ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Feb. 1998, p. 464–467 Vol. 42, No. 2
0066-4804/98/$04.00+0

Characterization of FOX-3, an AmpC-Type Plasmid-Mediated β-Lactamase from an Italian Isolate of *Klebsiella oxytoca*

ANNA MARCHESE,[1] GUILLAUME ARLET,[2]* GIAN CARLO SCHITO,[1] PHILIPPE H. LAGRANGE,[2] AND ALAIN PHILIPPON[2]

Istituto Di Microbiologia, 16132 Genoa, Italy,[1] and Service de Microbiologie, Hôpital Saint-Louis, 75475 Paris, France[2]

Received 10 April 1997/Returned for modification 12 August 1997/Accepted 24 November 1997

***Klebsiella oxytoca* 1731, which showed a wide spectrum of resistance to β-lactams, including cefoxitin, was isolated in 1994 from a patient in Genoa, Italy. This strain contained a plasmid-mediated AmpC β-lactamase with a pI of 7.25. Sequencing of the corresponding DNA of *K. oxytoca* 1731 revealed 96 and 97% identities of the deduced amino acid sequence with FOX-1 and FOX-2, respectively.**

Chromosomal group 1 β-lactamases (class C of Ambler) produced by gram-negative bacteria such as *Enterobacter* spp., *Serratia* spp., *Citrobacter* spp., and *Morganella* spp. can hydrolyze many β-lactam antibiotics, including cephamycins and extended broad-spectrum cephalosporins (8). In recent years *ampC* genes have been found mainly in conjugative plasmids and among *Klebsiella pneumoniae* isolates and occasionally among *Escherichia coli* isolates. *ampC* genes encode a variety of enzymes, including MIR-1 (20), CMY-1 (5), CMY-2 (4), BIL-1 (21, 28), MOX-1 (12), LAT-1 (25), FOX-1 (10), LAT-2 (9), FOX-2 (6), and ACT-1 (7); some of them are highly related to chromosomal AmpC of *Citrobacter freundii*, such as BIL-1, LAT-1, LAT-2, and CMY-2, or of *Enterobacter cloacae*, such as MIR-1 and ACT-1. Bacteria that harbor AmpC plasmids have antibiotic susceptibility patterns which are similar to those of strains overproducing chromosomally encoded β-lactamase (15).

In this study we characterize an AmpC-type plasmid-mediated β-lactamase isolated from *Klebsiella oxytoca* 1731 in Italy. This strain was isolated from a vaginal swab of one patient and was collected in 1994 during a European multicenter survey of the incidence of *Klebsiella* spp. carrying extended-spectrum β-lactamases in intensive care units (16). Furthermore, a strain of *K. pneumoniae* 1734 with the same resistance pattern was isolated from a urine specimen of another patient. Both patients were admitted to the same intensive care unit at the University Hospital of Genoa at the end of 1994. For treatment these patients received piperacillin, and one patient received in addition imipenem.

The minimal inhibitory concentrations (MICs) of β-lactams alone or in combination with clavulanate (4 μg/ml) were determined by the agar dilution technique recommended by the National Committee for Clinical Laboratory Standards (19). The antibiotic resistance phenotypes of the donor strains *K. oxytoca* 1731 and *K. pneumoniae* 1734 are shown in Table 1. The MICs of cefoxitin, cefotaxime, ceftazidime, and aztreonam remained unchanged despite the addition of clavulanate, whereas the MICs of the two penicillins (amoxicillin and ticarcillin) were substantially reduced.

* Corresponding author. Mailing address: Hôpital Saint-Louis, Laboratoire de Microbiologie, 1 ave. Claude Vellefaux, 75475 Paris Cedex 10, France. Phone: 33 (0)1 42 49 94 87. Fax: 33 (0)1 42 49 94 86. E-mail: a.philippon@chu-stlouis.fr.

Several *E. coli* J53-2 rif-R transconjugants were selected on MacConkey agar plates supplemented with rifampin (200 μg/ml) and cefoxitin (10 μg/ml) or ceftazidime (2 μg/ml) or ticarcillin (100 μg/ml). Because of the multiresistance profile of the two donor strains, plates supplemented with tetracycline (15 μg/ml) or kanamycin (25 μg/ml) were also prepared (18). All the transconjugants selected on these antibiotics showed a resistance phenotype similar to that of their respective donor strains. These profiles were characterized by resistance to expanded-spectrum cephalosporins and cefoxitin alone or in combination with clavulanate (Table 1) and to tetracycline and kanamycin.

Plasmid DNAs from *K. oxytoca* 1731 and two transconjugants were prepared by the alkaline extraction method (14). Analysis of plasmid DNA by electrophoresis in 0.8% agarose gels with Tris-borate-EDTA buffer revealed one large plasmid of about 130 kb common to all strains (data not shown). Analytical isoelectric focusing was performed in polyacrylamide gels with sonicated crude cell extracts as described previously (18). Two bands of β-lactamase activity (pI 5.4 and 7.25, respectively) were detected in *K. oxytoca* 1731 and its transconjugants.

The molecular characterization of the pI 5.4 β-lactamase produced by *K. oxytoca* 1731 was performed by PCR-restriction fragment length polymorphism, as previously described (3). With *Sau*3AI, *Bcl*I, *Bpm*I, *Hpa*II, *Hph*I, and *Mse*I as endonucleases, no point mutations were detected in comparison with the β-lactamase *tem-1* gene (pBR322). Therefore, this enzyme was identified as a TEM-1 β-lactamase and was probably responsible for the resistance to ticarcillin and amoxicillin and explains the reduction of the MICs of these two penicillins observed in the presence of clavulanate.

The characterization of the β-lactamase with a pI of 7.25 from the transconjugant EC1731 was determined after purification as described by Iaconis and Sanders (13). The substrate profile of the pI 7.25 β-lactamase of the transconjugant EC1731 was assessed by the spectrophotometric method (26) by using a Biochrom 4060 spectrophotometer (Pharmacia LKB Biotechnology) at 37°C and freshly prepared antibiotic solutions in 0.05 M phosphate buffer (pH 7.0). The molecular extinction coefficients were calculated as previously described (24), and the maximum rate of hydrolysis (V_{max}) was determined by the Lineweaver-Burk plot of initial velocity (v) at six different substrate concentrations. The maximal change in absorbance of the substrates was monitored as follows (in nm):

TABLE 1. In vitro β-lactam susceptibilities of *K. oxytoca* 1731 and *K. pneumoniae* 1734 and their transconjugants and the *E. coli* J53-2 recipient

β-Lactam	MIC (μg/ml) for:				
	K. oxytoca 1731	*E. coli* J53-2 R^+ (EC1731)	*K. pneumoniae* 1734	*E. coli* J53-2 R^+ (EC1734)	*E. coli* J53-2 R^-
Amoxicillin	1,024	1,024	1,024	1,024	2
+ Clavulanate	32	32	32	32	2
Ticarcillin	≥1,024	≥1,024	≥1,024	≥1,024	2
+ Clavulanate	32	32	32	32	2
Cefoxitin	64	64	64	64	4
+ Clavulanate	64	64	64	64	4
Cefotaxime	1	1	1	1	≤0.06
+ Clavulanate	1	1	1	1	≤0.06
Ceftazidime	16	16	16	16	0.25
+ Clavulanate	16	16	16	16	0.25
Aztreonam	4	1	4	1	≤0.06
+ Clavulanate	4	1	2	1	≤0.06
Cefepime	0.12	≤0.06	0.12	≤0.06	≤0.06
+ Clavulanate	≤0.06	≤0.06	0.12	≤0.06	≤0.06
Imipenem	0.12	0.12	0.12	0.12	0.12

cephaloridine, 260; cephalothin, 270; cefoxitin, 265; cefotaxime, 254; ceftazidime, 254; aztreonam, 292; benzylpenicillin, 232; and nitrocefin, 482. Specific activity was defined as micromoles of nitrocefin hydrolyzed per minute per microgram of protein. Protein concentration was determined by the method of Lowry et al. (17). The purified β-lactamase of pI 7.25 from EC1731 showed high rates of hydrolysis for cephaloridine and cephalothin and low rates of hydrolysis for benzylpenicillin, cefoxitin, cefotaxime, ceftazidime, and aztreonam (Table 2). The susceptibility to inhibition was determined by preincubating the enzyme with various concentrations of inhibitors for 10 min. Nitrocefin was then added as the substrate, and residual enzyme activity was measured. The concentration of inhibitors required for 50% inhibition of enzyme activity was defined as the IC_{50}. The purified β-lactamase of pI 7.25 from EC1731 was strongly inhibited by cloxacillin (IC_{50} = 0.02 μM) and aztreonam (IC_{50} = 0.0015 μM) and by relatively high concentrations of clavulanate (IC_{50} = 3 μM). The enzyme from EC1731 had kinetic parameters (K_m and V_{max} values) that were very similar to those of FOX-1 variants (10). The V_{max}s for cefoxitin, cefotaxime, ceftazidime, and aztreonam were very low. However, the high affinity of the enzyme for these antibiotics might compensate for the slow hydrolysis rates, and this might result in resistance as observed with *K. oxytoca* 1731. Very low values of V_{max} for cefoxitin (0.008 and 0.003) have also been described for FOX-1 variants (10). The IC_{50}s of clavulanate, cloxacillin, and aztreonam were in agreement with values reported for other plasmid-mediated AmpC-type β-lactamases (12, 20, 25) and were similar to values reported for FOX-1 (10).

To amplify the *ampC* gene of *K. oxytoca* 1731, degenerate oligonucleotide primers were designed from consensus sequences from the *ampC* genes of *E. coli*, *E. cloacae*, and *C. freundii* (A1, A2) and from the *ampC* genes of *Serratia marcescens* and those encoding MOX-1 and FOX-1 (B1, B2). The sequences of the primers are as follows: *ampC* A1, 5′ GGAATTCCTWTGCTGCGCBCTGCTGCT 3′; *ampC* A2, 5′ CGGGATCCCTGCCAGTTTTGATAAAA 3′; *ampC* B1, 5′ GGAATTCCTCASCGAGCAGACSCTGTT 3′; and *ampC* B2, 5′ CGGGATCCCCCGCACMTKAYRTAGGTGTGG 3′ (W = A or T; B = C, G, or T; S = G or C; M = A or C; K = G or T; Y = C or T; R = A or G). DNA sequencing was performed by the procedure of Sanger et al. (23) by using oligonucleotide primers, fluorescent dye-labeled dideoxynucleotides, *Taq* polymerase, and an ABI 373A DNA sequencer (Applied Biosystems, Foster City, Calif.).

The BLAST (1, 2) and FASTA programs were used to search databases for similar nucleotide and amino acid sequences. The Clustal V program (11) was used for the alignment of multiple protein sequences.

A fragment of 410 bp was obtained from *K. oxytoca* by PCR with *ampC* B1 and *ampC* B2 primers, and its sequence was determined. Comparison to sequences in the databases showed high sequence identities with the genes encoding FOX-1 and FOX-2 β-lactamases. Subsequent DNA amplifications of the *ampC* gene of *K. oxytoca* 1731 were performed with the new primer UT7 FOX (5′ TAATACGACTCACTATAG GGAAATGCAACAACGACGTGCG 3′) and LT3 FOX (5′ ATTAACCCTCACTAAAGGGAAATCACTCGGCCAACT GACT 3′). These primers contained the T7 (UT7 FOX) and the T3 (LT3 FOX) RNA polymerase promoter sequences. The DNA amplified by these primers encoded the entire mature protein of the FOX-1 β-lactamase. Two different PCR products, obtained from two separate PCRs, were sequenced by using the T7 and T3 primers and revealed a 1,149-bp open reading frame that had 96% nucleotide sequence identity to the plasmid-mediated β-lactamase FOX-1. The putative amino acid sequence was 96 and 97% identical to the amino acid sequences of plasmid-encoded FOX-1 and FOX-2, respectively (6, 10), and 75 to 74% identical to *Aeromonas sobria* chromosomal β-lactamases (22, 27) (Table 3). Comparison with sequences of eight plasmid-mediated class C β-lactamases revealed the presence of conserved motifs characteristic of serine β-lactamases, such as the box II (SVSK) and the box VII

TABLE 2. Kinetic constants of the FOX-3 β-lactamase produced by EC1731

Substrate	Relative V_{max} (%)	K_m (μM)	V_{max} (μmol/min/μg of protein)
Cephaloridine	100	363	1.11
Cephalothin	320	187	3.53
Benzylpenicillin	1.2	36	0.013
Cefoxitin	<1		<0.01
Cefotaxime	<1		<0.01
Ceftazidime	<1		<0.01
Aztreonam	<1		<0.01

TABLE 3. Amino acid sequence identities of FOX-3, eight plasmid-encoded β-lactamases, and the *A. sobria* class C β-lactamases AER14 and CEPS[a]

β-Lactamase	% Identity with:										
	FOX-3	FOX-2	FOX-1	CEPS	AER14	CMY-1	MOX-1	ACT-1	CMY-2	BIL-1	LAT-1
FOX-3	100	97	96	75	74	72	64	40	40	40	39
FOX-2		100	97	77	75	74	68	42	41	40	41
FOX-1			100	76	74	72	64	40	41	41	40
CEPS				100	74	73	65	43	43	42	42
AER14					100	73	65	37	43	43	42
CMY-1						100	89	40	41	40	40
MOX-1							100	32	37	36	36
ACT-1								100	75	74	73
CMY-2									100	99	98
BIL-1										100	97
LAT-1											100

[a] Data for AER14 and CEPS are from references 22 and 27, respectively.

with a KTG domain, and the class C typical motif YXN (Fig. 1). These results confirmed that this β-lactamase was of the AmpC type and was homologous to the FOX-1 β-lactamase isolated in Argentina (10) and the FOX-2 originating from Guatemala (6). We propose that this enzyme should be named FOX-3.

Because of the possibly related origins of such enzymes, the gene encoding the FOX-3 β-lactamase was detected in *K. pneumoniae* 1734 by using the same degenerate oligonucleotides (*ampC* B1 and *ampC* B2 primers). One hundred percent identity (410 bp) was observed with the corresponding sequence of *K. oxytoca*. The two patients from which the bacteria were isolated had no contact with Argentina or Guatemala or with people from those countries.

Nucleotide sequence accession number. The EMBL accession number for the nucleotide sequence reported in this paper is Y11068.

```
FOX-1     MQQRRAFALL TLGSLLLAPC TVARGEAPLT AAVDGIIQPM LKEYRIPGMA   50
FOX-2           L                S
FOX-3                            S        T                 I

FOX-1     VAVLKDGKAH YFNYGVANRE SGQRVSEQTL FEIGSVSKTL TATLGAYAAV  100
FOX-2
FOX-3

FOX-1     KGGFELDDKV SQHAPWLKGS AFDGVIMAEL ATYSAGGLPL QFPDEVDSND  150
FOX-2               H
FOX-3        V                 L                        K

FOX-1     KMRTYYRHWS PVYPAGIHRQ YSNPSIGLFG HLAANSLGQP FEQLMSQILL  200
FOX-2       Q    S
FOX-3       Q    S

FOX-1     PKLGLHHTYI QVPESAIANY AYGYSKEDKP VRVTPGVLAA EAYGIKTGSA  250
FOX-2                     M              I
FOX-3                     M              I

FOX-1     DLLKFIEANM GYQGDAALKT RIALTHTGFY SVGDMTQGLG WESYAYPLTE  300
FOX-2          V             S A                          V
FOX-3          A          LV S A             E          D  V

FOX-1     QALLAGNSPA VSFQANPVTR FAVPKAMGEQ RLYNKTGSIG GFGAYVAFVP  350
FOX-2
FOX-3      V

FOX-1     ARGIAIVMLA NRNYPIEARV KAAHAILSQL AE                     382
FOX-2
FOX-3
```

FIG. 1. Alignment of the deduced amino acid sequences of FOX-1, FOX-2, and FOX-3 β-lactamases. Only amino acid substitutions are reported for FOX-2 and FOX-3. Boldface letters indicate conserved amino acid motifs for the active site of serine β-lactamases and AmpC β-lactamases.

This investigation was supported in part by a FATMA grant (17/93.00241) from the Italian National Research Council.

REFERENCES

1. **Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman.** 1990. Basic local alignment search tool. J. Mol. Biol. **215:**403–410.
2. **Altschul, S. F., T. L. Madden, A. A. Schäffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman.** 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. **25:**3389–3402.
3. **Arlet, G., G. Brami, D. Decré, A. Flippo, O. Gaillot, P. H. Lagrange, and A. Philippon.** 1995. Molecular characterisation by PCR-RFLP of TEM β-lactamases. FEMS Microbiol. Lett. **134:**203–208.
4. **Bauernfeind, A., I. Stemplinger, R. Jungwirth, and H. Giamarellou.** 1996. Characterization of the plasmidic β-lactamase CMY-2, which is responsible for cephamycin resistance. Antimicrob. Agents Chemother. **40:**221–224.
5. **Bauernfeind, A., I. Stemplinger, R. Jungwirth, R. Wilhelm, and Y. Chong.** 1996. Comparative characterization of the cephamycinase bla_{CMY-1} gene and its relationship with other β-lactamase genes. Antimicrob. Agents Chemother. **40:**1926–1930.
6. **Bauernfeind, A., S. Wagner, R. Jungwirth, I. Schneider, and D. Meyer.** 1997. A novel class C β-lactamase (FOX-2) in *Escherichia coli* conferring resistance to cephamycins. Antimicrob. Agents Chemother. **41:**2041–2046.
7. **Bradford, P. A., C. Urban, N. Mariano, S. J. Projan, J. J. Rahal, and K. Bush.** 1997. Imipenem resistance in *Klebsiella pneumoniae* is associated with the combination of ACT-1, a plasmid-mediated AmpC β-lactamase, and the loss of an outer membrane protein. Antimicrob. Agents Chemother. **41:**563–569.
8. **Bush, K., G. A. Jacoby, and A. A. Medeiros.** 1995. A functional classification scheme for β-lactamases and its correlation with molecular structure. Antimicrob. Agents Chemother. **39:**1211–1233.
9. **Gazouli, M., L. S. Tzouvelekis, E. Prinarakis, V. Miriagou, and E. Tzelepi.** 1996. Transferable cefoxitin resistance in enterobacteria from Greek hospitals and characterization of a plasmid-mediated group 1 β-lactamase (LAT-2). Antimicrob. Agents Chemother. **40:**1736–1740.
10. **Gonzalez Leiza, M., J. C. Perez-Diaz, J. Ayala, J. M. Casellas, J. Martinez-Beltran, K. Bush, and F. Baquero.** 1994. Gene sequence and biochemical characterization of FOX-I from *Klebsiella pneumoniae*, a new AmpC-type plasmid-mediated β-lactamase with two molecular variants. Antimicrob. Agents Chemother. **38:**2150–2157.
11. **Higgins, D. G., A. J. Bleasby, and R. Fuchs.** 1992. Clustal V: improved software for multiple alignments. Cabios **8:**189–191.
12. **Horii, T., Y. Arakawa, M. Ohta, S. Ichiyama, R. Wacharotayankun, and N. Kato.** 1993. Plasmid-mediated AmpC-type β-lactamase isolated from *Klebsiella pneumoniae* confers resistance to broad-spectrum β-lactams, including moxalactam. Antimicrob. Agents Chemother. **37:**984–990.
13. **Iaconis, J. P., and C. C. Sanders.** 1990. Purification and characterization of inducible lactamases in *Aeromonas* spp. Antimicrob. Agents Chemother. **34:**44–51.
14. **Kado, C. I., and S. T. Liu.** 1981. Rapid procedure for detection and isolation of large and small plasmids. J. Bacteriol. **145:**1365–1373.
15. **Lawrence, E. B., and A. J. Godfrey.** 1991. β-lactam antibiotics: mode of action and bacterial resistance, p. 646. *In* V. Lorian (ed.), Antibiotics in laboratory medicine. Williams and Wilkins, Baltimore, Md.
16. **Livermore, D. M., and M. Yuan.** 1996. Antibiotic resistance and production of extended spectrum β-lactamases amongst *Klebsiella* spp. from intensive care units in Europe. J. Antimicrob. Chemother. **38:**409–424.
17. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein

measurement with the Folin phenol reagent. J. Biol. Chem. **193:**265–275.
18. **Marchese, A., G. Arlet, G. C. Schito, P. H. Lagrange, and A. Philippon.** 1996. Detection of SHV-5 extended-spectrum beta-lactamase in *Klebsiella pneumoniae* strains isolated in Italy. Eur. J. Clin. Microbiol. Infect. Dis. **15:**245–248.
19. **National Committee for Clinical Laboratory Standards.** 1995. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically, 3rd ed. Approved standard M7-A3. National Committee for Clinical Laboratory Standards, Villanova, Pa.
20. **Papanicolaou, G. A., A. A. Medeiros, and G. A. Jacoby.** 1990. Novel plasmid-mediated β-lactamase (MIR-1) conferring resistance to oxyimino- and α-methoxy β-lactams in clinical isolates of *Klebsiella pneumoniae*. Antimicrob. Agents Chemother. **34:**2200–2209.
21. **Payne, D. J., N. Woodford, and S. G. B. Amyes.** 1992. Characterization of the plasmid-mediated β-lactamase BIL-1. J. Antimicrob. Chemother. **30:**119–127.
22. **Rasmussen, B. A., D. Keeney, Y. Yang, and K. Bush.** 1994. Cloning and expression of a cloxacillin-hydrolyzing enzyme and a cephalosporinase from *Aeromonas sobria* AER 14M in *Escherichia coli*: requirement for an *E. coli* chromosomal mutation for efficient expression of the class D enzyme. Antimicrob. Agents Chemother. **38:**2078–2085.
23. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
24. **Seeberg, A. H., R. M. Tolxdorff-Neutzling, and B. Wiedemann.** 1983. Chromosomal β-lactamases of *Enterobacter cloacae* are responsible for resistance to third-generation cephalosporins. Antimicrob. Agents Chemother. **23:**918–925.
25. **Tzouvelekis, L. S., E. Tzelepi, A. F. Mentis, and A. Tsakris.** 1993. Identification of a novel plasmid-mediated β-lactamase with chromosomal cephalosporinase characteristics from *Klebsiella pneumoniae*. J. Antimicrob. Chemother. **31:**645–654.
26. **Waley, S.** 1984. A spectrophotometric assay of β-lactamase action on penicillins. Biochem. J. **139:**789–790.
27. **Walsh, T. R., L. Hall, A. P. MacGowan, and P. M. Bennett.** 1995. Sequence analysis of two chromosomally mediated inducible beta-lactamases from *Aeromonas sobria*, strain 163a, one a class D penicillinase, the other an AmpC cephalosporinase. J. Antimicrob. Chemother. **36:**41–52.
28. **Woodford, N., D. J. Payne, A. P. Johnson, M. J. Weinbren, R. M. Perinpanayagam, R. C. George, B. D. Cookson, and S. G. B. Amyes.** 1990. Transferable cephalosporin resistance not inhibited by clavulanate in *Escherichia coli*. Lancet **336:**253.