In this work, we have studied the mechanism of resistance in 16 clinical isolates of *E. coli* resistant to the combination of amoxicillin and clavulanate but susceptible to cephalothin.

MATERIALS AND METHODS

Bacterial strains, medium, and antibiotics. Sixteen clinical isolates of amoxicillin-clavulanate-resistant and cephalothin-susceptible *E. coli* were collected during a 1-year period (1991 to 1992) from different units and different patients in two hospitals (Hôpital Broussais and Hôpital Saint-Joseph). All strains were grown with aeration at 37°C in Mueller-Hinton broth or on Mueller-Hinton agar plates (Sanofi Diagnostics Pasteur). MICs of antibiotics were determined on Mueller-Hinton agar with a multiple-inoculum replicator with ca. 10⁴ CFU per spot. Conjugation and transformation were done in *E. coli* BM694 (17) and *E. coli* DH5 α (Clontech, Palo Alto, Calif.), respectively. The following antibiotics were provided by

the indicated suppliers: amoxicillin, clavulanic acid, and BRL42715, Smith-Kline Beecham Pharmaceuticals; cephalothin, Lilly France SA; cephaloridine, Glaxo Pharmaceuticals, Ltd.; piperacillin and tazobactam, Lederle Laboratories; sulbactam, Pfizer Inc.; mezlocillin, Bayer; and oxacillin and ampicillin, Bristol Laboratories.

β -Lactamase assays. Crude β -lactamase preparations were obtained from the different strains after centrifugation (30,000 $\times$ g, 45 min) of sonified cells. Analytical isoelectric focusing was performed by the method of Matthew et al. (21). β -Lactamase activity was determined spectrophotometrically at 30°C with a double-beam spectrophotometer (model 550S; Perkin-Elmer Corp.). The wavelengths of maximal absorption differences were as previously described (18, 30). K_m values were calculated by linear regression analysis of Woolf-Augustinsson-Hofstee plots (v versus v/s). The concentrations of inhibitors required to inhibit 50% of the β -lactamase activity (IC₅₀s) were measured after 10 min of preincubation, using 100 μ M ampicillin as the substrate and equal amounts of enzyme units in each case.

Identification of the TEM β -lactamase. After separation on a sodium dodecyl sulfate–15% polyacrylamide gel of the proteins present in the crude β -lactamase preparations of the different strains, polyclonal anti-TEM antibodies (kindly provided by J. Davies) and immunoblotting were used to identify the β -lactamase as previously described (1).

Oligotyping and sequencing of DNA amplified by PCR. Colony hybridizations were performed as follows. Nylon filters (0.45- μ m pore size) (Hybond-N; Amersham) were placed on Mueller-Hinton agar and inoculated with 10 μ l of 18-h broth cultures. After 6 h of incubation, colonies were lysed and DNA was fixed as described previously (19). The oligonucleotides used as probes are listed in Table 1. Oligonucleotides were used without purification and completely deblocked. Oligonucleotide probes (10 pmol) were ³²P end labeled by using T4 polynucleotide kinase and 5'-[γ -³²P]ATP (3,000 Ci/mmol) for 45 min. Hybridization with oligonucleotides was done at 37°C

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TABLE 1. Sequences of oligonucleotides used for oligotyping and amplification

Oligonucleotide	Sequence ^a	Position ^b
Probes		
Gln-37	5'-d(ACC CAA CTG ATC TTC AG)-3'	325
Lys-37	T	
Arg-241	5'-d(CGT GGG TCT CGC GGT ATC)-3'	919
Cys-241	T	
Ser-241	A	
Amplification primers		
A	5'-d(GTATGGATCCTCAACATTTCCGTGTCG)-3'	207
B	5'-d(ACCAAGCTTAATCAGTGAGGCA)-3'	1067

^a Underlined nucleotides indicate *Bam*HI and *Hind*III sites on primers A and B, respectively.

^b Each number corresponds to the position of the first 5' base of the nucleotide according to the numbering of Sutcliffe (33).

overnight. Washings were done at 5°C below the T_d for 5 min followed by one final wash at 1°C below the T_d for 2 min. *E. coli* GUER and SAL (4, 36) were used as negative controls for Arg-241 hybridizations and as positive controls for Ser-241 (TEM-30) and Cys-241 (TEM-31) hybridizations.

If after oligotyping either an ambiguity remained or no change was found, a PCR amplification which covered the entire *bla*T gene was done, using primers A and B shown in Table 1. Amplification was performed as previously described (7) after extraction of the plasmid harboring the β-lactamase gene. The presence of a restriction site in each oligonucleotide used for amplification (Table 1) allowed, after gene cleaning and digestion with the appropriate restriction enzymes, ligation of the PCR product into M13mp18 and M13mp19 (23). After transformation into *E. coli* JM101, DNA sequencing was carried out by the dideoxy chain termination method of Sanger et al. (29). If a mutation was found, sequencing of a second M13mp19 or M13mp18 clone was always performed. Other molecular methods used in this study were as previously described (27).

RESULTS

Identification of the β-lactamases and susceptibility testing.

Sixteen clinical isolates of *E. coli* were chosen because of their unusual susceptibility profiles on the antibiogram: resistance to amoxicillin, carbenicillin, and amoxicillin-clavulanate and susceptibility to cephalothin (MIC ≤ 8 μg/ml). Examination of the β-lactamase content (Fig. 1) showed that eight strains produced a β-lactamase with a pI of 7.4 which comigrated with the reference OXA-1 (10) and did not react with the anti-TEM antibodies (data not shown). For these strains, although the

associations of the different inhibitors with amoxicillin showed that BRL42715 was the most active, the concentrations of the other inhibitors chosen were not sufficient to restore susceptibility to amoxicillin (Table 1). By using the crude β-lactamase extracts from these strains and the OXA-1 reference enzyme, it was shown that the rate of hydrolysis of oxacillin was 130% for the reference enzyme (hydrolysis of penicillin G was taken as 100%), while the relative hydrolysis for the enzymes of the clinical isolates ranged from 113 to 140%. With the same extracts, 50% inhibition by clavulanate was, respectively, 2.7 μM for the reference OXA-1 β-lactamase and within a range of 2.3 to 3 μM for the β-lactamases of the different clinical isolates. These overall results suggest that the enzymes present in the clinical isolates were very similar, if not identical, to the OXA-1 reference enzyme.

From the eight other strains, the β-lactamase genes responsible for resistance to amoxicillin-clavulanate were transferable by conjugation to *E. coli* BM694 in two cases (Table 2), and in five cases DH5α could be transformed to amoxicillin-clavulanate resistance by electroporation. For only one strain (*E. coli* 86325) the resistance to amoxicillin-clavulanate could not be transferred by conjugation or transformation. The pIs of the β-lactamases present in the transformants or the transconjugants were 5.2 for five strains and 5.4 for two strains (Fig. 1; Table 2), while a β-lactamase with a pI of 5.2 was present in *E. coli* 86325. Extracts of these strains all reacted with the anti-TEM antibodies (data not shown). Compared with the amounts of inhibitor required to reduce to 8 μg/ml the MIC of amoxicillin for *E. coli* BM694 producing TEM-1, larger quantities of inhibitor were needed for the clinical isolates as well as the transconjugants producing the different TEM derivatives. However, smaller quantities of inhibitor were needed when the β-lactamase inhibitor BRL42715 was used. Similarly, at the fixed concentrations of inhibitors chosen, the MICs of amoxicillin for these strains were either not reduced or only slightly reduced with BRL42715, which remained the most effective inhibitor.

Properties and point mutations of the TEM β-lactamases conferring resistance to augmentin. Except for ampicillin, the relative rates of hydrolysis of the different substrates by the TEM β-lactamases responsible for amoxicillin-clavulanate resistance appeared generally lower than those observed with TEM-1 (Table 3). The *K_m* values for penicillin G and piperacillin were 4- to 20-fold higher for all the other enzymes than for TEM-1. Similarly, the IC₅₀s were 5- to 400-fold higher, depending on the enzyme and the inhibitor tested. This explains why larger quantities of inhibitors were necessary to restore the susceptibility to amoxicillin. Among the inhibitors tested, BRL42715 appeared to be the best inhibitor of these enzymes,

FIG. 1. Analytical isoelectric focusing of β-lactamases. Lanes 1 and 12, TEM-1, pI 5.4; lanes 2 and 9, TEM-101, pI 5.2; lanes 3 to 8, β-lactamases from strains 92734, 10476, 86941, 98041, 13162, and 86325, respectively; lanes 10 and 11, β-lactamase from strains 59904 and 92741, respectively; lane 13, OXA-1, pI 7.4; lane 14, one representative extract of the eight *E. coli* strains harboring exclusively OXA-1.

TABLE 2. MICs of amoxicillin and different combinations of amoxicillin with β -lactamase inhibitors

<i>E. coli</i> strain ^a	pI ^b	MIC (μ g/ml) ^c of:							
		Inhibitor with fixed concn of amoxicillin (8 μ g/ml)				Amoxicillin with fixed concn of inhibitor			
		BRL	CLA	TAZ	SUL	BRL (2 μ g/ml)	CLA (2 μ g/ml)	TAZ (4 μ g/ml)	SUL (8 μ g/ml)
+OXA-1 ^d	7.4	0.12–0.25	16–32	64–128	32–64	0.5–2	64–256	256–1,024	64–512
BM694 $\times$ TEM-1	5.4	0.5	2	8	32	2	8	256	$\geq 1,024$
10476	5.2	32	64	≥ 128	64	512	$\geq 1,024$	$\geq 1,024$	$\geq 1,024$
10476 $\times$ DH5 α	5.2	16	32	128	64	512	$\geq 1,024$	$\geq 1,024$	$\geq 1,024$
92734	5.2	≥ 16	32	64	64	256	1,024	1,024	1,024
92734 $\times$ DH5 α	5.2	≥ 16	16	64	64	512	$\geq 1,024$	$\geq 1,024$	$\geq 1,024$
86941	5.2	16	16	64	64	256	$\geq 1,024$	512	$\geq 1,024$
86941 $\times$ BM694	5.2	8	8	64	32	64	512	512	256
13162	5.2	8	16	64	32	64	1,024	1,024	512
13162 $\times$ BM694	5.2	8	32	64	32	64	1,024	1,024	512
98041	5.2	16	32	64	≥ 128	32	$\geq 1,024$	1,024	$\geq 1,024$
98041 $\times$ DH5 α	5.2	4	32	64	≥ 128	16	$\geq 1,024$	512	$\geq 1,024$
86325	5.2	2	16	64	128	4	256	512	512
92741	5.4	4	32	64	64	16	1,024	256	1,024
92741 $\times$ DH5 α	5.4	0.5	16	16	32	4	512	32	256
59904	5.4	1	16	64	≥ 128	8	$\geq 1,024$	$\geq 1,024$	$\geq 1,024$
59904 $\times$ DH5 α	5.4	1	16	128	≥ 128	4	1,024	$\geq 1,024$	$\geq 1,024$

^a MICs were also tested after transformation in *E. coli* DH5 α or conjugation in *E. coli* BM694 as indicated ($\times$).

^b pI of the enzyme involved in amoxicillin-clavulanate resistance.

^c BRL, BRL42715; CLA, clavulanic acid; TAZ, tazobactam; SUL, sulbactam. MICs of BRL and CLA alone were ≥ 16 , that of TAZ was >128 , and that of SUL was ≥ 32 . The MICs of amoxicillin alone were all $\geq 1,024$.

^d Eight *E. coli* clinical isolates harboring OXA-1. Ranges of MICs are given.

and this correlated with the MICs of the amoxicillin-BRL42715 combinations.

In all the transformants and transconjugants, plasmids of variable sizes, of more than 50 kb, were found (data not shown). All these TEM enzymes derived from TEM-1, since by using the oligotyping method and Gln-37 oligonucleotide a Gln was found at position 37 (according to the numbering system of Sutcliffe [33]). When the Arg-241, Cys-241, and Ser-241 oligonucleotides were used, a Ser was found at position 241 instead of Arg in TEM enzymes of strains 92734, 10476, and 86941, and a Cys-241 was found in strain 13162 (Table 3). This was confirmed by sequence analysis of PCR-amplified fragments from strains 86941 and 13162. These results agreed well with the pI of 5.2 of these TEM enzymes since serine and cysteine, which contain uncharged groups, were substituted for arginine, which is a basic positively charged amino acid. Since no changes with these oligonucleotides were observed in the other strains studied, sequencing of their β -lactamase TEM genes was carried out after amplification by PCR and cloning into M13mp18 and M13mp19. In all cases, a Gln was found at position 37 of TEM, confirming again that these enzymes derived from TEM-1. Four types of amino acid substitutions were found. In strains 59904 and 92741, there were different substitutions at the same position: Leu (CTG) and Val (GTG), respectively, for Met-67 (ATG) (Table 3). Since Leu and Val, just as Met, are uncharged, the pI remained that of TEM-1. In each of the TEM enzymes of strains 98041 and 86325, two substitutions were found: Asp (GAT) for Asn-272 (AAT) in both enzymes and Leu (CTG) for Met-67 in 98041 and Val (GTG) for Met-67 in 86325. The replacement of the noncharged Asn by the acid Asp explains why a pI of 5.2 was found for these TEM-1 derivatives.

DISCUSSION

Sixteen clinical isolates of *E. coli* were chosen for analysis because of their particular susceptibility phenotype: resistance

to amoxicillin and amoxicillin-clavulanate and susceptibility to cephalothin. Eight strains produced a β -lactamase which comigrated with OXA-1 and showed resistance to the combination of amoxicillin with different β -lactamase inhibitors, except BRL42715, as previously described (8). It was previously shown that the OXA-1 enzyme was not as well inhibited as TEM-1 by different β -lactamase inhibitors (8, 13, 15). For clavulanate, as shown again in this study, this could be explained by a lesser affinity of OXA-1 than that of TEM-1 (6).

The other eight strains appeared to be more interesting, since they showed point mutations of the TEM-1 enzyme. A correlation was found between the increased IC₅₀ of the β -lactamase inhibitors and their inefficiency to restore the activity of amoxicillin. The point mutations were also responsible for an apparent increase in K_m of the penicillins tested. Four strains produced TEM-1 derivatives which we called TEM-30 and TEM-31 for the purpose of clarity as far as the TEM classification is concerned, with a Ser or a Cys substituted for Arg-241 (ABL244 [2]), respectively. These mutations are similar to the one previously described in clinical isolates, also called TRI-1 and TRI-2 (36), and an in vitro mutant (20). All the other TEM-1 derivatives differed at position 67 (ABL69 [2]) with, instead of Met-67, Leu in a derivative called TEM-33 and Val in a derivative called TEM-34. These latter derivatives resemble one previously described by Blazquez et al. (4) and called IRT-3, although the mutation described was the substitution of Ile for Met. We propose to rename it TEM-32. The derivatives called TEM-35 and TEM-36 had two point mutations, with a Leu-67 or Val-67 associated with a substitution of Asp for Asn-272 (ABL276 [2]), explaining their pI of 5.2. Mutations in position 67 associated with resistance to different inhibitors were also previously described after in vitro mutagenesis of the TEM-1 gene (11, 24) and in an in vitro mutant of OHIO-1 in which a Leu replaced Met-67 (5). Interestingly, it seemed that, at least for tazobactam and BRL42715, the IC₅₀s were significantly lower when a mutation occurred at position 67 than at position 241.

TABLE 3. Sources and characteristics of TEM-1 derivatives on various substrates

Strain(s)	Relative rate of hydrolysis of:					K_m (μ M)		IC ₅₀				pI	Mutated amino acid(s)	β -Lactamase
	Peni- cillin G	Ampi- cillin	Mezlo- cillin	Pipera- cillin	Cephalo- ridine	Peni- cillin G	Pipera- cillin	Cephalo- ridine	Clavulanic acid	Tazo- bactam	Sul- bactam	BRL 42715		
DH5 α (TEM-1)	100	115	97	88	65	38	47	1,169	0.05	0.02	1.4	0.002	5.4	TEM-1
92734, 86947, and 10476 ^a	100	152	28	42	4	335	914	1,073	4	2.3	81	0.7	5.2	TEM-30 ^c
13162	100	248	45	70	11	365	ND ^d	681	22	3.6	150	0.9	5.2	TEM-31 ^c
59904	100	155	61	33	9	120	456	1,224	4	0.4	36	0.011	5.4	TEM-33
92741	100	154	83	58	36	152	316	931	2	0.5	16	0.014	5.4	TEM-34
98041	100	148	58	59	31	157	361	1,304	17	0.7	62	0.03	5.2	TEM-35
86325	ND	ND	ND	ND	ND	ND	ND	ND	2.9	1.2	20	0.006	5.2	TEM-36

^a Mean results obtained for the enzymes extracted from the different strains.^b Determined after oligotyping (strains 92734, 86941, 10476, and 13162) and sequencing (strains 86941 and 13162).^c Also designated TRI-1 (TEM-30) and TRI-2 (TEM-31) (4, 36).^d ND, not determined.^e Determined after sequencing.

Finally, it is clear that mutants of the TEM enzyme involved in resistance to β -lactamase inhibitors are different from those responsible for the emergence of the extended-spectrum β -lactamases (16) and can be found in the clinical setting. Moreover, and in contrast to what was observed for some of the extended-spectrum β -lactamases such as TEM-3 or TEM-7 (12), for which lower IC₅₀s of the inhibitors (in particular sulbactam) were observed, higher IC₅₀s of all inhibitors were associated with the mutations found in all TEM derivatives described in this study. In this context, it should be noted that the mutation at position 67 is adjacent to Ser-68 (ABL70 [2]), which is part of the Ser-X-X-Lys motif and essential for the catalytic activity of the β -lactamase (14). The mutation at position 241, which is very near the mutations at positions 235 to 237 found in some ESbla TEM derivatives, is located at the beginning of the β -4 strand surrounding the active site cavity of the β -lactamase (9, 14). Since Asp-272 is distant from this cavity (14), it would not be expected to play a major role, as shown by the absence of significant changes between the IC₅₀s of the different inhibitors for TEM-33 and TEM-35 and for TEM-34 and TEM-36.

If such enzymes became very common, they could render extremely hazardous the use of combinations of β -lactam antibiotics with inhibitors of β -lactamase to treat serious infections.

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