ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Nov. 1995, p. 2580–2582 Vol. 33, No. 11
0066-4804/95/$04.00+0

First Characterization of Inhibitor-Resistant TEM (IRT) β-Lactamases in *Klebsiella pneumoniae* Strains

J. LEMOZY,[1] D. SIROT,[2]* C. CHANAL,[2] C. HUC,[1] R. LABIA,[3] H. DABERNAT,[1] AND J. SIROT[2]

Laboratoire de Microbiologie, CHR PURPAN, 31059 Toulouse Cédex,[1] Laboratoire de Bactériologie, Faculté de Médecine, 63001 Clermont-Ferrand,[2] and Museum National d'Histoire Naturelle, UA 401 Centre National de la Recherche Scientifique, Paris,[3] France

Received 18 May 1995/Returned for modification 2 August 1995/Accepted 8 September 1995

Two clinical strains of *Klebsiella pneumoniae*, TP 01 and TP 02, presented resistance to amoxicillin-clavulanate and were fully susceptible to cephalothin. These strains produced two β-lactamases, SHV-1 and a TEM enzyme with a pI of 5.2. The previously described changes Arg-244→Cys and Arg-244→Ser in IRT-1 and IRT-2 (A. Belaaouaj, C. Lapoumeroulie, M. M. Caniça, G. Vedel, P. Nevot, R. Krishnamoorthy, and G. Paul, FEMS Microbiol. Lett. 120:75–80, 1994) were found in TEM enzymes from the TP 01 and TP 02 strains, respectively. This is the first report of inhibitor-resistant TEM (IRT) in species other than *Escherichia coli* from the family *Enterobacteriaceae*.

Most *Klebsiella pneumoniae* strains are moderately resistant to amino- and carboxy-penicillins by synthesis of a class A chromosomally mediated penicillinase (SHV-1), and clavulanic acid has a synergistic effect when associated with these penicillins. These strains are generally susceptible to cephalothin and all other cephalosporins. Some *K. pneumoniae* isolates which are highly resistant to penicillins because of the production of plasmid-mediated TEM-like penicillinases also have reduced susceptibility to cephalosporins.

Previous reports have established that the susceptibility of *Escherichia coli* to β-lactamase inhibitors can be affected by the overproduction of TEM-1 β-lactamase (14, 18). In *K. pneumoniae*, overproduction of either TEM-1 or SHV-1 increased resistance to amoxicillin-clavulanic acid and to cephalothin (16).

In this report, we describe the characterization of the β-lactamases produced by two clinical isolates of *K. pneumoniae* that exhibited high-level resistance to amoxicillin-clavulanate and susceptibility to cephalothin.

K. pneumoniae TP 01 and TP 02 were isolated from the urine and respiratory tracts of patients hospitalized in the geriatric and pneumology services, respectively, of a teaching hospital in Toulouse. The MICs of amoxicillin, amoxicillin-clavulanate, ticarcillin, ticarcillin-clavulanate, piperacillin, piperacillin-tazobactam (clavulanic acid and tazobactam were at fixed concentrations of 2 μg/ml and 4 μg/ml, respectively), and cephalothin were determined by dilution in Mueller-Hinton agar (Sanofi Diagnostics Pasteur, Marnes-la-Coquette, France) with an inoculum of 10^4 CFU per spot.

A *K. pneumoniae* strain (CF 004) producing only the chromosomal SHV-1 enzyme, a *K. pneumoniae* strain (CF 014) producing both SHV-1 and TEM-1, and a *K. pneumoniae* strain (CF 024) producing SHV-1 enzyme at a high level were studied as comparisons for the MIC determinations (Table 1).

K. pneumoniae CF 004 (SHV-1) was characterized by susceptibility to all β-lactams tested except for amoxicillin and ticarcillin. The MIC for both of these drugs was 128 μg/ml. The resistance patterns of *K. pneumoniae* TP 01 and TP 02 were characterized by high MICs of amoxicillin-clavulanate (with clavulanate at a fixed concentration of 2 μg/ml) of 256 and 512 μg/ml, respectively, while that for TEM-1-producing strain CF 014 is only 32 μg/ml. MICs of ticarcillin-clavulanate for inhibitor-resistant TEM (IRT)-producing strains TP 01 and TP 02 (32 to 64 μg/ml) and the TEM-1-producing strain (32 μg/ml) were similar.

Susceptibility to piperacillin is less reduced for IRT-producing strains (MICs, 8 and 32 μg/ml) than for the TEM-1-producing strain (MIC, 64 μg/ml). Susceptibilities to the combination of piperacillin and tazobactam were similar for IRT- and TEM-producing strains (MICs, 2 to 4 μg/ml).

In addition to amoxicillin-clavulanate resistance, the two IRT-producing strains exhibited a susceptibility to cephalothin (MIC, 4 μg/ml) similar to that observed for the SHV-1-producing strain. The MIC of cephalothin for the TEM-1-producing strain is higher (8 μg/ml).

Strain CF 024 producing SHV-1 at a high level was characterized by increased MICs of all the β-lactams listed in Table 1 for this strain.

The five *K. pneumoniae* strains presented similar susceptibilities to cefoxitin (MICs, 2 to 8 μg/ml), cefotaxime (MICs, 0.03 to 0.06 μg/ml), and imipenem (MICs, 0.06 to 0.5 μg/ml).

Mating experiments for strains TP 01 and TP 02 were performed with recipient *E. coli* HB101. Transconjugants were selected on agar containing rifampin (100 μg/ml) and amoxicillin (100 μg/ml) or tetracycline (10 μg/ml).

The bla_{TEM} genes were transferred from strains TP 01 and TP 02 to *E. coli* HB101 at a low frequency ($\approx 10^{-6}$). They were located on a very large plasmid with a size of >180 kb which is alone in the two wild strains (data not shown). The β-lactam resistance phenotypes of the *E. coli* transconjugants obtained (Table 1) were similar to those reported for the *E. coli* clinical strains SAL and GUER, which produce IRT-1 and IRT-2, respectively (25).

Isoelectric focusing performed with polyacrylamide gels containing ampholines with a pH range of 3.5 to 10.0 (22) showed that the β-lactamases produced by the two strains focused at a pI of 5.2.

Single-stranded DNA templates for sequencing were generated by PCR performed at an asymmetric ratio of amplification primers A and B (5, 19). The dideoxynucleotide chain termi-

* Corresponding author. Mailing address: Laboratoire de Bactériologie, Faculté de Médecine, 28 Place Henri-Dunant, 63001 Clermont-Ferrand, France.

TABLE 1. MICs of β-lactams for different *K. pneumoniae* strains and *E. coli* transconjugants

Species and strain (enzyme)	MIC (μg/ml)						
	Amoxicillin		Ticarcillin		Piperacillin		Cephalothin
	Alone	With clavulanic acid[a]	Alone	With clavulanic acid[a]	Alone	With tazobactam[b]	
K. pneumoniae[c]							
TP 01 (IRT-1)	512	256	128	32	8	4	4
TP 02 (IRT-2)	>1,024	512	256	64	32	4	4
CF 014 (TEM-1)	>1,024	32	>1,024	32	64	2	8
CF 024 (high-level SHV-1)	>1,024	128	>1,024	256	>256	>64	64
CF 004 (SHV-1)	128	2	128	4	4	2	4
E. coli[d]							
CF 01 (IRT-1)	128	64	16	8	1	1	4
CF 02 (IRT-2)	1,024	512	256	32	16	1	4

[a] At a fixed concentration of 2 μg/ml.
[b] At a fixed concentration of 4 μg/ml.
[c] All *K. pneumoniae* strains also produced the chromosomally encoded SHV-1 β-lactamase.
[d] *E. coli* HB101 transconjugants.

nation method of Sanger et al. (20) was applied to purified PCR products with the Sequenase version 2.0 kit (Amersham-France, Les Ulis, France) as previously described (5). Complete sequencing of the TEM genes of *K. pneumoniae* TP 01 and TP 02 was performed.

The nucleotide substitutions relative to those in other bla_{TEM} genes and the deduced amino acid changes observed in the TEM β-lactamases from TP 01 and TP 02 were compared to those in IRT-1 and IRT-2 (Table 2). The deduced amino acid sequence of strain TP 01 showed an amino acid replacement with respect to the TEM-1 sequence (1): Arg-244→Cys, corresponding to nucleotide change C→T at position 929. In strain TP 02, the replacement observed at the same position is Arg-244→Ser, corresponding to nucleotide change C→A at the same position, 929. Analysis of the nucleotide sequences revealed some silent substitutions relative to the bla_{TEM-1A} sequence at known positions: 226, 346, 436, 604, 682, and 925. Positions 346, 604, 682, and 925 differed between the two genes, with mutation Arg-244→Cys encoding IRT-1 enzymes and mutation Arg-244→Ser encoding the IRT-2 enzymes.

Since 1989, TEM-derived β-lactamases which exhibit reduced activities against β-lactam antibiotics and resistance to β-lactamase inhibitors (IRT enzymes) have been reported only in *E. coli* strains (2–4, 9, 10, 22, 24–26).

The frequency of common plasmid-mediated β-lactamase genes, especially the TEM-1 genes, and their ability to mutate to code for mutant enzymes led to the supposition that *Enterobacteriaceae* IRT mutants occurred in species of *Enterobacteriaceae* other than *E. coli*, such as *K. pneumoniae*, a species predominantly involved in the production of extended-spectrum β-lactamase mutants (17, 21).

In both *E. coli* and *K. pneumoniae*, high-level resistance to amoxicillin-clavulanate associated with susceptibility to cephalothin is an unusual resistance phenotype. However, to distinguish additional production of a TEM-1 enzyme at various levels from production of an IRT mutant, it may sometimes be required to take into account the resistance levels to other β-lactams, such as piperacillin, cephalothin, or cefamandole (data not shown), which increase according to the TEM-1 production level. Strains with high levels of SHV-1 enzyme exhibit increased resistance levels to all penicillins and to cephalothin and reduced susceptibilities to ceftazidime (16). This phenotype resistance is noticeably different from that conferred by production of an IRT enzyme.

The molecular characterization of the two genes encoding the β-lactamases revealed a mutation of arginine residue 244, which participates in the active-site binding of substrates and clavulanate and plays a specific role in the inactivation process. Its substitution by cysteine (IRT-1) or serine (IRT-2) removes the ionic bond to the substrate in the active site, which results in reduced affinities of these mutant enzymes for clavulanate

TABLE 2. Nucleotides and corresponding amino acid substitutions in the bla_{TEM} and bla_{IRT} genes

Gene	Nucleotide at position (amino acid change)[a]:								Source or reference(s)
	226	317	346	436	604	682	925	929	
bla_{TEM-1A}	C	C	A	C	G	T	G	C	23
bla_{TEM-1B}	T	C	A	T	T	T	G	C	6, 8
bla_{TEM-2}	C	A (Lys)	G	T	G	C	A	C	6, 8
bla_{IRT-1}	ND[b]	C	A	T	T	T	G	T (Cys)	3
bla_{IRT-1b}	C	C	G	T	G	C	A	T (Cys)	This study
bla_{IRT-2}	ND	C	G	T	G	C	A	A (Ser)	3
bla_{IRT-2b}	C	C	A	T	T	T	G	A (Ser)	This study
Amino acid in TEM-1 (position)[c]	Phe (6)	Gln (39)	Glu (48)	Gly (78)	Ala (134)	Thr (160)	Gly (242)	Arg (244)	

[a] Nucleotide numbering is according to Sutcliffe (23).
[b] ND, not done.
[c] Numbering according to Ambler et al. (1).

and reduced efficiency of enzymatic activity for these mutants (7, 11–13, 15).

In the TP 01 strain, the gene encoding the IRT-1 enzyme differed from that reported by Belaaouaj et al. (2) by four silent mutations at positions 346 (A→G), 604 (T→G), 682 (T→C), and 925 (G→A). These four silent mutations were observed in the $bla_{\text{TEM-2}}$ gene, while at these positions, the IRT-1-encoding gene previously described (2) is identical to the $bla_{\text{TEM-1B}}$ gene. In the TP 02 strain, the gene encoding the IRT-2 enzyme differed at the same four positions in the inverse manner from that previously reported, since $bla_{\text{IRT-2}}$ in *K. pneumoniae* TP 02 is identical to $bla_{\text{TEM-1B}}$, except at position 226 (T→C). We suggest that the two genes described in this study be designated $bla_{\text{IRT-1b}}$ and $bla_{\text{IRT-2b}}$.

The first two IRT enzymes found in *K. pneumoniae* were the same as the first two IRT enzymes identified in *E. coli*. The IRT-1 (TEM-31) enzyme is rarely detected, since it has been found in only one strain (strain SAL) (2), while the IRT-2 (TEM-30) enzyme was frequently encountered (≈20% of IRT-producing strains), as demonstrated in a recent study (9). After this first characterization of IRT in *K. pneumoniae* strains, we can expect the emergence of other IRT variants in this species, in other species of *Enterobacteriaceae*, and in other TEM-containing organisms, such as *Haemophilus influenzae.*

We thank Rolande Perroux and Marlène Jan 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. **Belaaouaj, A., C. Lapoumeroulie, M. M. Caniça, G. Vedel, P. Nevot, R. Krishnamoorthy, and G. Paul.** 1994. Nucleotide sequences of the genes coding for the TEM-like β-lactamases IRT-1 and IRT-2 (formerly called TRI-1 and TRI-2). FEMS Microbiol. Lett. **120:**75–80.
3. **Blazquez, J., M.-R. Baquero, R. Canton, I. Alos, and F. Baquero.** 1993. Characterization of a new TEM-type β-lactamase resistant to clavulanate, sulbactam, and tazobactam in a clinical isolate of *Escherichia coli*. Antimicrob. Agents Chemother. **37:**2059–2063.
4. **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.
5. **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.
6. **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.
7. **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.
8. **Goussard, S., and P. Courvalin.** 1991. Sequence of the genes *blaT-1B* and *blaT-2*. Gene **102:**71–73.
9. **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.
10. **Henquell, C., D. Sirot, C. Chanal, C. De Champs, P. Chatron, B. Lafeuille, P. Texier, J. Sirot, and R. Cluzel.** 1994. Frequency of inhibitor-resistant TEM (IRT) β-lactamases in *Escherichia coli* isolates from urinary tract infections. J. Antimicrob. Chemother. **34:**707–714.
11. **Imtiaz, U., E. Billings, J. R. Knox, E. K. Manavathu, S. A. Lerner, and S. Mobashery.** 1993. Inactivation of class A β-lactamases by clavulanic acid: the role of arginine-244 in a proposed nonconcerted sequence of events. J. Am. Chem. Soc. **115:**4435–4442.
12. **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 Å resolution. FEBS Lett. **299:**135–142.
13. **Manavathu, E. K., S. A. Lerner, and S. Mobashery.** 1991. Effect of amino acid substitution at position 241 of TEM-1 β-lactamase on inactivation by β-lactamase inactivators, abstr. 16, p. 101. *In* Program and abstracts of the 31st Interscience Conference on Antimicrobial Agents and Chemotherapy. American Society for Microbiology, Washington, D.C.
14. **Martinez, J. L., M. F. Vicente, A. Delgado-Iribarren, J. C. Perez-Diaz, and F. Baquero.** 1989. Small plasmids are involved in amoxicillin-clavulanate resistance in *Escherichia coli*. Antimicrob. Agents Chemother. **33:**595.
15. **Oliphant, A. R., and K. Struhl.** 1989. An efficient method for generating proteins with altered enzymatic properties: application to β-lactamase. Proc. Natl. Acad. Sci. USA **86:**9094–9098.
16. **Petit, A., H. B. Yaghlane-Bouslama, L. Sofer, and R. Labia.** 1992. Does high level production of SHV-type penicillinase confer resistance to ceftazidime in *Enterobacteriaceae*? FEMS Microbiol. Lett. **92:**89–94.
17. **Philippon, A., G. Arlet, and P. H. Lagrange.** 1994. Origin and impact of plasmid-mediated extended-spectrum beta-lactamases. Eur. J. Clin. Microbiol. Infect. Dis. **13:**17–29.
18. **Reguera, J. A., F. Baquero, J. C. Penez-Diaz, and J. L. Martinez.** 1991. Factors determining resistance to β-lactam combined with β-lactamase inhibitors in Escherichia coli. J. Antimicrob. Chemother. **27:**569–575.
19. **Saiki, R. K., D. H. Gelfand, S. Stoffel, S. J. Scharf, R. Higuchi, G. T. Horn, K. B. Mullis, and H. A. Erlich.** 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science **239:**487–491.
20. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
21. **Sirot, D.** 1995. Extended-spectrum plasmid-mediated β-lactamases. J. Antimicrob. Chemother. **36**(Suppl. A)**:**19–34.
22. **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.
23. **Sutcliffe, G.** 1978. Nucleotide sequence of the ampicillin resistance gene of *Escherichia coli* plasmid pBR 322. Proc. Natl. Acad. Sci. USA **75:**3737–3741.
24. **Thomson, C. J., and S. G. B. Amyes.** 1992. TRC-1: emergence of clavulanic acid-resistant TEM β-lactamase in a clinical strain. FEMS Microbiol. Lett. **91:**113–118.
25. **Vedel, G., A. Belaaouaj, L. Gilly, R. Labia, A. Philippon, P. Nevot, and G. Paul.** 1992. Clinical isolates of *Escherichia coli* producing TRI β-lactamases: novel TEM-enzymes conferring resistance to β-lactamase inhibitors. J. Antimicrob. Chemother. **30:**449–462.
26. **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.