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Properties of IRT-14 (TEM-45), a Newly Characterized Mutant of TEM-Type β-Lactamases

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IRT-14 (TEM-45) is a new mutant TEM-type β-lactamase that was isolated from clinical *Escherichia coli* P37 and that confers resistance to broad-spectrum penicillins with reduced sensitivity to β-lactamase inhibitors. The MICs of amoxicillin alone and of amoxicillin combined with 2 μg of clavulanic acid or 2 μg of tazobactam per ml were 4,096, 2,048, and 1,024 μg/ml, respectively. The strain was susceptible to cephalosporins, aztreonam, moxalactam, and imipenem. The enzyme was purified to homogeneity, and values of the kinetic parameters k_{cat}, K_m, and k_{cat}/K_m were determined for different substrates. This enzyme, with a pI of 5.2, was found to have reduced affinity for broad-spectrum penicillins and cephalosporins. The values of 50% inhibitory concentrations of clavulanic acid, sulbactam, tazobactam, and brobactam are correlated with the higher K_ms for substrates. The resistance of *E. coli* P37 to mechanism-based inactivators results from a higher level of production of the TEM-derived enzyme due to the G-to-T substitution at position 162 (G-162→T) in the promoter region of bla_{TEM} and from the structural modifications resulting from the Met-69→Leu and Arg-275→Gln substitutions that characterize IRT-14 β-lactamase.

Among the mechanisms which contribute to the development of resistance to β-lactam–β-lactamase inhibitor combinations in *Escherichia coli*, hyperproduction of unmodified TEM-type β-lactamase and modification of the outer membrane protein limiting the uptake of the antibiotic combination were the first to be described (30). Since 1990, the effect of β-lactamase inactivators has been compromised by the emergence of mutant TEM-type β-lactamases (35, 36), collectively designated inhibitor-resistant TEM (IRT) (3, 5, 7).

In France, a recent hospital study of clinical *E. coli* isolates indicates that the frequency of IRTs was around 3% (4), a value comparable to those in other European reports (19, 33). So far, 13 bla_{TEM} genes conferring resistance to β-lactamase inhibitors (bla_{IRT}) have been isolated and characterized from clinical isolates, and the encoded enzymes were designated IRT-1 to IRT-10 (TEM-30 to TEM-39) (3, 5, 7, 18, 37), TEM-40, TEM-41 (33), and IRT_2-2 (TEM-44) (6). Although *E. coli* was the only species previously found to harbor bla_{IRT} genes, recently *Klebsiella pneumoniae* and *Proteus mirabilis* strains have also been found to produce IRTs (6, 26).

We have recently described the nucleotide sequence for the entire bla_{IRT-14} gene (EMBL database accession number, X95401), including the promoter region (9). In this sequence, which is closely related to the bla_{TEM-2} sequence framework, three mutations may be implicated in the resistance to β-lactamase inhibitors: the G-to-T substitution at position 162 (G-162→T) is localized in the promoter region, and, in the structural region, the A-407→T and the G-1020→A substitutions cause, respectively, the Met-69→Leu and the Arg-275→Gln changes.

The purpose of this work was to study the resistance of the strain *E. coli* P37 and the properties of the new enzyme IRT-14 (TEM-45) and to correlate the properties with the gene structure.

MATERIALS AND METHODS

Bacterial strains. The strain producing IRT-14, namely *E. coli* P37, was isolated from a patient with a urinary tract infection at Cochin Hospital, Paris, France. The susceptibility pattern of the strain was determined by a disc diffusion method (13) in Mueller-Hinton agar (Sanofi Diagnostics Pasteur, Marnes-la-Coquette, France) and was interpreted according to the guidelines of the Comité de l'Antibiogramme de la Société Française de Microbiologie (1).

In this study, the *E. coli* strain producing TEM-1 (R111) (24) was used as a control for determining MICs. For analytical isoelectric focusing, the β-lactamases TEM-1 (R111), TEM-2 (RP4) (28), IRT-1 (TEM-31), and IRT-2 (TEM-30) (3) were used as known standards along with the mutant enzyme.

Antimicrobial agents and measurement of MICs. The following standard powders were used to prepare antimicrobial solutions for estimating MICs and/or kinetic parameters: benzylpenicillin (Sarbach); amoxicillin, ticarcillin, and clavulanic acid (SmithKline Beecham); piperacillin and tazobactam (Lederlé); methicillin, oxacillin, and aztreonam (Bristol-Myers); cephaloridine, ceftazidime, and cefuroxime (Glaxo); cefoperazone and sulbactam (Pfizer); cefoxitin and imipenem (Merck Sharp & Dohme); cephalothin (Panpharma); cefotaxime (Roussel); mezlocillin (Bayer); moxalactam (Lilly); mecillinam and brobactam (Leo); and ceftriaxone (Roche). The determination of MICs was carried out by an agar dilution procedure on Mueller-Hinton agar (Diagnostics Pasteur) with a Steers multiple inoculator and an inoculum of 10^4 CFU per spot according to standard guidelines (13).

Purification and kinetic studies of the mutant β-lactamase. The *E. coli* P37 culture was grown overnight in 6 liters of brain heart infusion broth (Difco Laboratories, Detroit, Mich.) with constant shaking at 37°C. Crude cell extract was obtained by sonication of 54 g of wet bacteria. The β-lactamase was purified as shown in Table 1 according to a procedure previously described (7, 36), and purity was verified by sodium dodecyl sulfate-gel electrophoresis (25). The detection of β-lactamase and the determination of its pI by analytical isoelectric focusing in polyacrylamide gels (pH range, 3.5 to 9.5) were performed as reported elsewhere (27), the β-lactamase activity being localized by the use of an iodine-starch method in agar gel (23).

The kinetic parameters were determined at 37°C and pH 7.0 by computerized microacidimetric assay with a pH stat apparatus (22). One unit of β-lactamase activity is defined as the amount of enzyme hydrolyzing 1 μmol of benzylpenicillin per min at pH 7.0 and 37°C.

Plasmid isolation. Attempts to transfer the plasmid encoding the *bla* gene were unsuccessful. Plasmid DNA was prepared from the clinical strain by a cleared-lysate procedure with a purification step involving cesium chloride-ethidium bromide density gradient ultracentrifugation (31). The minimal size of the plasmid DNA was assessed as follows. Plasmid DNA digested with *Eco*RI,

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TABLE 1. Purification of IRT-14 β-lactamase from *E. coli* P37 and β-lactamase activities

Purification step	Total protein (mg)	Total activity (U)	Sp act (U/mg)	Purification (fold)	Yield[a] (%)
Crude extract	2,200	1,214	0.550	1.0	100
$(NH_4)_2SO_4$ precipitation	375	1,140	3.00	5.5	94
Ion exchange	46.8	440	9.4	17.0	36
Size exclusion chromatography	3.5	530	150	273	44
Preparative IEF[b]	0.18	200	1,110	2,018	16

[a] Yield is the units of enzyme activity recovered after the indicated purification step divided by the units in the total crude extract times 100.
[b] IEF, isoelectric focusing.

*Bgl*I, and *Sca*I (Boehringer Mannheim, Meylan, France) was electrophoresed on a 1.5% (wt/vol) agarose gel, stained with ethidium bromide, and visualized under UV light. The fragment sizes were determined by comparison with standard size markers (markers I, II, IV, and VII from Boehringer Mannheim), and the minimal size of the whole plasmid was deduced by the addition of the sizes of these fragments.

RESULTS

Resistance pattern of IRT-14 β-lactamase. Strain P37 was resistant to amoxicillin and ticarcillin and combinations of these with β-lactamase inhibitors but remained susceptible to other β-lactams. It was also resistant to kanamycin (APH3′ resistance phenotype), tetracyclines, chloramphenicol, co-trimoxazole, and nalidixic acid. A reduced susceptibility to fluoroquinolones and rifampin was also observed. The resistance pattern, evaluated in terms of the MICs of β-lactams, is shown in Table 2. These results confirmed the resistance of strain P37 to broad-spectrum penicillins and the restricted potentiating effect of β-lactamase inhibition on penicillins. We obtained a twofold decrease in the MIC with clavulanic acid and a fourfold decrease with tazobactam in combination with amoxicillin, ticarcillin, or piperacillin. Under identical experimental conditions, TEM-1 was susceptible to the effect of β-lactamase inhibitors and the synergy was more pronounced with clavulanic acid than with tazobactam. The MICs of mecillinam, cephalosporins, aztreonam, moxalactam, and imipenem were such as to confirm the susceptibility of the strain.

TABLE 2. β-lactam MICs for *E. coli* P37 producing IRT-14 and the control strain

Antibiotic[a]	MIC (μg/ml) for:	
	E. coli K12 J53 TEM-1 (R111)	*E. coli* P37 IRT-14
Amx	>4,096	4,096
Amx + clavulanate	64	2,048
Amx + tazobactam	128	1,024
Tic	>4,096	1,024
Tic + clavulanate	128	512
Tic + tazobactam	256	256
Pip	128	16
Pip + clavulanate	2	8
Pip + tazobactam	16	4
Mecillinam	8	0.25
Cephalothin	16	4
Cefoxitin	4	8
Cefotaxime	0.03	0.03
Ceftazidime	0.03	0.125
Aztreonam	0.015	0.03
Moxalactam	0.015	0.06
Imipenem	0.125	0.125

[a] The concentration of both clavulanate and tazobactam was 2 μg/ml. Amx, amoxicillin; Tic, ticarcillin; Pip, piperacillin.

Functional characterization of purified IRT-14 β-lactamase. Analytical isoelectric focusing of both the crude bacterial extract and the purified enzyme from strain P37 revealed a single band with a pI of 5.2. The four-step purification procedure that had been employed to recover 0.18 mg of IRT-14 β-lactamase, with a purity of more than 95%, is summarized in Table 1. The enzyme was purified 2,018-fold and had a final specific activity of 1,110 U/mg.

The kinetic parameters k_{cat} and K_m and the k_{cat}/K_m ratio for IRT-14 are given in Table 3. The enzyme activity levels of IRT-14 with all β-lactams except ticarcillin were found to be lower than those of the wild-type TEM-1 β-lactamase, as indicated by the k_{cat} values. Except for cephaloridine, the affinities and catalytic efficiencies of IRT-14 for broad-spectrum penicillins and cephalosporins were also found to be reduced in comparison with those of the wild-type TEM-1 β-lactamase. The K_i values of IRT-14 for β-lactamase inhibitors were compared. Brobactam (0.8 μM) and tazobactam (0.46 μM) appeared to have greater affinities than clavulanic acid (4.1 μM) and sulbactam (10.6 μM). For the latter two inhibitors, the TEM-1 K_is (10) were lower: 0.8 μM for clavulanic acid and 0.9 μM for sulbactam. The 50% inhibitory concentration (IC_{50}) values for IRT-14 are shown in Table 4. The major difference between the IC_{50}s for IRT-14 and those for TEM-1 is in the dramatic loss of sensitivity of the former to clavulanic acid.

Plasmid characterization. The minimal size of the plasmid from the clinical strain was estimated to be ca. 33 kb.

DISCUSSION

The resistance of clinical *E. coli* strains to β-lactams and mechanism-based inactivators for class A β-lactamases is expanding (9, 18). The initial descriptions of resistance involving spontaneous mutations in the bla_{TEM} genes were made at the beginning of 1990, after almost 10 years of use of β-lactamase inhibitors (35, 36). Currently, more than 10 IRTs have been identified, and their emergence has been reported in Spain, France, and Great Britain (3, 5, 7, 18, 33, 37). They have been placed in group 2br in the recent classification of β-lactamases (8). The finding that IRTs are also found in *Klebsiella pneumoniae* (26) and *Proteus mirabilis* (6) suggests that IRT variants may also emerge in other species of *Enterobacteriaceae.*

E. coli P37 exhibits high levels of resistance to broad-spectrum penicillins, and the loss of the synergistic effect between the β-lactamase inactivators and the inactive penicillins is also observed for this strain. In addition, the strain is susceptible to cephalothin and mecillinam. Such a resistance profile is characteristic of organisms producing other IRTs (7, 36). Furthermore, a comparison of MIC values reveals that the inhibitory action of tazobactam seems to be slightly greater than that of clavulanic acid. Both strain P37 and strain PEY (IRT-4) have comparably high levels of resistance to β-lactam–β-lactamase

TABLE 3. Kinetic parameters for TEM-1 and IRT-14 β-lactamases

Substrate	TEM-1[a]			IRT-14[b]			
	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1} s^{-1}$)	k_{cat}[c] (s^{-1})	K_m[d] (μM)	k_{cat}/K_m ($\mu M^{-1} s^{-1}$)	Efficiency[e] (%)
Benzylpenicillin	1,542	24	64.2	700	140	5	7.8
Amoxicillin	1,295	43	30.1	725	205	3.5	11.6
Ticarcillin	46	10	4.6	82	1,440	0.06	1.3
Piperacillin	1,326	43	30.8	730	365	2.0	6.5
Mezlocillin	ND[f]	ND	ND	740	670	1.1	ND
Cephalothin	200	260	0.77	69	660	0.1	13
Cephaloridine	1,049	950	1.1	630	980	0.64	58.2
Cefoperazone	ND	ND	ND	360	1,300	0.28	ND

[a] Data are from reference 7. Kinetic values for TEM-1 and IRT-14 were determined under the same technical conditions.
[b] k_{cat} and K_m values for IRT-14 for mecillinam, aztreonam, cefotaxime, ceftriaxone, cefoxitin, ceftazidime, cefuroxime, and moxalactam could not be determined.
[c] The standard deviation for the analysis was ±5%. For methicillin, oxacillin, and imipenem k_{cat} values could not be determined; K_i values were <1 μM for these substrates.
[d] The standard deviations for these determinations were ±10% for $K_m < 100$ μM and ±20% for $K_m > 100$ μM.
[e] Efficiency of IRT-14 relative to that of TEM-1, which was fixed at 100.
[f] ND, not determined.

inhibitor combinations, as indicated by comparable MIC values.

As an enzyme, IRT-14 is similar to other IRTs in terms of the overall reduction of catalytic efficiency (k_{cat}/K_m). This reduction results from the combination of a major loss of affinity and reduction of hydrolysis for most β-lactams. The increased IC_{50} values of β-lactamase inhibitors for IRT-14 enzyme also reflect the lower affinity levels of the characterized enzyme compared to those of TEM-1. The affinities of IRT-14 for cephalothin and cephaloridine are as small as those for other IRT enzymes.

At the protein level, IRT-14 is closer to TEM-1, since TEM-1 and TEM-2 β-lactamases are distinguished by a characteristic amino acid change at position 39 (glutamine in TEM-1 versus lysine in TEM-2), with a consequent pI difference (pI, 5.4 for TEM-1 and 5.6 for TEM-2). However, a comparison of the nucleotide sequence of the bla_{IRT-14} gene (9) with those of the three wild-type bla_{TEM} genes (16) shows that the gene encoding IRT-14, with five nucleotide variations, appears to be closer to the bla_{TEM-2} gene than to the bla_{TEM-1A} and bla_{TEM-1B} genes (Table 5). Both the T→C variation at nucleotide position 32 in the promoter region and the variation at nucleotide position 317 (Lys-39→Gln) in the structural gene are at common polymorphic sites encountered in IRTs (9) and in extended-spectrum β-lactamase genes (20). They are not likely to account for the observed resistance of IRT-14 to the effect of β-lactamase inhibitors. The T-32→C substitution would be expected to reduce the level of β-lactamase production (12, 16), and amino acid 39 does not seem to be involved in catalysis (21).

Contrasting with these neutral sequence variations, the substitution at position 162 falls within the functional −10 Pribnow box. This G→T transversion renders the −10 consensus sequence of the IRT-14 β-lactamase gene more similar to the optimal promoter of *E. coli*, 5′-TATAAT-3′ (17), and has already been shown to increase the level of production of the *E. coli malT* gene (11). In a preliminary study, the promoter of IRT-14 was shown to be more efficient than the parental promoter, with a similarity to the consensus sequence type, and was shown to contribute, via a higher level of production of the mutant enzyme, to the resistance phenotype for the suicide inhibitors (data not shown). We have also found this important specific variation of the promoter region in IRT-4, IRT-7, and IRT-8, all of which have two substitutions in the structural gene at nucleotide positions 69 and 276 (9).

TABLE 4. IC_{50} values of β-lactamase inhibitors

Inhibitor	IC_{50} (μM)[a] for β-lactamase:					
	TEM-1[b]	IRT-1[b]	IRT-2[b]	IRT-3[c]	IRT-4[d]	IRT-14
Clavulanic acid	0.034	31	9.4	12	41	22.5
Sulbactam	19	300	260	160	435	104
Tazobactam	0.74	12	2.9	5	3.2	1.48
Brobactam	0.12	7.4	0.6	ND[e]	0.93	0.3

[a] All IC_{50}s were determined by microacidimetry with penicillin as substrate, with the exception of IRT-3, whose measurements were carried out spectrophotometrically at 25°C with nitrocefin as substrate.
[b] Data from reference 36.
[c] Data from reference 5.
[d] Data from reference 7.
[e] ND, not determined.

TABLE 5. Nucleotide and amino acid substitutions in genes encoding TEM-1, TEM-2, and IRT-14

Nucleotide[a] (aa)[b] position	Nucleotide (aa) in gene (β-lactamase)[c]:			
	bla_{TEM-1A} (TEM-1) (Tn*3*)	bla_{TEM-1B} (TEM-1) (Tn*2*)	bla_{TEM-2} (TEM-2) (Tn*1*)	bla_{IRT-14} (IRT-14)
Promoter region				
32	C	C	T	C
162	G	G	G	T
175	A	G	A	A
Coding region				
226	C (Phe)	T	C	C
317 (39)	C (Gln)	C	A (Lys)	C (Gln)
346	A (Glu)	A	G	G
407 (69)	A (Met)	A	A	T (Leu)
436	C (Gly)	T	T	T
604	G (Ala)	T	G	G
682	T (Thr)	T	C	C
925	G (Gly)	G	A	A
1020 (275)	G (Arg)	G	G	A (Gln)

[a] Nucleotide numbering is as described by Sutcliffe (34).
[b] Amino acid (aa) positions are numbered in accordance with the Ambler numbering system (2).
[c] Data for TEM-1 and TEM-2 and transposons are from reference 16; data for IRT-14 are from reference 9.

The characteristic resistance of IRT-14 to clavulanic acid results principally from two amino acid substitutions: Met-69→Leu and Arg-275→Gln. The codon at position 69 is a hot spot for IRT mutations. The replacement of Met-69, produced by site-directed mutagenesis (29), was already described in vivo and either occurred alone or was associated with a second variation (7, 9, 18, 37). Met-69, which forms the back wall of the oxyanion pocket in parental β-lactamases, is important because of its side chain on the three-dimensional structure of TEM-type β-lactamases or variants that impair β-lactam binding (14, 29). The Arg-275→Gln substitution represents a new mutation in the TEM enzymes. This neutral-for-basic amino acid change accounts for the pI of 5.2 for IRT-14 enzyme. Another mutation, Arg-275→Leu, which also lowered the pI to 5.2, has been described earlier for IRT-9 (18). In the parental TEM β-lactamases, Arg-244 plays a role in maintaining active-site integrity and is restricted in its movements by the side chains of other amino acids, particularly Arg-275 and Asn-276 (14, 15). The replacement of a guanidinium group by an amide group due to an Arg-275→Gln substitution is expected to destabilize the active site and overall enzyme turnover. Therefore, the high level of resistance of strain P37 is likely to reflect the combined effect of both the Met-69→Leu and Arg-275→Gln substitutions in IRT-14 β-lactamase. Similar conclusions were reached for IRT-4 with the Met-69→Leu and Asp-276→Asn substitutions (7, 14, 32).

The characterization of this new inhibitor-resistant TEM-type β-lactamase (IRT-14) should provide further insight into the understanding of the heterogeneity of IRT types and into the structure-activity correlation of mutated enzymes. The diversity of IRT enzymes is expanding as a consequence of microbial adaptation to antibiotic stress, a phenomenon of importance in the therapeutic field.

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