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Molecular Characterization of Nine Different Types of Mutants among 107 Inhibitor-Resistant TEM β-Lactamases from Clinical Isolates of *Escherichia coli*

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DNA-DNA hybridization and sequencing were performed to determine the molecular basis of resistance to clavulanic acid in 107 inhibitor-resistant TEM (IRT) enzymes produced by *Escherichia coli* clinical isolates. These β-lactamases derived from TEM-1 enzyme focused at pI 5.2 ($n = 68$) or 5.4 ($n = 39$) and were very poorly inhibited by clavulanic acid compared with TEM-1 enzyme. Results showed that the amino acid sequences of 84 of the 107 enzymes differ from TEM-1 by one or two substitutions previously described: Arg-244→Ser (IRT-2) in 22 strains, Met-69→Leu (TEM-33) in 17 strains, Met-69→Val (TEM-34) in 14 strains, Met-69→Ile (IRT-3) in 6 strains, Met-69→Leu associated with Asn-276→Asp (IRT-4) in 13 strains, and Met-69→Val associated with Asn-276→Asp (TEM-36) in 12 strains. A new combination, Met-69→Ile with Asn-276→Asp, was found in 20 strains and was called IRT-8. Two IRT enzymes not previously described were characterized. The substitution Met-69→Val associated with a novel substitution Arg-275→Leu occurred in one strain. The combination Met-69→Leu and Asn-276→Asp was associated with the novel substitution Trp-165→Arg in two strains. These two novel enzymes were called IRT-9 and IRT-10, respectively. The implication of these novel mutated positions, 165 and 275, in resistance to inactivation by clavulanate was supported by crystallographic data on the TEM-1 enzyme and results of site-directed mutagenesis. Molecular characterization of these mutants showed great diversity among the genes coding for inhibitor-resistant TEM enzymes produced by clinical *E. coli* isolates.

The plasmid-mediated TEM-1 and TEM-2 β-lactamases are the most widespread among gram-negative bacteria. The clinical use of extended-spectrum cephalosporins, such as cefotaxime and ceftazidime, has been followed since 1983 by the emergence of extended-spectrum β-lactamases, often derived from TEM-1 and TEM-2 by a small number of mutations, mainly at positions 104, 164, and 238 (positions numbered by the Ambler numbering system [1, 11]). These extended-spectrum β-lactamases are susceptible to the β-lactamase inhibitors clavulanic acid, sulbactam, and tazobactam (13). More recently, a novel evolution of TEM β-lactamases has generated inhibitor-resistant TEM (IRT) enzymes in *Escherichia coli* clinical isolates (22, 23). These mutants harbored substitutions at other locations (69, 244, and 276) which decrease the affinities for β-lactam substrates and alter the way in which the enzymes interact with suicide inhibitors such as clavulanic acid (2, 3, 7, 15, 20, 24).

A previous study of 2,972 clinical *E. coli* isolates collected during the first half of 1993 has shown a total frequency of 4.9% of IRT-producing isolates among *E. coli* isolated from urine specimens (9). These IRT-producing isolates were resistant to amoxicillin and ticarcillin alone and in combination with clavulanic acid. They produced β-lactamases of pI 5.4 or 5.2. DNA-DNA hybridization confirmed that the β-lactamases involved in this resistance mechanism were derived from TEM-1 and contained variations at positions 69, 244, and 276. As a follow-up to this study, we used oligotyping and sequencing to identify the molecular basis of this resistance in 107 isolates.

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MATERIALS AND METHODS

Bacterial strains. Bacterial strains reported in this study were 107 clinical isolates of IRT-producing *E. coli* cultured in 1993 from urinary tract infections at Clermont-Ferrand, France. These IRT β-lactamases were derived from the TEM-1 enzyme, as indicated by probing with a TEM-1-specific probe (9). The isoelectric points of β-lactamase activity in cell extracts were 5.2 ($n = 68$) and 5.4 ($n = 39$).

Oligotyping. Oligotyping for detection of point mutations in the bla_{TEM-1} gene (the *bla* gene encoding the parental penicillinase TEM-1) was performed for the 107 isolates as previously described (14) with 15 oligonucleotide probes listed in Table 1. These probes were centered on three positions: 69 ($n = 7$), −244 ($n = 6$), and 276 ($n = 2$). Probes centered on position 244 (nucleotide position 929 according to Sutcliffe [21]) were synthesized in duplicate to take into account the near-silent mutation at nucleotide 925 which differs in bla_{TEM-1} and bla_{TEM-2} genes. Control strains and the DNA-DNA hybridization method have been previously described (20).

Sequencing of DNA amplified by PCR. Single-stranded DNA templates were generated for sequencing by PCR (18) performed with an asymmetric ratio for the two amplification primers A and B (5). The dideoxynucleotide chain-termination method of Sanger (19) was applied to purified PCR products with the Sequenase version 2.0 kit (Amersham-France, les Ulis, France) as previously described (5). Complete sequencing was performed for 26 of 107 isolates.

Inhibition of β-lactamase activity by clavulanic acid. For β-lactamase assays, bacteria growing exponentially at 37°C in Trypticase-soy-yeast extract medium were harvested and cell-free lysates were prepared by sonication. β-Lactamase activity of crude sonic extracts was determined with nitrocefin as the substrate at a final concentration of 20 μM. Two representative isolates in each group of mutants were chosen. The enzyme concentrations used in this assay were adjusted to produce complete hydrolysis of nitrocefin in 15 min at 37°C. Hydrolysis was measured at 486 nm. A preparation of the TEM-1 enzyme from strain UA 274 was used as the reference. Inhibition studies were carried out by preincubation of β-lactamase extracts and 10 μM of clavulanic acid (final concentration) for 5 min at 37°C, and then nitrocefin was added.

RESULTS AND DISCUSSION

Molecular characterization. Colony hybridization was applied to 107 isolates producing IRT enzymes. When an ambiguity remained after oligotyping, complete sequencing of the

TABLE 1. Sequences of oligonucleotides used for hybridization

Probe[a]	Sequence[b]	Position[c]
Met-69	5′-d(TTT CCA ATG ATG AGC ACT)-3′	398
Leu-69	.....C.....	398
Leu-69	.....T.....	398
Val-69	.....G.....	398
Ile-69	.....T.....	398
Ile-69	.....A.....	398
Ile-69	.....C.....	398
Arg-244	5′-d(CGT GGG TCT CGC GGT ATC)-3′	920
Cys-244	.....T.....	920
Ser-244	.....A.....	920
Arg-244[d]	5′-d(CGT GGA TCT CGC GGT ATC)-3′	920
Cys-244[d]	.....T.....	920
Ser-244[d]	.....A.....	920
Asn-276	5′-d(AT GAA CGA AAT AGA CAG)-3′	1014
Asp-276	.....G.....	1014

[a] Designated according to the amino acid substituted in the TEM enzyme. The number refers to the position of the amino acid in the Ambler system (1).
[b] The 17 or 18 bases are centered on positions where mutations lead to the amino acid substitutions indicated.
[c] Corresponds to the position of the first 5′ base of the oligonucleotide according to the numbering system of Sutcliffe (21).
[d] Corresponds to the nucleotide sequence of the bla_{TEM-2} gene.

clavulanate-resistant bla_{TEM} gene was performed. The nucleotide substitutions relative to bla_{TEM-1a} gene and the deduced amino acid changes observed in the 107 IRT are listed in Table 2. We have adopted the more-explicit denomination "IRT" used by Vedel et al. (23), Blazquez et al. (3), and Brun et al. (4) to classify these enzymes.

Analysis of results showed that IRT enzymes differ from TEM-1 by one, two, or three amino acid substitutions. In 84 strains, these substitutions are located at the positions previously described, 69, 244, and 276.

(i) Position 244. A mutation at position 244 was found in 22 enzymes. Complete sequencing was performed for 8 of 22 genes. Substitution Arg-244→Cys described for IRT-1 (TEM-31) (2) was never detected in our study by oligotyping. Substitution Arg-244→Ser described for IRT-2 (TEM-30) (2) was found in 22 isolates.

(ii) Position 69. A mutation at position 69 was found in 37 enzymes. Complete sequencing was performed for 11 of 37 genes. The substitution Met-69→Ile, as described for IRT-3 (TEM-32), was found in six enzymes. Ile was coded by the codon ATA, as previously described (3), in four enzymes. The complete sequence of IRT-3 described by Blazquez et al. (3) revealed a second mutation Met-182→Thr, which is not involved to resistance to inhibitors. The two mutants completely sequenced in our group did not reveal this additional mutation. Ile-69 was coded by the codon ATT in two enzymes. Ile-69 coded by the codon ATC was never detected. The single substitution Met-69→Leu (codon CTG), described for TEM-33 (IRT-5) (24) was found in 17 isolates, and Met-69→Val described for TEM-34 (IRT-6) (24) was detected in 14 enzymes. Five mutants hybridized both with normal probe Met-69 and with probes corresponding to Val-69 ($n = 3$) or Ile-69 ($n = 2$), suggesting the simultaneous presence of TEM-1 and IRT-6 or IRT-3. DNA sequencing confirmed the association of a bla_{TEM-1} gene with a bla_{IRT} gene coding for Val or Ile at position 69.

(iii) Combination of positions 69 and 276. The combination of positions 69 and 276 was found in 25 enzymes. Complete sequence was determined for 3 of 25 genes. The combination of substitutions Met-69→Leu with Asn-276→Asp described for IRT-4 (TEM-35) (4, 24) was present in 13 enzymes. Leu-69 was coded by the codon CTG, as described by Zhou et al. (24), in nine variants and by the codon TTG in four variants. Twelve enzymes were identical to TEM-36 (IRT-7) (24), with substitutions Met-69→Val and Asn-276→Asp.

Three new types of mutants were detected in the 23 remaining strains.

(i) IRT-8 enzyme. The IRT-8 enzyme produced by 20 isolates harbored the substitutions Met-69→Ile (codon ATA) and Asn-276→Asp. The combination of these two mutations (confirmed by complete sequencing of one strain) was not already described.

TABLE 2. Amino acid substitutions in 107 IRT

β-Lactamase	No. of isolates (no. sequenced)	Mutated amino acid (codon) at position:					pI	Reference(s)
		69	165	244	275	276		
TEM-1		Met (ATG)	Trp (TGG)	Arg (CGC)	Arg (CGA)	Asn (AAT)	5.4	21
IRT-1 (TEM-31)*	0			Cys (TGC)			5.2	2
IRT-2 (TEM-30)*	22 (8)			Ser (AGC)			5.2	2
IRT-3 (TEM-32)*	4 (2)	Ile (ATA)					5.4	3
	2 (2)	Ile (ATT)					5.4	This study
IRT-4 (TEM-35)*	9 (1)	Leu (CTG)				Asp (GAT)	5.2	4, 24
	4 (1)	Leu (TTG)				Asp (GAT)	5.2	This study
TEM-33* (IRT-5)	17 (3)	Leu (CTG)					5.4	24
TEM-34* (IRT-6)	14 (4)	Val (GTG)					5.4	24
TEM-36* (IRT-7)	12 (1)	Val (GGT)				Asp (GAT)	5.2	24
IRT-8 (TEM-37)	20 (1)	Ile (ATA)				Asp (GAT)	5.2	This study
IRT-9 (TEM-38)	1 (1)	Val (GTG)			Leu (CTA)		5.2	This study
IRT-10 (TEM-39)	2 (2)	Leu (CTG)	Arg (CGG)			Asp (GAT)	5.4	This study

[a] Asterisks indicate the names proposed by Zhou et al. (24).

TABLE 3. Percentage of neutralized β-lactamase activity with clavulanic acid in crude sonic extracts for TEM variants compared with TEM-1 β-lactamase

β-Lactamase	Sp act (mU/mg)[a]	% Neutralized activity[b]
TEM-1	1,900	88
IRT-2	130	5
IRT-3	70	14
IRT-4	250	14
IRT-5	1,500	16
IRT-6	150	16
IRT-7	120	5
IRT-8	40	22
IRT-9	70	5
IRT-10	70	9
IRT-6 + TEM	350	54

[a] One unit equals 1.0 mole of substrate hydrolyzed by 1.0 ml of extract per min.
[b] At the concentration of 10 μM clavulanic acid in the presence of 20 μM nitrocefin.

(ii) IRT-9 enzyme. One enzyme harboring a substitution Met-69→Val, without change in positions 244 and 276, had a pI of 5.2 instead of the 5.4 usually observed for these mutants. Sequencing of the gene coding for this enzyme revealed that IRT-9 enzyme differed from TEM-1 by the known substitution Met-69→Val (nucleotide position −407; A→G) and by the novel substitution Arg-275→Leu (nucleotide position −1020; G→T). The change of arginine, which is a basic positively charged amino acid, for leucine, an uncharged amino acid, explains the decreased pI value.

(iii) IRT-10 enzyme. Two enzymes of pI 5.4 showed an oligotype (Leu-69, Asp-276) that did not match their pI value. The substitution Met-69→Leu does not affect the pI of the enzyme, but the substitution of Asn-276, which is an uncharged amino acid, to the acid Asp, is responsible for a more acidic pI value (5.2) than that of TEM-1 (5.4). The genes of these mutant enzymes have been sequenced and found to be identical. The nucleotide and deduced amino acid sequences confirmed the substitutions Leu-69 and Asp-276 and revealed an additional point mutation at nucleotide position 695, leading to the amino acid change Trp-165→Arg. Petit et al. (17) reported that the substitution Trp-165→Arg in TEM-1 conferred an increased value of the pI (5.4 to 5.6) to the variant enzyme. So, the double substitution at positions 276 and 165 explains the unmodified pI value of 5.4 of this new TEM mutant.

TEM numbers. TEM-37, TEM-38 and TEM-39 could be provisionally assigned to these three novel enzymes, IRT-8, IRT-9 and IRT-10, respectively.

DNA-DNA hybridization with probes centered on position 244 revealed that the silent mutation at nucleotide position 925 (G→A), which differs in bla_{TEM-1} and bla_{TEM-2} genes (6, 8, 21), occurred in 80% of genes coding for these IRT enzymes. However, the relationship with bla_{TEM-2} gene was not obvious when we considered the silent mutations occurring at positions 226, 346, 436, 604, and 682 (data not shown).

Inhibition profiles. The specific activities of β-lactamases resistant to inhibitors were variable but always lower than that observed for TEM-1 enzyme.

The results of the inhibition study (average of results obtained for two representative isolates in each group) are expressed as percentage decrease of β-lactamase activity against nitrocefin remaining after preincubation with clavulanic acid (Table 3).

Results showed that all IRT enzymes were less sensitive than TEM-1 to inhibition by clavulanic acid in the conditions of this experiment. The activity of TEM-1 β-lactamase against nitrocefin as the substrate was reduced by 88% by clavulanic acid, whereas those of IRT enzymes were reduced by only 5 to 22%. When an IRT enzyme (IRT-6) was associated with a normal TEM-1, the activity was neutralized 54%. The combined specific activity (350) is less than that of TEM-1 alone (1,900) and greater than that of IRT-6 alone (150).

Structure-function relationships. TEM-1 is a serine enzyme which is irreversibly inhibited by suicide inhibitors. Analysis of X-ray crystallographic structure and site-directed mutagenesis have provided information on the interactions between suicide inhibitors (clavulanic acid) and the substrate active site of the enzyme.

The arginyl residue 244, at the beginning of strand β4, participates in the active-site binding of substrates and clavulanate and plays a specific role in the inactivation process (10, 12). Its substitution by serine or cysteine removes the ionic bond to the substrate in the active site and interferes with the process of inactivation, thereby leading to the reduced affinities of these mutant enzymes for clavulanate and less efficient inactivation by it (7, 10, 15).

Replacement of methionine 69, just adjacent to serine 70, by aliphatic amino acids as leucine, isoleucine, or valine, induces minor structural changes but leads to an increased resistance to clavulanic acid (16). Delaire et al. (7) suggest that the substitution at this position modifies the orientation of small molecules, such as clavulanic acid, in the active site so that such mutant enzymes cannot undergo inactivation as readily as the wild-type enzyme.

Zhou et al. (24) do not attribute a major role in clavulanate resistance to the substitution of Asn-276. In the same manner, the role of substitution of Arg-275 in the IRT-9 mutant could be contested. However, crystallographic data indicate that residues in the C-terminal α helix such as Asn-276 and Arg-275 restrict the mobility of the Arg-244 side chain and so play a role in maintaining the integrity of the active site (12). Substitution of Asn-276 is suspected to play the same role as that of Arg-244 in resistance to inhibition by clavulanic acid (4). To date, mutations observed at these two positions have never been observed alone, so it is difficult to affirm their role in inhibitor resistance.

In the IRT-10 mutant, the substitution Trp-165→Arg, which affects the pI value, might not be expected to play a major role in the resistance to inhibition, since it was associated with Val-69 and Asp-276, already implicated in this mechanism. However, using site-directed mutagenesis, Petit et al. (17) have shown that the replacement of tryptophan 165 by arginine modulates the catalytic properties of the enzyme, and in particular, decreases its sensitivity to inhibition by clavulanic acid.

Conclusion. Molecular characterization of these mutants showed the great diversity of genes coding for IRT enzymes. Examination of silent mutations, which do not affect the amino acid sequence, is useful in tracing the evolution of these genes and suggests that the genes coding for IRT enzymes have evolved from different progenitors, either relative to bla_{TEM-2} gene or to bla_{TEM-1} genes. IRT enzymes could be derived from the most frequently encountered TEM-1 enzyme. However, the variability of mutations observed for nucleotides 226, 346, 436, 604, and 672 (silent mutations) suggests that the genes coding for "wild-type" TEM β-lactamases vary much more than those previously described (bla_{TEM-1a}, bla_{TEM-1b}, and bla_{TEM-2} [6, 8, 21]).

The diversity of mutant enzymes, which appeared during a very short period in nonrepetitive and nonlinked strains (hospital strains isolated in different wards and community strains) cannot be explained by an epidemic phenomenon, as is often

observed for extended-spectrum β-lactamases. These results suggest an independent emergence of these TEM variants under antibiotic selective pressure.

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