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Molecular Epidemiology of TEM-3 (CTX-1) β-Lactamase

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A total of 33 clinical isolates encoding TEM-3 (CTX-1) from four French hospitals were studied. The strains belonged to seven species, *Klebsiella pneumoniae* (*n* = 24), *Escherichia coli* (*n* = 3), *Serratia marcescens* (*n* = 2), *Citrobacter freundii* (*n* = 1), *Enterobacter aerogenes* (*n* = 1), *Enterobacter cloacae* (*n* = 1), and *Klebsiella oxytoca* (*n* = 1). All the strains harbored an Inc7 or M self-transferable plasmid with a size of approximately 85 kilobases. The plasmids had closely related *Eco*RI, *Hin*cII, *Hin*dIII, and *Pvu*II restriction endonuclease-generated patterns and conferred resistance to all β-lactams, except cephamycins and imipenem; to tetracycline, because of the presence of the genes bla_{tem-3} and *tetC*, respectively, as determined by hybridization with specific probes; and to sulfonamide. Depending on the presence or absence and level of expression of the genes *aacA4*, *aadA*, and *dfrI* and of insertion element IS*15*, four types of plasmids could be distinguished. Plasmid pCFF04, the prototype plasmid encoding TEM-3, was widespread and appeared, by Southern hybridization, as the progenitor of the other types of replicons. The plasmid epidemic responsible for dissemination of TEM-3 in clinical isolates of members of the family *Enterobacteriaceae* may have originated in *S. marcescens* since pCFF04 was first detected in this species.

In 1983, Knothe et al. (9) described the first transferable resistance to broad-spectrum cephalosporins in clinical isolates of *Klebsiella pneumoniae* and *Serratia marcescens*. This new resistance phenotype was due to production of a broad-spectrum β-lactamase, SHV-2, which evolved from the chromosomal SHV-1 enzyme (8). Since 1984, strains of *K. pneumoniae* that produce a novel, transferable, broad-spectrum β-lactamase have been isolated in Clermont-Ferrand hospitals (21). The enzyme was designated CTX-1 because of its high hydrolytic activity against cefotaxime. It was later shown to be derived from the TEM-2 enzyme (23) and was therefore redesignated TEM-3. In an earlier study (22), we screened 490 members of the family *Enterobacteriaceae* isolated between 1984 and 1987 that exhibited an antibiotic resistance phenotype similar to that of the first TEM-3-producing strains (21). We concluded that β-lactam resistance via synthesis of TEM-3 spread into seven different bacterial genera and was due to dissemination of a single 85-kilobase (kb) Inc7 or M plasmid, pCFF04. Here we report the molecular characterization of the plasmids isolated in 33 clinical isolates representative of the TEM-3-producing strains obtained from Clermont-Ferrand hospitals and three other geographically distant French hospitals.

MATERIALS AND METHODS

Bacterial strains and plasmids. Twenty-eight strains of members of the family *Enterobacteriaceae* (22) were selected to represent all species of bacteria involved, different times of isolation over a 3-year period, different wards in which infection with TEM-3-producing bacteria occurred, and different biotypes (API 50 CH; API System S.A., Montalieu-Vercieu, France) of the most important species of *K. pneumoniae* (Table 1). Five other TEM-3-producing strains of *Enterobacteriaceae* were obtained from three different French hospitals geographically distant from Clermont-Ferrand (Table 2). Nalidixic acid- or rifampin-resistant mutants of *Escherichia coli* K-12 C600 (E. Wollman) were used in transfer experiments, and *Escherichia coli* BM21 harboring plasmid pIP135 (Tra$^+$ Inc7 or M Gm^r Sm^r Sp^r Su^r Tc^r; 79.3 kb) was used for incompatibility studies (11).

Media. Brain heart infusion broth (Difco Laboratories, Detroit, Mich.) and Mueller-Hinton agar (Diagnostics Pasteur, Marnes-La-Coquette, France) were used. All incubations were done at 37°C.

Genetic techniques. Conjugation (12) and incompatibility testing (3) were performed as described previously. Antibiotic concentrations for selection were as follows: cefotaxime, 4 μg/ml; gentamicin, 4 μg/ml; kanamycin, 20 μg/ml; nalidixic acid, 50 μg/ml; rifampin, 300 μg/ml.

Assay for aminoglycoside-modifying enzymes. The bacterial extracts were prepared (4) and the enzymes were assayed by the phosphocellulose paper-binding technique (5) as described previously. The final concentration of aminoglycosides in the assay mixture was 66.7 μg/ml, and the reaction was allowed to proceed for 30 min at 30°C.

Isolation and cleavage of DNA. Plasmid DNA was extracted by the alkaline lysis method (13) and digested with *Eco*RI, *Hin*cII, *Hin*dIII, and *Pvu*II restriction endonucleases. Restriction fragments were visualized after horizontal electrophoresis in 0.8% agarose gels with *Eco*RI-, *Hin*dIII-, and/or *Pst*I-digested bacteriophage λ DNA as the molecular size standard.

Hybridization. Derivation of DNA probes used for hybridization are listed in Table 3. Plasmid DNA was labeled with ^{32}P in vitro (14) and was hybridized to DNA immobilized on nytran filters (11). Autoradiography was performed by exposing the filters to Fuji X-ray film (Diffusion Image et Son, Paris, France).

Enzymes and reagents. Restriction endonucleases (Amersham Corp., Arlington Heights, Ill.) were used according to the recommendations of the manufacturer. [1-^{14}C]acetyl coenzyme A, [α-^{32}P]ATP (triethylammonium salt), and [γ-^{32}P]ATP (triethylammonium salt) were obtained from the Radiochemical Centre (Amersham, England). Antibiotics

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TABLE 1. Properties of the TEM-3-producing *Enterobacteriaceae* isolated in Clermont-Ferrand hospitals

Resistance markers cotransferred with TEM-3 β-lactamase[a]	Strain	Biotype	Date of isolation
Ak Km Nt Su Tc Tm	*Serratia marcescens* CF15	ND[b]	1984 (June)
	Klebsiella pneumoniae CF14	9	1984 (July)
	Klebsiella pneumoniae CF21	9	1985
	Klebsiella pneumoniae CF22	4	1985
	Klebsiella pneumoniae CF23	4	1985
	Klebsiella pneumoniae CF24	4	1985
	Klebsiella pneumoniae CF25	5	1986
	Klebsiella pneumoniae CF26	5	1986
	Klebsiella pneumoniae CF27	6	1986
	Enterobacter cloacae CF106	ND	1986
	Escherichia coli CF142	ND	1986
	Serratia marcescens CF105	ND	1986
	Klebsiella oxytoca CF107	ND	1986
	Citrobacter freundii CF118	ND	1986
Ak Cm Km Nt Su Tc Tm	*Klebsiella pneumoniae* CF104[c]	5	1985
	Klebsiella pneumoniae CF28	11	1986
	Escherichia coli CF102	ND	1986
Ak Cm Km Nt Su Tc Tm Tp	*Klebsiella pneumoniae* CF29	4	1985
	Klebsiella pneumoniae CF30	4	1986
	Klebsiella pneumoniae CF31	4	1986
	Klebsiella pneumoniae CF32	5	1986
	Enterobacter aerogenes CF153	ND	1986
Ak Gm Km Nt Su Tc Tm Tp	*Klebsiella pneumoniae* CF33	4	1985
Ak Cm Gm Km Nt Su Tc Tm Tp	*Klebsiella pneumoniae* CF34	4	1985
Ak[d] Gm Km Nt Su Tc Tm	*Klebsiella pneumoniae* CF35	7	1985
Ak[d] Cm Gm Km Nt Su Tc Tm	*Klebsiella pneumoniae* CF36	4	1986
Ak[d] Cm Gm Km Nt Su Tc Tm Tp	*Klebsiella pneumoniae* CF37	4	1986
Sm Sp Su Tc Tp	*Klebsiella pneumoniae* CF38	4	1987

[a] Abbreviations: Ak, amikacin; Cm, chloramphenicol; Gm, gentamicin; Km, kanamycin; Nt, netilmicin; Sm, streptomycin; Sp, spectinomycin; Su, sulfonamide; Tc, tetracycline; Tm, tobramycin; Tp, trimethoprim.
[b] ND, Not determined.
[c] Strain harboring plasmid pCFF04.
[d] MIC of amikacin was of 8 μg/ml instead of 64 μg/ml for the other amikacin-resistant isolates.

were provided by the following laboratories: Bristol Laboratories, Syracuse, N.Y. (amikacin, kanamycin A and C, and kanamycin); Eli Lilly & Co., Indianapolis, Ind. (tobramycin); Hoechst-Roussel Pharmaceuticals Inc., Somerville, N.J. (cefotaxime); Kyowa Laboratories, Kokaï, Japan (fortimicin); Meiji, Yokohama, Japan (dibekacin); Merrell-Dow, Winnersh, United Kingdom (rifampin); Parke, Davis & Co., Detroit, Mich. (paromomycin); Pfizer Inc., New York, N.Y. (streptomycin); Schering Corp., Bloomfield, N.J. (gentamicin C1 and C2, gentamicin, and netilmicin); The Upjohn Co., Kalamazoo, Mich. (neomycin B); and Winthrop Laboratories, Div. Sterling Drug Inc., New York, N.Y. (nalidixic acid).

RESULTS

Transfer of cefotaxime resistance. Transfer of cefotaxime resistance by conjugation to *E. coli* after selection on cefo-

TABLE 2. Properties of the TEM-3-producing *Enterobacteriaceae* isolated in different French hospitals

Resistance markers cotransferred with TEM-3 β-lactamase[a]	Strain	Biotype	Hospital (city in France)	Date of isolation
Ak Km Nt Su Tc Tm	*Klebsiella pneumoniae* SE8601	12	Bellevue (Saint-Etienne)	1986
	Klebsiella pneumoniae TP8701	3	Purpan (Toulouse)	1987
	Escherichia coli TP8702	ND[b]	Purpan (Toulouse)	1987
Ak Cm Km Nt Sm Sp Su Tc Tm Tp	*Klebsiella pneumoniae* AX3471	10	Rangueil (Toulouse)	1987
Ak Cm Gm Km Nt Sm Sp Su Tc Tm Tp	*Klebsiella pneumoniae* BZ9211	2	Rangueil (Toulouse)	1987

[a] See footnote *a* of Table 1 for abbreviations.
[b] ND, Not determined.

TABLE 3. Derivation of DNA probes

Plasmid	Vector	Genotype[a]	Insert	Reference or source
pCFF04		bla_{tem-3} *aacA4 tetC sul*	Entire plasmid	21
pBR322		bla_{tem-1} *tetC*	561-bp *Pst*I-*Ssp*I	1
pFE872		*dfrI*	560-bp *Hpa*I	L. Elwell
pBR322		*tetC* bla_{tem-1}	929-bp *Bst*NI	16
pIP1088	pBR322	IS*15* bla_{tem-1} *tetC*	670-bp *Sal*I	10
pSH77		*aadA*	491-bp *Rsa*I	6
pAZ505		*aacA4*	344-bp *Dde*I-*Ava*I	25
pSK606		*aacC1*	127-bp *Bgl*II-*Hin*fI	J. Davies
pSL18	pUC18	*tetA* bla_{tem-2}	750-bp *Sma*I	S. Levy
pRT11	pRT11	*tetB*	1,271-bp *Hin*cII	S. Levy

[a] Genetic symbols are as described by Novick et al. (19).

taxime or kanamycin was obtained with all the clinical isolates of *Enterobacteriaceae* studied (Table 1 and 2). The transconjugants were resistant to cefotaxime and other broad-spectrum cephalosporins, except cephamycins (cefoxitin and moxalactam) and imipenem. They were also resistant to sulfonamide and tetracycline but not to chloramphenicol. Based on resistance to aminoglycosides and trimethoprim, four types of transconjugants were obtained (Table 4). Twenty-seven transconjugants were resistant to amikacin, kanamycin, netilmicin, and tobramycin but not to gentamicin (type I). This resistance pattern was due to synthesis of a 6′-aminoglycoside acetyltransferase type IV [AAC(6′)-IV] (see results of hybridizations below). Three other transconjugants (type II) were also resistant to amikacin, with an MIC of 8 μg/ml instead of 64 μg/ml for transconjugants of type I, and were, in addition, resistant to gentamicin (MIC, 8 μg/ml). They also produced an AAC(6′)-IV, as determined by the phosphocellulose paper-binding assay, but no phosphotransferase or adenylyltransferase. The mechanism of resistance to gentamicin in these strains therefore remains unexplained. A single transconjugant (type III) was resistant to streptomycin and spectinomycin by synthesis of a 3″,9-adenylyltransferase [AAD(3″)(9)] (see results of hybridizations below). It was also resistant to trimethoprim. The last two transconjugants (type IV) displayed an aminoglycoside resistance pattern which resulted from production of both AAC(6′)-IV and AAD(3″)(9). Except for two transconjugants obtained from *K. pneumoniae* BZ9211 and *Enterobacter aerogenes* CF153 harboring an additional plasmid, all the transconjugants harbored a single plasmid of approximately 85 kb belonging to incompatibility group 7 or M. The plasmid transfer frequency was 10^{-4} from *K. pneumoniae* to *Escherichia coli* but was less than or equal to 10^{-6} from *S. marcescens* to *Escherichia coli*, *K. pneumoniae*, or *Enterobacter aerogenes* and from *Enterobacter aerogenes* to *S. marcescens*.

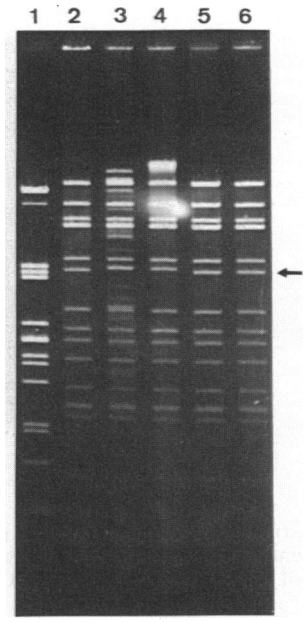

FIG. 1. Comparison of the *Eco*RI fragment patterns of plasmids encoding TEM-3. Lanes: 1, bacteriophage λ DNA digested with *Pst*I; 2, pCFF04; 3 to 6, plasmids of transconjugants of *K. pneumoniae* BZ9211 (lane 3), *K. pneumoniae* AX3471 (lane 4) *K. pneumoniae* SE8601 (lane 5), and *Escherichia coli* TP8702 (lane 6). The arrow indicates *Eco*RI fragment 5.

Analysis of plasmid DNA by digestion with restriction endonucleases. Plasmid DNA was prepared from all transconjugants (13) and digested with *Eco*RI, *Hin*cII, *Hin*dIII, or *Pvu*II (Fig. 1 provides part of this analysis). The size of pCFF04 was estimated to be 85 kb (Fig. 1, lane 2). The restriction patterns of the plasmids were indistinguishable, except for the two strains of type IV. These strains harbored a plasmid which had a size increase of approximately 100 base pairs (bp) in *Eco*RI fragment 5 (Fig. 1, lanes 3 and 4). Because of the cotransfer of a plasmid conferring gentamicin and trimethoprim resistance, additional fragments were present in one of these two transconjugants (Fig. 1, lane 3). The transconjugant from *Enterobacter aerogenes* CF153 (Fig. 2B, lane 6′) also harbored a second plasmid that was apparently cryptic.

Analysis of plasmid DNA by hybridization. Plasmid pIP135, the prototype Inc7 or M plasmid (11), was included in this analysis (Fig. 2).

(i) Comparison of the Inc7 or M plasmids. Comparison of the *Eco*RI DNA fragment patterns showed that pIP135 and plasmids encoding TEM-3 shared fragments 1 to 16 and B. Fragments A (10.7 kb) and C (2.5 kb) of pIP135 were absent in TEM-3-encoding plasmids, which, in contrast, contained

TABLE 4. Resistances cotransferred with TEM-3 to *Escherichia coli* by conjugation

Resistance phenotype	Resistances cotransferred[a]	No. of strains (n = 33)	Origin of the donors (hospital or city)	Species
I	Ak Km Nt Su Tc Tm	27	Clermont-Ferrand, Saint-Etienne, Toulouse	Various species[b]
II	Ak Gm Km Nt Su Tc Tm	3	Clermont-Ferrand	*Klebsiella pneumoniae*
III	Sm Sp Su Tc Tp	1	Clermont-Ferrand	*Klebsiella pneumoniae*
IV	Ak Km Nt Sm Sp Su Tc Tm	2	Toulouse Rangueil	*Klebsiella pneumoniae*

[a] See footnote *a* of Table 1 for abbreviations.

[b] Donor strains of resistance phenotype I were as follows: *C. freundii* (n = 1), *Enterobacter aerogenes* (n = 1), *Enterobacter cloacae* (n = 1), *Escherichia coli* (n = 3), *K. oxytoca* (n = 1), *K. pneumoniae* (n = 18), and *S. marcescens* (n = 2).

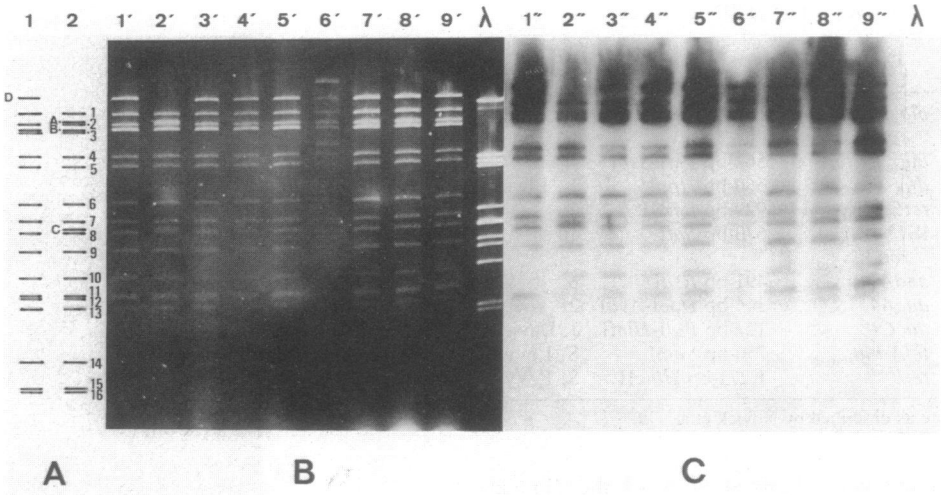

FIG. 2. Comparison of the *Eco*RI fragment patterns of Inc7 or M plasmids and corresponding hybridization with plasmid pCFF04. (A) Schematic representation of pCFF04 (lane 1) and pIP135 (lane 2). The nomenclature of the fragments is as described previously (11); D, 19-kb *Eco*RI fragment of pCFF04. (B) Lanes: 1′, pCFF04; 2′, pIP135; 3′ and 4′, *S. marcescens*; 5′, *Enterobacter cloacae*; 6′, *Enterobacter aerogenes*; 7′, 8′, and 9′, transconjugants of *K. pneumoniae* belonging to types I, II, and III, respectively. Bacteriophage λ DNA digested with *Pst*I was used as a molecular size standard. (C) Corresponding hybridization with probe pCFF04.

fragment D (19 kb). Plasmid pCFF04 was used as a probe and hybridized with all the *Eco*RI fragments of pIP135 and of plasmids encoding TEM-3, regardless of their origin. As expected, pCFF04 did not hybridize to the fragments which corresponded to the additional plasmids in the transconjugants of *Enterobacter aerogenes* CF153 (Fig. 2B, lane 6′) and *K. pneumoniae* BZ9211 (Fig. 1, lane 3; data not shown).

(ii) Localization of the resistance determinants and of IS*15*. The presence of bla_{tem-1}, *tetA*, *tetB*, *tetC*, *dfrI*, *aadA*, *aacC1*, and *aacA4* and of IS*15* in all types of transconjugants was tested by dot blot hybridization (data not shown) by using intragenic probes (Table 3). They were localized by Southern hybridization in one strain of each class of phenotype (Fig. 3 provides part of this analysis, the results of which are summarized in Table 5). The *Eco*RI fragment D of all the plasmids, except pIP135, hybridized with an intragenic probe for bla_{tem-1}. The probe specific for *tetC* hybridized with *Eco*RI fragment 4 of all the plasmids studied.

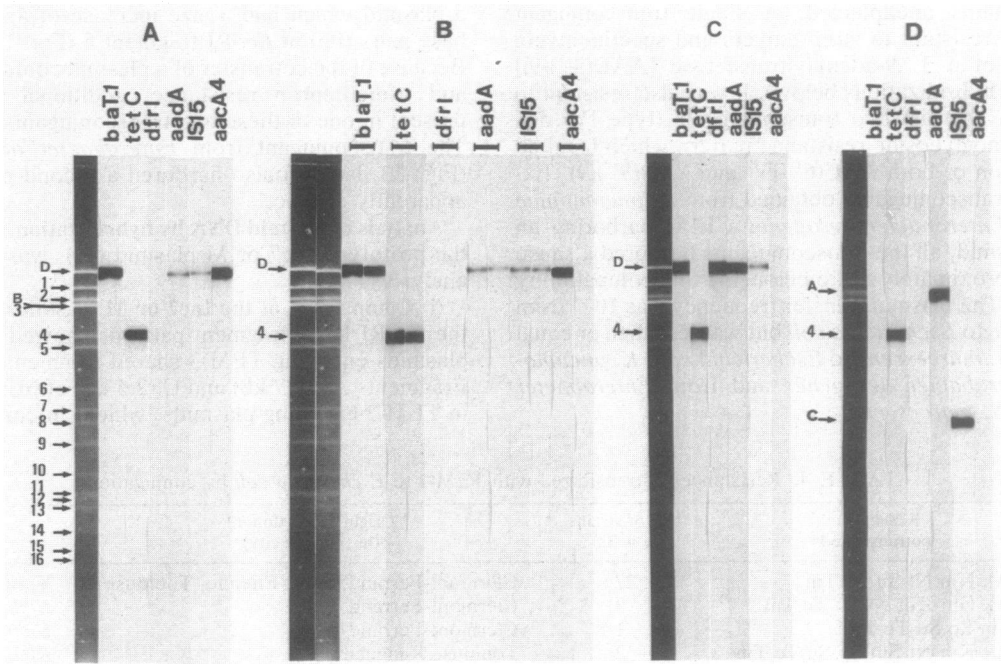

FIG. 3. Hybridization of Inc7 or M *Eco*RI fragment patterns with intragenic probes. (A) Plasmid pCFF04, prototype of plasmids of type I transconjugants. (B) Plasmids of type II transconjugants. (C) Plasmid of type III transconjugant. (D) Plasmid pIP135. In vitro ^{32}P-labeled intragenic probes are indicated on the top line. Letter and number designations are as described in the legend to Fig. 2A.

TABLE 5. Localization of the resistance determinants and of IS*15* by hybridization

Probe	Signals of the indicated *Eco*RI fragments of the following resistance phenotypes[a]:								
	I		II		III		pIP135		
	D	4	D	4	D	4	A	C	4
bla_{tem-1}	++	−	++	−	++	−	−	−	−
$tetC^{b}$	−	++	−	++	−	++	−	−	++
dfrI	−	−	−	−	++	−	−	−	−
aadA	+	−	+	−	++	−	++	−	−
IS*15*	+	−	+	−	+	−	−	++	−
aacA4	++	−	++/−[c]	−	−	−	−	−	−
aacC1	−	−	−	−	−	−	++	−	−

[a] Symbols: ++, strong signal; +, weak signal; −, no signal.
[b] No hybridization was observed with *tetA* and *tetB* probes.
[c] Depending on the plasmid studied.

Probes for *tetA* and *tetB* did not hybridize. Plasmid DNA extracted from the type III transconjugant (Sm^r Sp^r Su^r Tc^r Tp^r) hybridized with an intragenic *dfrI* probe on *Eco*RI fragment D. By using a probe specific for *aadA*, strong hybridizations were found on *Eco*RI fragment D of the plasmid from the type III transconjugant and on *Eco*RI fragment A of pIP135 (Gm^r Sm^r Sp^r Su^r Tc^r). Weaker hybridizations were also found on *Eco*RI fragment D of plasmids of types I (Ak^r Km^r Nt^r Su^r Tc^r Tm^r) and II (Ak^r Gm^r Km^r Nt^r Su^r Tc^r Tm^r). Plasmids of the three resistance types weakly hybridized with a probe for IS*15* on *Eco*RI fragment D, while *Eco*RI fragment C of pIP135 strongly hybridized. Plasmid pCFF04 and two of the three plasmids extracted from type II transconjugants and obtained with *K. pneumoniae* CF36 and CF37 hybridized with an intragenic probe for *aacA4* on *Eco*RI fragment D. Only pIP135 (*Eco*RI fragment A) hybridized with the *aacC1* probe.

(iii) Evolutionary relationships among the Inc7 or M plasmids. Fragment A, which hybridized with probes *aadA* and *aacC1*, and fragment C, which hybridized with IS*15*, are contiguous in pIP135 (11) and share homology with pCFF04 (Fig. 2). Fragment D, which contains bla_{tem-3}, also hybridized with *aadA* (strongly when streptomycin-spectinomycin resistance was expressed phenotypically and weakly when it was not expressed) and also hybridized weakly to the IS*15* probe but not to *aacC1*. It therefore seems that acquisition of bla_{tem-3} by an Inc7 or M plasmid resulted in the loss of *aacC1*; an *Eco*RI site; presumably, part of IS*15*; and in some cases, part of *aadA*.

DISCUSSION

We compared plasmids that confer resistance to cefotaxime by synthesizing TEM-3 β-lactamase from strains of various origins. We focused our attention on the study of the epidemiology of this mechanism of resistance because it first appeared in France (2, 7, 22) and we were therefore able to trace its subsequent spread. In the 33 strains we studied, cefotaxime resistance was self-transferable to *Escherichia coli* or to other members of the family *Enterobacteriaceae* at high frequencies and was encoded by a plasmid of ca. 85 kb belonging to incompatibility group 7 or M. The plasmids mediating resistance to cefotaxime were isolated from different bacterial hosts (Tables 1 and 2) and from different locations (Tables 1 and 2). All the transconjugants displayed the same resistance phenotype, except six transconjugants that were obtained with strains of *K. pneumoniae* from two different sources (Table 4). The fragment patterns of the plasmids digested with different restriction endonucleases were indistinguishable, except for two plasmids from strains isolated in Toulouse which encoded an AAD(3'')(9) and had a slight size increase in an *Eco*RI fragment. They all hybridized with the prototype plasmid pCFF04 which encoded both TEM-3 and AAC(6')-IV (Fig. 2). It therefore seems that dissemination of the apparently new TEM-3 resistance determinant in France was caused by the spread of an epidemic plasmid. This observation is consistent with that of Kitzis et al. (7). During this 3-year period of time, we also observed evolution of the epidemic plasmid: acquisition of gentamicin resistance by an yet unknown mechanism and/or replacement of gene *aacA4* by a gene specifying the same enzymatic activity but which did not cross-hybridize to the probe (one plasmid of type II); acquisition of *aadA* and *dfrI*, possibly Tn*7* (plasmid of type III); and acquisition of *aadA* (plasmids of type IV). The heterogeneity of AAC(6')-IV enzymes and the corresponding *aacA4* genes has been reported previously (17, 24, 25). It has been shown that insertion of *aacA4* genes in plasmids could occur at a particular hot spot (17, 18, 25). In two instances, the *aacA4* gene was adjacent to a truncated or an active *aadA* gene (18, 25). A similar organization in plasmids of types II and IV could account for the differences observed by hybridization and the fact that resistance determinants are clustered on the DNA molecule. Our study confirms the relative stability of plasmids of incompatibility group 7 or M (11, 15) and their capacity to be responsible for plasmid epidemics (20, 26). Cefotaxime is commonly used in France, often in combination with amikacin. Plasmid pCFF04 encodes an AAC(6')-IV which confers resistance to aminoglycosides, especially amikacin. Therefore, against strains encoding this type of aminoglycoside-modifying enzyme, cefotaxime is the only active antibiotic of the combination. It is likely that broad-spectrum cephalosporins constitute the selective pressure for the emergence and subsequent spread of TEM-derived, broad-spectrum β-lactamases.

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