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Replicon sequence typing of IncF plasmids carrying virulence and resistance determinants

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Objectives: IncF plasmids are frequently encountered in clinical enterobacterial strains associated with the dissemination of relevant antimicrobial resistance and virulence genes. These plasmids are usually heterogeneous in size and carry multiple replicons, and technical difficulties can impair the comparison and detection of related plasmids by restriction fragment length polymorphism analysis. We devised a rapid sequence-based typing scheme to categorize the members of this plasmid family into homogeneous groups.

Methods: We compared the available IncF replicon sequences, identifying the combination of the different IncF replicon alleles as the discriminating characteristic of these plasmid scaffolds. An IncF typing method based on PCR amplification and sequence typing of the IncF replicons was devised. A collection of IncF plasmids carrying resistance and/or virulence genes, identified in strains from different sources and geographical origins, was tested with this typing system.

Results: We devised a replicon sequence typing (RST) scheme discriminating IncF plasmid variants. This system was tested on the collection of IncF plasmids, demonstrating that it was useful for the discrimination of plasmids carrying the same resistance gene (i.e. the $bla_{\text{CTX-M-15}}$ gene), but also recognized strictly related virulence plasmids (i.e. IncFIme plasmids). The PCR-based replicon typing (PBRT) system was also updated by including new primer pairs to allow the identification of the *Salmonella*, *Klebsiella* and *Yersinia* IncF plasmids.

Conclusions: The ability to recognize and sub-categorize IncF plasmids by RST in homogeneous groups on the basis of their phylogenetic relatedness can be helpful in analysing their distribution in nature and discovering their evolutionary origin.

Keywords: β-lactamases, CTX-M, incompatibility groups, ESBLs, plasmid-mediated resistance

Introduction

IncF plasmids contribute to the fitness of the bacterial host by providing virulence and antimicrobial resistance determinants.[1] In particular, *Salmonella enterica*, *Shigella* spp. and enterohaemorrhagic *Escherichia coli* (EHEC), enteropathogenic *E. coli* (EPEC) and enteroinvasive *E. coli* (EIEC) are characterized by specific virulence traits, such as bacteriocins, siderophores, cytotoxins and adhesion factors, that are encoded by IncF plasmids, designated in these species as virulence plasmids.[1] The IncF plasmids have been associated with the abrupt worldwide emergence of clinically relevant extended-spectrum β-lactamases (ESBLs), such as CTX-M-15,[2–7] but also with the spread of plasmid-mediated AmpC genes (bla_{CMY} and bla_{DHA}) and quinolone and aminoglycoside resistances encoded by the genes *aac(6′)-Ib-cr*, *qnr*, *qepA*, *armA* and *rmtB*.[8–12]

The IncF family contains a varied assortment of plasmids, whose relatedness and nomenclature are often complex. IncF plasmids are limited by host range to the family Enterobacteriaceae and rely on both self-encoded and host-encoded factors for duplication. IncF plasmids need DNA gyrase, DnaB, DnaC, DnaG, single strand binding and DNA polymerase III proteins for their replication.[13]

IncF plasmids are usually low copy number plasmids, >100 kb in size, and often carry more than one replicon promoting the initiation of replication. The multi-replicon status has been described to be one means by which plasmids with a narrow host range can accomplish broad host range replication. For instance, plasmid pGSH500 from a clinical isolate of *Klebsiella pneumoniae* has a moderately broad host range and contains two functional replicons, one being related to the narrow host range IncFII plasmids and the second to the broad host range IncN plasmids.[14] A plasmid might occasionally be replaced or driven away by incoming plasmids carrying an incompatible replicon. Thus, the multi-replicon status can allow the acquisition of plasmids carrying incompatible replicons when replication is

driven by a compatible one. The replicon that is not responsible for the control of replication can undergo a succession of genetic alterations.[15] The classic multi-replicon IncF plasmid contains the FII replicon (also designated as FIIA) regulated by CopA, a constitutively synthesized 90 nt antisense-RNA, which is normally silent,[13] and FIA and FIB replicons, which function only in enteric bacteria and are regulated by iterons, in *cis*-negative binding sites of the replication protein RepA.[16] In plasmids F and p307, the FII replicon is substituted by a non-functional FIC replicon.[16] By contrast, plasmids R1 and R100 of *E. coli* contain only one functional FII replicon, which was one of the best-studied examples of replication control by inhibition of leader peptide translation in the early 1990s.[17,18] It has been demonstrated that FII replicons are free to diverge when associated with FIA and/or FIB replicons since they do not participate in the initiation of replication of the plasmid, generating new compatible variants that can be used to overcome the incompatibility barrier with incoming IncF plasmids. A few mismatches in the kissing loop of the antisense RNA are sufficient to eliminate the incompatibility effect among two closely related FII replicons.[14]

In summary, the IncF plasmids possess great versatility of intracellular adaptation by the rapid evolution of the regulatory sequences of the replicons. These characteristics have been a factor in the great success of IncF plasmids in enteric bacteria. IncF plasmids are widely diffused in clinically relevant Enterobacteriaceae, representing one of the most frequently encountered plasmid types. For instance, the FII replicon was detected in >50% of *E. coli* from faeces of healthy, antibiotic-free humans and faecal flora from healthy birds in the USA.[19] Addiction systems encoded by these plasmids also contributed to the promotion of plasmid spread and adaptation to the host. IncF plasmids carrying virulence- and resistance-linked determinants can be positively selected by antimicrobials, thus implementing their adaptive strategies.[20]

The mosaic structure of these plasmids impairs the application of a classical multilocus sequence typing-based approach. Most parts of the plasmid scaffold are not present in all IncF plasmids or are acquired and lost as a consequence of the fusion of the different replicons in the multi-replicon status. For instance, the genes controlling the partitioning of plasmids in daughter cells are not present in all IncF scaffolds, and addiction systems are physically linked to particular replicons (the *pemI*/*pemK* genes are linked to the FII replicon, while *vagC*-*vagG* genes are linked to the FIA replicon).[21] In this study, we analysed the FII, FIA, FIB and FIC replicon sequences available at GenBank, proposing an IncF replicon sequence typing (RST) scheme with the aim of classifying all members of the large IncF family by a replicon sequence-based approach. This scheme was based on the *in silico* analysis of 90 IncF plasmids of various origins and sources available at GenBank. This scheme may provide a novel IncF plasmid nomenclature, based on replicon sequence types and assortment. The typing scheme was experimentally tested on 36 IncF virulence and resistance plasmids obtained from our collections of enteric bacteria isolated in six countries.

Materials and methods

Comparative analysis of the IncF replicons

An *in silico* comparative analysis of IncF replicons was performed at GenBank using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi), using as template the replication region of the FII, FIA and FIB replicons located between the primer pairs described in the PCR-based replicon typing (PBRT) method.[22] Additional iterative BLAST searches using the phylogenetically most distant family members were carried out in order to cover a maximum sequence space without corruption. For each FII, FIA and FIB family, we calculated a phylogenetic tree by neighbour-joining (NJ) analysis, tested with bootstrap values (500 replicates), using the CLC Free Workbench 4 program. Plasmids analysed *in silico*, their virulence and resistance characteristics and GenBank accession numbers are reported in Table 1.

Multiple sequence alignments of the FII, FIA, FIB, FIC, FII_K, FII_Y and FII_S alleles were performed using DNAMAN software (Lynnon BioSoft, Vaudreuil, Quebec, Canada) set for Quick Alignment with a gap penalty of 7, K-tuple of 3, number of top diagonals of 5, and a window size of 5.

Plasmids

A collection of 36 strains of *E. coli*, *S. enterica*, *K. pneumoniae*, *Yersinia pestis* and *Yersinia pseudotuberculosis* isolated in the UK, Italy, Spain, Albania, Algeria and Iran has been studied previously.[22–28] Plasmids were assigned to the IncF group by PBRT.[22] Twenty IncF plasmids carried one or more of the $bla_{CTX\text{-}M\text{-}9}$, $bla_{CTX\text{-}M\text{-}15}$, $bla_{SHV\text{-}12}$, $bla_{CMY\text{-}2}$, $bla_{TEM\text{-}1}$ and $bla_{OXA\text{-}1}$ β-lactamase genes and the remaining plasmids did not contain any β-lactam resistance gene (Table 2). Plasmid DNAs purified with the Invitrogen PureLink™ HiPure Plasmid Filter Midiprep Kit were analysed by restriction fragment length polymorphism (RFLP) by PstI restriction.

IncF RST

Template DNAs for replicon amplification were prepared by generating total DNA of higher purity by the Wizard Genomic DNA Purification System (Promega, Madison, WI, USA), starting from 2 mL of Luria-Bertani broth cultures containing a suitable concentration of antibiotic for selection. A total of 200–400 ng of DNA per amplification reaction was used as template. All PCR amplifications were performed using the primers shown in Table 3 with the following amplification scheme (except for the primer pairs FII FW–FII RV and RepAFII FW–RepAFII RV; see below): 1 cycle of denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 30 s and elongation at 72°C for 1 min; amplification was concluded with an extension programme of 1 cycle at 72°C for 5 min. For primers FII FW–FII RV the same amplification scheme was used except that annealing was performed at 54°C instead of 60°C.

Amplicons were purified using the Wizard PCR Preps DNA Purification System (Promega) and sequenced using fluorescent dye-labelled dideoxy nucleotides with an ABI 3730 automatic DNA sequencer (Perkin-Elmer, Foster City, CA, USA). Alleles were assigned by submitting the amplicon sequence to the web site www.pubmlst.org/plasmid/. Novel IncF replicon alleles identified in this study were released under accession numbers HM751874 (B21), HM751875 (F32), HM751876 (A4), HM751877 (F31), HM751878 (B20), HM751880 (K5) and HM751881 (B22) (Figure S1, available as Supplementary data at *JAC* Online).

Characterization of the repA gene in pZM3 and IncF/97 plasmids

The *repA* genes of the pZM3 and IncF/97 plasmids were amplified using RepAFII FW–RepAFII RV (Table 3), yielding amplicons of 5700 and 1700 bp, respectively, with the following amplification scheme: 1 cycle of denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 30 s and elongation at 72°C for 5 min; amplification was concluded with an extension program of 1 cycle at 72°C for 5 min. The amplicons were cloned in pCR2.1 cloning vector (Invitrogen, Milan, Italy) and sequenced using universal primers and primer walking, by fluorescent dye-labelled dideoxy-nucleotides with an

Table 1. Characteristics of the IncF plasmids in GenBank and assignation of the IncF alleles and FAB formulae

IncF plasmid name	Strain origin	Virulence and resistance key features on plasmid	Allele numbers for replicons			FAB formula	EMBL no.
			FII, FIC, FII_S, FII_K, FII_Y	FIA	FIB		
pRSB107	uncultured bacterium	aerobactin, MDR	F1	A1	B1	F1:A1:B1	AJ851089
pFK3-140	*K. pneumoniae*	colicin, iron acquisition system, MDR	F1	A2	B2	F1:A2.B2	FJ876827
pU302L	*S. enterica* Typhimurium	MDR	absent	A1	B1	F−:A1:B1	AY333434
R100	*S. flexneri*	MDR	F2	absent	absent	F2:A−:B−	AP000342
pC15-1a	*E. coli*	MDR, $bla_{CTX\text{-}M\text{-}15}$	F2	absent	absent	F2:A−:B−	AY458016
pEK499	*E. coli* O25	MDR, $bla_{CTX\text{-}M\text{-}15}$	F2	A1	absent	F2:A1:B−	EU935739
pEK516	*E. coli*	MDR, $bla_{CTX\text{-}M\text{-}15}$	F2	absent	absent	F2:A−:B−	EU935738
pO26-L	*E. coli* EHEC	haemagglutinin	F2	absent	absent	F2:A−:B−	FJ449539
p53638_75	*E. coli* O144	colicin	F2	absent	absent	F2:A−:B−	CP001065
pEG356	*S. sonnei*	$bla_{CTX\text{-}M\text{-}24}$	F2	absent	absent	F2:A−:B−	FN594520
pKF3-70	*K. pneumoniae*	putative iron transport system (*eitABCD*), $bla_{CTX\text{-}M\text{-}14}$	F2	absent	absent	F2:A−:B−	FJ494913
pAPEC-O2-ColV	*E. coli* ExPEC	iron uptake	F2	absent	B1	F2:A−:B1	AY545598
pSFO157	*E. coli* EHEC	haemagglutinin, haemolysin, T2SS	F3	A3	B3	F3:A3:B3	AF401292
pAPEC-O2-R	*E. coli* APEC	MDR	F4	absent	absent	F4:A−:B−	AY214164
pO111_3	*E. coli* EHEC	catalase-peroxidase, cytopathic protease, haemolysin, polysaccharide deacetylase	F5	absent	absent	F5:A−:B−	AP010963
pETEC_74	*E. coli* ETEC	Ser-protease associated with CS1 fimbriae	F6	absent	absent	F6:A−:B−	CP000799
pSU316	*E. coli*	partially sequenced	F7	ND	ND	ND	M26937
pTUC100	*E. coli*	microcin J25	F8	ND	ND	ND	AY091607
pAA	*E. coli* EAEC	AAF, dispersine, putative iron transport system (*eitABCD*)	F9	absent	absent	F9:A−:B−	FN554767
pSU221	*E. coli*	partially sequenced	F10	ND	ND	ND	M28098
pETEC_80	*E. coli* ETEC	enterotoxin A, CS3 fimbriae	F11	absent	absent	F11:A−:B−	CP000795
pEntH10407	*E. coli* ETEC	LT- and STIa-enterotoxins	F12	absent	absent	F12:A−:B−	AP010910
pB171	*E. coli* EPEC	type IV pili, glutamate decarboxylase	F13	absent	B4	F13:A−:B4	AB024946
pMAR7	*E. coli* EPEC	type IV pili, glutamate decarboxylase	F13	absent	B23	F13:A−:B23	DQ388534
pSU212	*E. coli*	partially sequenced	F14	ND	ND	ND	X55895
pCoo	*E. coli* ETEC	CS1 fimbriae, polysaccharide deacetylase, Ser-protease associated with CS1 fimbriae	F15	absent	absent	F15:A−:B−	CR942285
pETEC_35	*E. coli* ETEC	no features	F16	absent	absent	F16:A−:B−	CP000796
pO86A1	*E. coli* EHEC	AAF	F17	absent	B5	F17:A−:B5	AB255435
pAPEC-O1-ColBM	*E. coli* ExPEC	iron uptake, serum survival, haemagglutinin, macrolide ABC efflux	F18	absent	B6	F18:A−:B6	DQ381420
pAPEC-1	*E. coli* APEC	iron uptake (missing *eitABCD*), serum survival gene, haemagglutinin, haemolysin, colicin V, macrolide ABC efflux	F18	absent	B1	F18:A−:B1	CP000836
pVir68	*E. coli* NTEC	F17b fimbriae, cytotoxin necrotizing factor 2, *cdt* cytolethal distending toxin gene, haemolysin, adhesin	F18	A2	B7	F18:A2:B7	CP001162
pVM01	*E. coli* APEC	iron uptake (missing *eitABCD*), serum survival gene, haemagglutinin, colicin, macrolide ABC efflux	F18	absent	B1	F18:A−:B1	EU330199
pAPEC-O103-ColBM	*E. coli* ExPEC	iron uptake (missing *eitABCD*), serum survival gene, haemagglutinin, haemolysin, colicin, macrolide ABC efflux	F18	absent	B8	F18:A−:B8	CP001232
55989p	*E. coli* EAEC	AAF	F19[a]	absent	B9	F19:A−:B9	CU928159

pSE11-2	*E. coli*	fimbrial operon	F20[a]	absent	absent	F20:A−:B−	AP009242
pCROD1	*Citrobacter rodentium*	colicin, fimbrial operon	F21	absent	absent	F21:A−:B−	FN543503
pIP1206	*E. coli*	MDR, *qepA*, iron acquisition system	F22	A1	B1	F22:A1:B1	AM886293
pO157_ ATCC 43894	*E. coli* EHEC	T2SS, haemolysin, catalase-peroxidase, cytopathic Ser-protease, antibiotic-induced enterocolitis	F23	absent	B3	F23:A−:B3	GU363949
pO157_ TW14359	*E. coli* EHEC	T2SS, haemolysin, antibiotic-induced enterocolitis	F23	absent[b]	B3	F23:A−:B3	CP001369
pO157_ EC4115	*E. coli* EHEC	T2SS, haemolysin, catalase-peroxidase	F23	absent[b]	B3	F23:A−:B3	CP001163
pO157_ Sakai	*E. coli* EHEC	T2SS, haemolysin, catalase-peroxidase, cytopathic Ser-protease, antibiotic-induced enterocolitis	F23	absent	B3	F23:A−:B3	AB011549
pO157_ EDL933	*E. coli* EHEC	T2SS, haemolysin, catalase-peroxidase, cytopathic Ser-protease, antibiotic-induced enterocolitis	F23	absent	B3	F23:A−:B3	AF074613
p1658/97	*E. coli*	bla_{SHV-5}	F24	absent	B1	F24:A−:B1	AF550679
pSMS35_130	*E. coli*	MDR, colicin, haemolysin, iron transport (*sitABCD*)	F24	absent	B1	F24:A−:B1	CP000971
pECOS88	*E. coli* ExPEC	colicin, macrolide ABC efflux, haemolysin, iron uptake (missing *eitABCD*)	F24	absent	B1	F24:A−:B1	CU928146
pCVM29188_146	*S. enterica* Kentucky	iron uptake (missing *eitABCD*), macrolide drug exporter, serum survival gene	F24	absent	B1	F24:A−:B1	CP001122
pSD1_197	*Shigella dysenteriae*	T3SS	F25	absent	absent	F25:A−:B−	CP000035
pWR100 partial	*S. flexneri*	T3SS	F26	ND	ND	ND	AF177050
pSS_046	*S. sonnei*	T3SS	F27	absent	absent	F27:A−:B−	CP000039
pSF5	*S. flexneri*	no features	F27	absent	absent	F27:A−:B−	AY879342
pCP301	*S. flexneri*	T3SS	F27	absent	absent	F27:A−:B−	AF386526
pBS512_211	*Shigella boydii*	T3SS	F27	absent	absent	F27:A−:B−	CP001062
pSB4_227	*S. boydii*	no features	F27	absent	absent	F27:A−:B−	CP000037
pINV_F6	*S. flexneri*	partially sequenced	F27	ND	ND	ND	AY206448
p53638_226	*E. coli* EIEC	phosphoglycerate transport system, colicin Js, T3SS, fimbrial operon	F28	absent	absent	F28:A−:B−	CP001064
pUTI89	*E. coli* UPEC	ColIa immunity, *colIA*, enterotoxin, salmochelin	F29	absent	B10	F29:A−:B10	CP000244
pEC14_114	*E. coli* ExPEC	ColIa immunity, *colIA*, enterotoxin, iron acquisition system	F29	absent	B10	F29:A−:B10	GQ398086
pECSF1	*E. coli*	ColIa immunity, *colIA*, enterotoxin, iron acquisition system	F29	absent	B10	F29:A−:B10	AP009379
p1ESCUM	*E. coli* UPEC	ColIa immunity, *colIA*, enterotoxin, iron acquisition system	F29	absent	B10	F29:A−:B10	CU928148
pO26_2	*E. coli* EHEC	no features	F30	absent	absent	F30:A−:B−	AP010955
pO26_1	*E. coli* EHEC	haemolysin, adherence factor, polysaccharide deacetylase, catalase-peroxidase	absent	absent	B11	F−:A−:B11	AP010954
pSE11-3	*E. coli*	fimbrial operon	absent	absent	B12	F−:A−:B12	AP009243
pO103	*E. coli* EHEC	T2SS, polysaccharide deacetylase, haemolysin	absent	absent	B13	F−:A−:B13	AP010959
pO113	*E. coli* EHEC	serine protease autotransporter, mucinase, haemolysin	absent	absent	B14	F−:A−:B14	AY258503
pO55	*E. coli* EPEC	T2SS, LEE	absent	absent	B15	F−:A−:B15	CP001847
Plasmid F	*E. coli*		C1	A2	B16	C1:A2:B16	AP001918
p307	*E. coli*	partially sequenced	C2	ND	ND	ND	M16167
pSLT-BT	*S. enterica* Typhimurium	*Salmonella* plasmid virulence (*spv*), plasmid encoded fimbriae (*pef*), resistance to complement killing (*rck*)	S1	absent	B17	S1:A−:B17	FN432031
pSLT	*S. enterica* Typhimurium	*Salmonella* plasmid virulence (*spv*), plasmid encoded fimbriae (*pef*), resistance to complement killing (*rck*)	S1	absent	B17	S1:A−:B17	AE006471
pSPCV	*S. enterica* Paratyphi C	*Salmonella* plasmid virulence (*spv*), plasmid-encoded fimbriae (*pef*)	S1	absent	B18	S1:A−:B18	CP000858
pSVC50	*S. enterica* Choleraesuis	*Salmonella* plasmid virulence (*spv*)	S2	absent	B19	S2:A−:B19	AY509003
pKDSC50	*S. enterica* Choleraesuis	*Salmonella* plasmid virulence (*spv*)	S2	absent	B19	S2:A−:B19	AB040415

Continued

Table 1. *Continued*

IncF plasmid name	Strain origin	Virulence and resistance key features on plasmid	Allele numbers for replicons			FAB formula	EMBL no.
			FII, FIC, FII_S, FII_K, FII_Y	FIA	FIB		
pOU7519	*S. enterica* Choleraesuis	*Salmonella* plasmid virulence (*spv*)	S2	absent	B19	S2:A−:B19	EU219534
pOU1115	*S. enterica* Dublin	*Salmonella* plasmid virulence (*spv*)	S3	absent	absent	S3:A−:B−	DQ115388
pCT02021853	*S. enterica* Dublin	*Salmonella* plasmid virulence (*spv*)	S3	absent	absent	S3:A−:B−	CP001143
pOU1113	*S. enterica*	*Salmonella* plasmid virulence (*spv*)	S4	absent	absent	S4:A−:B−	AY517905
pKPN3	*K. pneumoniae*	copper and silver resistance	K1	absent	absent	K1:A−:B−	CP000648
pKPN4	*K. pneumoniae*	MDR, bla_{SHV-12}	K1	absent	absent	K1:A−:B−	CP000649
pKF3-94	*K. pneumoniae*	no features	K2	absent	absent	K2:A−:B−	FJ876826
pKpQIL	*K. pneumoniae*	bla_{KPC-3}	K2	absent	absent	K2:A−:B−	GU595196
pGSH500	*K. pneumoniae*	partially sequenced	K3	ND	ND	ND	AJ009980
pKP91	*K. pneumoniae*	no features	K4	absent	absent	K4:A−:B−	CP000966
pPB1	*Y. pseudotuberculosis*	T3SS (*yop*)	Y1	absent	absent	Y1:A−:B−	CP001049
pCD1	*Y. pestis*	T3SS (*yop*)	Y1	absent	absent	Y1:A−:B−	CP001594
new_pCD	*Y. pestis* Angola	T3SS (*yop*)	Y1	absent	absent	Y1:A−:B−	CP000902
pCD	*Y. pestis* Pestoides F	T3SS (*yop*)	Y1	absent	absent	Y1:A−:B−	CP000669
pYV	*Y. pseudotuberculosis*	T3SS (*yop*)	Y1	absent	absent	Y1:A−:B−	BX936399
pYVe227	*Y. enterocolitica*	T3SS (*yop*)	Y2	absent	absent	Y2:A−:B−	AF102990
pG8786	*Y. pestis*	phospholipase D, F1 capsular protein	Y3	absent	absent	Y3:A−:B−	AJ698720
pMT	*Y. pestis* Pestoides F	phospholipase D, F1 capsular protein	Y3	absent	absent	Y3:A−:B−	CP000670

MDR, multidrug resistance (MDR indicates the presence of genes conferring resistance to aminoglycosides, sulphonamides, tetracyclines, trimethoprim and β-lactams); the presence of genes encoding ESBLs, carbapenemases, macrolide ABC efflux systems and plasmid-mediated quinolone resistance is indicated in the respective lines; T2SS, type II secretion system; T3SS, type III secretion system; CS fimbriae, clusters encoding pili of the CS1 and CS3 types; iron acquistion systems include *iutA*, *iucABCD* (encoding the aerobactin siderophore system), *sitABCD* (ABC iron transport system), *iroBCDEN* (salmochelin siderophore system) and *eitABCD* (putative iron transport system) clusters except when specifically indicated; AAF, aggregative adherent fimbriae; LT, heat-labile enterotoxin; STIa, heat-stable enterotoxin; LEE, locus for enterocyte effacement; APEC, avian pathogenic *E. coli*; NTEC, necrotoxigenic *E. coli*.

[a]This plasmid shows mismatches with respect to the FII RV primer sequence.

[b]FIA replicon interrupted by the insertion of IS*629*.

Table 2. Characteristics of the IncF plasmids analysed in this study

Plasmid/strain name	Species	Country	Year	Resistance genes	Allele numbers for replicons: FII, FII_S, FII_K, FII_Y	FIA	FIB	FAB formula	Reference
pZM3	*S. enterica* Wien	Algeria	1970	MDR	F1	A1	B1	F1:A1:B1	28
IncFI/97	*S. enterica* Typhimurium	Albania	1997	MDR	F1	A1	B1	F1:A1:B1	28
TP160	*S. enterica* Typhi	—	—	—	F1	A1	B1	F1:A1:B1	28
TP181	*S. enterica* Typhimurium	Iran	1975	—	F1	A1	B1	F1:A1:B1	28
R136	*S. enterica* Typhimurium	—	—	—	F2	neg	neg	F2:A−:B−	22
R1-16	*S. enterica* Paratyphi B	—	—	—	F2	neg	neg	F2:A−:B−	22
p48165T	*E. coli*	Italy	2006	MDR, $bla_{CTX\text{-}M\text{-}15}$	F2	neg	neg	F2:A−:B−	22
p169967	*E. coli*	UK	2002	MDR, $bla_{CTX\text{-}M\text{-}15}$	F2	A1	neg	F2:A1:B−	27
p177243	*E. coli*	UK	2003	MDR, $bla_{CTX\text{-}M\text{-}15}$	F2	A1	neg	F2:A1:B−	27
t-ST4	*S. enterica* Typhimurium	Italy	1997	MDR	F2	neg	B17	F2:A−:B17	25
p45913	*E. coli*	Italy	2006	MDR, $bla_{CTX\text{-}M\text{-}15}$	F22	A1	B20	F22:A1:B20	23
p171897	*E. coli*	UK	2003	MDR, $bla_{CTX\text{-}M\text{-}15}$	F22	A1	B20	F22:A1:B20	27
p108137	*E. coli*	UK	1995	MDR, $bla_{CMY\text{-}2}$	F22	A1	B20	F22:A1:B20	27
p45545	*E. coli*	Italy	2006	MDR, $bla_{CTX\text{-}M\text{-}15}$	F31	A4	B1	F31:A4:B1	23
p162237	*E. coli*	UK	2001	MDR, $bla_{CTX\text{-}M\text{-}15}$	F31	A4	B10	F31:A4:B10	27
p177273	*E. coli*	UK	2003	MDR, $bla_{CTX\text{-}M\text{-}15}$	F31	A4	B1	F31:A4:B1	27
p759-D-T[a]	*E. coli*	Spain	1996	MDR, $bla_{CTX\text{-}M\text{-}9}$	F24	neg	B21	F24:A−:B21	26
p836-D-T[a]	*E. coli*	Spain	1996	MDR, $bla_{CTX\text{-}M\text{-}9}$	F24	neg	B21	F24:A−:B21	26
p876-D-T[a]	*E. coli*	Spain	1997	MDR, $bla_{CTX\text{-}M\text{-}9}$	F24	neg	B21	F24:A−:B21	26
p1383-D-T	*E. coli*	Spain	1999	—	F32	neg	B1	F32:A−:B1	26
p1185-D-T	*E. coli*	Spain	1998	—	F18	neg	B1	F18:A−:B1	26
p1249-D-T[a]	*E. coli*	Spain	1998	MDR, $bla_{CTX\text{-}M\text{-}9}$	F18	neg	B1	F18:A−:B1	26
p1290-D-T	*E. coli*	Spain	1998	—	F18	neg	B1	F18:A−:B1	26
p1292-D-Tc	*E. coli*	Spain	1998	—	F18	neg	B1	F18:A−:B1	26
p1384-D	*E. coli*	Spain	1999	—	F18	neg	B16	F18:A−:B16	26
S.82/10	*S. enterica* Enteritidis	Italy	1995	—	S1	neg	B22	S1:A−:B22	22
17829	*K. pneumoniae*	Italy	2008	$bla_{CTX\text{-}M\text{-}15}$, $bla_{SHV\text{-}12}$	F2, K5	neg	neg	F2:A−:B−, K5:A−:B−	24
p17829Tf	*E. coli* transformant	—	—	$bla_{CTX\text{-}M\text{-}15}$, $bla_{SHV\text{-}12}$	F2	neg	neg	F2:A−:B−	24
17830	*K. pneumoniae*	Italy	2008	$bla_{CTX\text{-}M\text{-}15}$	F2, K5	neg	neg	F2:A−:B−, K5:A−:B−	24
17830Tf	*E. coli* transformant	—	—	$bla_{CTX\text{-}M\text{-}15}$	F2	neg	neg	F2:A−:B−	24
17834	*K. pneumoniae*	Italy	2008	$bla_{CTX\text{-}M\text{-}15}$	F2, K5	neg	neg	F2:A−:B−, K5:A−:B−	24
p17834Tf	*E. coli* transformant	—	—	$bla_{CTX\text{-}M\text{-}15}$	F2	neg	neg	F2:A−:B−	24
71697	*K. pneumoniae*	Italy	2008	$bla_{CTX\text{-}M\text{-}15}$	F2, K5	neg	neg	F2:A−:B−, K5:A−:B−	24
pYPISS1	*Y. pestis*	—	—	—	Y1	neg	neg	Y1:A−:B−	this study
pYPISS2	*Y. pseudotuberculosis*	Italy	2009	—	Y1	neg	neg	Y1:A−:B−	this study
pYPISS3	*Y. pseudotuberculosis*	Italy	—	—	Y1	neg	neg	Y1:A−:B−	this study

MDR, multidrug resistance.
[a]A co-integrate IncF and IncHI2 plasmid.

ABI 3730 automatic DNA sequencer (Perkin-Elmer). The regions containing the *copA* gene in the pZM3 and IncF/97 plasmids were obtained by PCR amplification using the FII FW–F_D RV primer pair (Table 3), generating amplicons of 600 and 1440 bp, respectively, which were fully sequenced.

Results and discussion

In silico analysis of IncF replicons

An *in silico* analysis comparison was performed by BlastN on DNA sequences available at GenBank for: (i) the *copA* region of the FII replicon; (ii) the region comprising the iterons and the replication protein *repE* gene of the FIA replicon; and (iii) the replication protein *repB* gene of the FIB replicon. These regions corresponded to the sequences targeted by the PBRT method proposed by Carattoli *et al.*[22] in 2005, were enclosed within the probes used by the hybridization-based replicon typing method proposed by Couturier *et al.*[29] in 1988 and were part of the incompatibility determinants used in the conjugation-based phenotypic classification of plasmids, defined by Datta and Hedges[30] in 1971. Therefore, these regions are the targets currently used for the identification of the IncF plasmids.

Table 3. Primers used in this study

Name	DNA sequence	EMBL accession no.	Amplicon size (bp)	Reference
FII FW	5′-CTGATCGTTTAAGGAATTTT-3′	AP000342	258–262[a]	this study
FII RV	5′-CACACCATCCTGCACTTA-3′			this study
FIA FW	5′-CCATGCTGGTTCTAGAGAAGGTG-3′	J01724	462	22
FIA RV	5′-GTATATCCTTACTGGCTTCCGCAG-3′			22
FIB FW	5′-TCTGTTTATTCTTTTACTGTCCAC-3′	M26308	683	this study
FIB RV	5′-CTCCCGTCGCTTCAGGGCATT-3′			22
FIB_S FW[b]	5′-TGCTTTTATTCTTAAACTATCCAC-3′	FN432031	683	this study
FIC FW	5′-GTGAACTGGCAGATGAGGAAGG-3′	AH003523	262	22
FIC RV	5′-TTCTCCTCGTCGCCAAACTAGAT-3′			22
FII_S FW	5′-CTAAAGAATTTTGATGGCTGGC-3′	AE006471	259–260[a]	this study
FII_S RV	5′-CAGTCACTTCTGCCTGCAC-3′			this study
FII_Y FW	5′-TGGYAGGGAACTGGTTCTG-3′	CP001590	227	this study
FII_Y RV	5′-GTRAGTCACACCTTCCCGC-3′			this study
FII_K FW	5′-TCTTCTTCAATCTTGGCGGA-3′	CP000648	142–148[a]	this study
FII_K RV	5′-GCTTATGTTGCACRGAAGGA-3′			this study
F_D RV[c]	5′-GGAATGTAGCACCCGAT-3′	AM886293	700	this study
RepAFII FW[d]	5′-CCAAACGTATTACCGCCAGGTAA-3′	AP000342	809	this study
RepAFII RV[d]	5′-CGGTTACGTGACAGAATCATGCGC-3′			this study

[a]Variable size amplicons can be obtained for these replicons.
[b]Used in a pair with FIB RV.
[c]Used in a pair with FII FW for the identification of an intact FII replicon in plasmids showing a second deleted FII replicon.
[d]These primers were used for the amplification of the entire *repA* gene of the FII replicon of the IncFIme plasmids, interrupted by the insertion of IS elements. In these cases the size of the amplicon can vary. They can be used for further characterization of plasmids carrying the FII replicon.

The comparative analysis of DNA sequences of 90 IncF plasmids of various origins and sources available at GenBank demonstrated the existence of several alleles for each FII, FIA, FIB and FIC replicon. Multiple alignments of selected replicon sequences, representative of the various alleles identified in GenBank, produced the phylogenetic trees that are shown in Figures 1 and 2. The largest branch obtained in the phylogenetic tree of the FII and FIC replicons showed a nucleotide identity of >90%, including all the replicons previously assigned by conjugation to the IncFII, IncFIII, IncFIV, IncFV and IncFVI groups, but also the FII replicons from the virulence plasmids of *Shigella flexneri* and *Shigella sonnei* (here designated as FII_{Sh}). Separate branches (nucleotide identities <80%) were generated by FII replicons identified in *Salmonella* spp. (here designated as FII_S), *Y. pestis*, *Y. pseudotuberculosis* and *Yersinia enterocolitica* (here designated as FII_Y) and *K. pneumoniae* (here designated as FII_K) virulence plasmids (Figure 1).

The FIA and FIB replicons demonstrated lower sequence variability than the FII replicons, with the exception of some FIB replicons, here designated as *Salmonella* FIB, which were exclusively associated with the *Salmonella* virulence plasmids (Figure 2).

The observation that FII replicons are more divergent than FIA and FIB replicons can be explained by assuming that, in the multi-replicon status, the FII replicons are more free to diverge, evolving towards the establishment of novel incompatibility groups.[17,18]

Update of the PBRT scheme

Several newly described IncF plasmids, including the *Shigella*, *Salmonella*, *Yersinia* and *Klebsiella* virulence plasmids, showed mismatches with the previously described PBRT primer pairs, and therefore some of these IncF plasmids may be non-typeable by the former scheme. To increase the sensitivity of the PBRT to all the members of this plasmid family, new primers were devised for detecting the FII (FII FW and FII RV), FIB (a new forward primer, FIB FW, was devised), *Salmonella* FIB (a new forward primer, FIB_S FW, was devised), *Salmonella* FII (FII_S FW and FII_S RV), *Yersinia* FII (FII_Y FW and FII_Y RV) and *Klebsiella* FII (FII_K FW and FII_K RV) replicons (Table 3). The previously described FIA and FIC primer pairs were well conserved with respect to GenBank entries and were used in this study without modification with respect to the former PBRT scheme (Table 3).

IncF RST

The DNA sequences of the replicons included among the nucleotide positions of the primers listed in Table 3 were used to set up the RST scheme for IncF plasmids (www.pubmlst.org/plasmid/). The sequence variants identified in GenBank for each respective replicon were assigned to an allele number. Thirty different alleles (named F1 to F30) were identified for the FII replicon in the sequence corresponding to the *copA* gene; 3 (A1–A3) and 20 (B1–B19 and B23) alleles were assigned for the FIA and FIB replicons, respectively (Figure S1). Only two alleles (C1 and C2) were identified for the FIC replicon. Four (S1–S4), four (K1–K4) and three (Y1–Y3) alleles were identified for the FII replicons of *Salmonella*, *Klebsiella* and *Yersinia* plasmids, respectively (Figure S1).

Because of the multi-replicon nature of these plasmids, each plasmid can be identified using the FAB (FII, FIA, FIB)

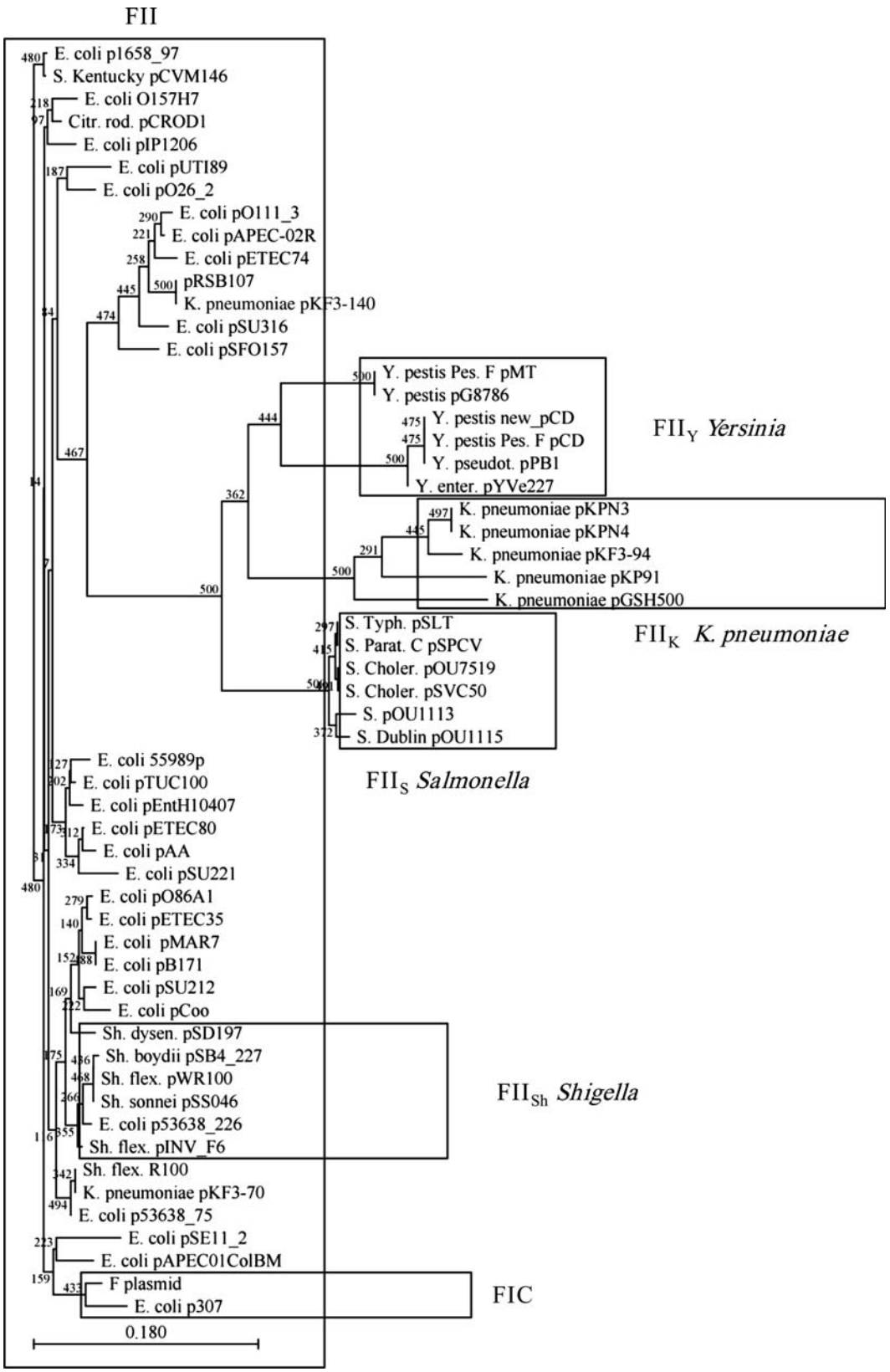

Figure 1. Phylogenetic tree of the FII and FIC replicons. The dendrogram was constructed using an NJ algorithm. Bootstrap values for 500 replicates are indicated. The distribution of FII plasmids according to bacterial species is highlighted with boxes that identify the separate branches of *Salmonella* spp., *Yersinia* spp., *K. pneumoniae* and *Shigella* spp.

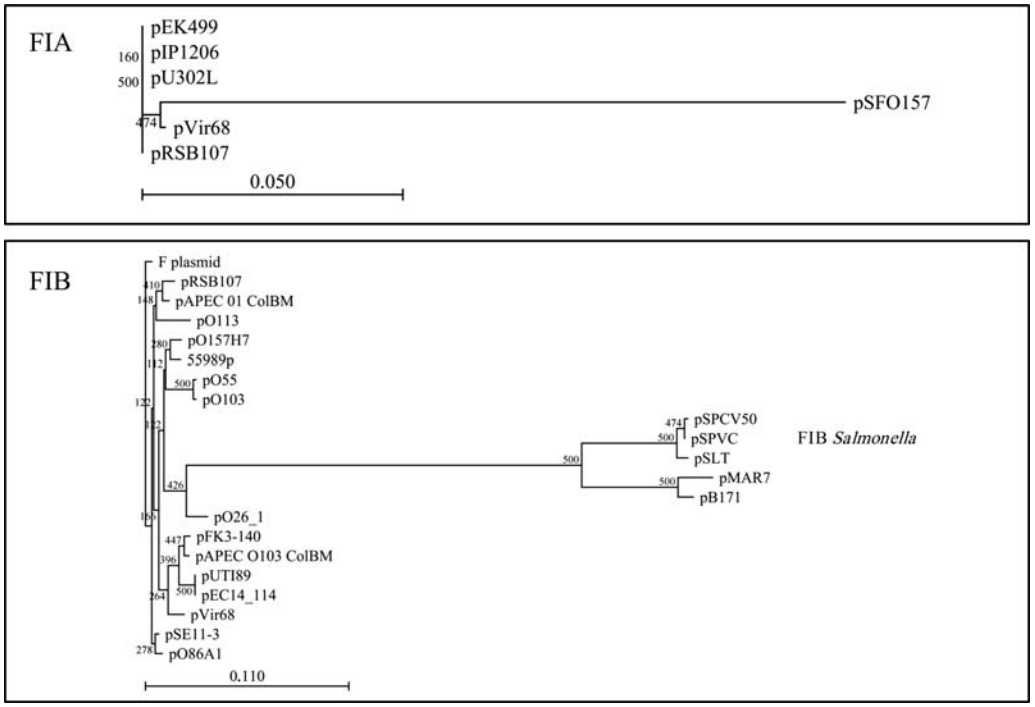

Figure 2. Phylogenetic tree of the FIA (top) and FIB (bottom) replicons. The dendrogram was constructed using an NJ algorithm. Bootstrap values for 500 replicates are indicated.

formula by the allele type and number identified for each replicon (like a serotype formula). For instance, the formula F1:A1:B1 was assigned to plasmid pRSB107 (AJ851089)—the first fully sequenced multi-replicon IncF plasmid—showing the allele F1 for the FII replicon, A1 for the FIA replicon and B1 for the FIB replicon, respectively. The formula F2:A−:B− was assigned to plasmid R100 (AP000342), the prototypic reference IncF plasmid carrying only the FII replicon, which was assigned to allele F2. The A− and B− symbols indicate the absence of the FIA and FIB replicons, respectively. Alleles were determined and deposited at the www.pubmlst.org/plasmid/ web site for all the IncF plasmids available to date at GenBank and representative plasmids and their respective FAB formulae are reported in Table 1.

Atypical IncF plasmids were found in GenBank; the pIP1206 (AM886293) and pSD1_197 (CP000035) plasmids showed two copies of the FII replicon, one truncated and one functional. In these cases the allele assignation was determined only for the functional FII replicon. On the basis of the pIP1206 DNA sequences, the F_D RV primer was devised and used with the FII FW primer (Table 3) to amplify the intact FII replicon. The pAA plasmid (FN554767) contained two identical copies of the FII replicons and therefore the allele assignation was univocal in this case (Table 1).

Characterization by RST of resistance and virulence IncF plasmids

A collection of 36 IncF plasmids from *E. coli*, *S. enterica*, *Y. pestis*, *Y. pseudotuberculosis* and *K. pneumoniae* strains were typed by RST (Table 2). The replicons from these plasmids were amplified using the primers shown in Table 3 and the DNA sequences of the alleles were determined. All were positive for the FII replicon, 21 also carried FIB and 12 also carried FIA replicons. Ten plasmids showed a multi-replicon status with the three FII, FIA and FIB replicons.

From this plasmid collection, the new alleles F31, F32, A4, B20, B21, B22 and K5 were identified (Figure S1).

Plasmids encoding the CTX-M-15 ESBL were placed in five groups on the basis of their FAB formula; F2:A−:B−, F2:A1:B−, F22:A1:B20, F31:A4:B10 and F31:A4:B1. This result is in agreement with previously reported characterizations of IncF plasmids carrying the $bla_{CTX\text{-}M\text{-}15}$ gene, which are not a homogeneous group of plasmids, as suggested by the observed heterogeneity of restriction patterns, sizes and DNA sequences.[2,23,27,31] Three of the CTX-M-15 encoding plasmids (p162237, p177273 and p45545) from our collection,[23,27] showing the new F31 allele, carried two copies of the FII replicon (demonstrated by Southern blot hybridization on PstI-restricted plasmid DNA; data not shown), as previously described for the pIP1206 plasmid,[32] and were noted since they produced three C/T heteroduplexes in the raw sequencing data. For these plasmids, FII FW–F_D RV PCR (Table 3) was used to amplify the intact FII replicon for allele assignation.

The *K. pneumoniae* strains (17829, 17830, 17834 and 71697) producing CTX-M-15 isolated in Italy in 2008[24] (Table 2) showed the coexistence in the same bacterial cell of two unrelated IncF plasmids. These strains carried a K5:A−:B− plasmid and the F2:A−:B− plasmid, the latter encoding CTX-M-15.[24] These IncF plasmids clearly showed a compatible phenotype within the same bacterial cell. The F2:A−:B− plasmid was a derivative of the R100 plasmid and was similar to plasmid pEK516, previously

identified in the ST131 epidemic *E. coli* clone from the UK (EU935738), and to pC15-1a from *E. coli* CTX-M-15 producers, which caused a large outbreak in Canada (AY458016).[21,33] Plasmids p48165T, p169967 and p177243 from *E. coli* from Italy and the UK[23,27] also showed this formula, suggesting that the R100 derivative carrying CTX-M-15 has spread worldwide and is probably one of the most frequent $bla_{CTX\text{-}M\text{-}15}$-carrying plasmids circulating among Enterobacteriaceae. The example of the $bla_{CTX\text{-}M\text{-}15}$ plasmids showing the F2:A−:B− formula demonstrated that the same scaffold can be identified in the fully sequenced plasmids available in GenBank (Table 1) and in the clinical strains under study (Table 2). The K5:A−:B− plasmid is probably a *Klebsiella* virulence plasmid whose function is still unknown, but can easily be recognized in the different strains of our *K. pneumoniae* collection, showing the novel K5 allele (Table 2 and Figure S1).

Four IncF plasmids in our collection, pZM3, IncFI/97, TP160 and TP181, were representative of IncFIme (me, Middle East) plasmids, which contributed to the epidemic spread of *S. enterica* serotype Wien, causing protracted outbreaks in Europe, the Middle East and North Africa during the early 1970s.[28] All of them were assigned the formula F1:A1:B1, the same as that of pRSB107 (Table 1). These plasmids were previously demonstrated to maintain very well conserved plasmid scaffolds for 25 years, also evolving by the acquisition of different resistance determinants,[28] and the RST results confirmed their phylogenetic relatedness. Interestingly, a careful analysis of the *repA* replicase gene of the FII replicon of these plasmids revealed that a complete Tn*3* transposon, carrying the $bla_{TEM\text{-}1}$ gene, interrupted the *repA* gene of pZM3 at 535 bp from the ATG codon, while the insertion of an IS*26* element caused a deletion of the *repA* gene of plasmid IncFI/97 at position 374 from the ATG codon (accession numbers HM769901 and HM769900, respectively). The insertion of mobile elements within the FII replicon did not impair plasmid replication, because of the presence of the FIA and FIB replicons, which assured the replication of these plasmids. These results highlight the role played by the multi-replicon status in the versatility of IncF plasmids, whose complex structure sustained their maintenance also when mobile elements impaired the functionality of some crucial replication determinants.

The discriminatory power of RST seems sufficient to discern unrelated plasmids, but similar plasmid scaffolds can also be recognized. Further examples of conserved plasmids that can be recognized by RST are represented by: (i) the F18:A−:B1 pAPEC-1 (CP000836) and pVM01 (EU330199) plasmids identified in APEC strains isolated in the USA and Australia, respectively; (ii) the five F23:A−:B3 plasmids from different EHEC strains; (iii) the F29:A−:B10 ColIa plasmids identified in uropathogenic *E. coli* (UPEC) and extraintestinal pathogenic *E. coli* (ExPEC) strains; and (iv) the various *Shigella*, *Salmonella*, *Klebsiella* and *Yersinia* virulence plasmids that can be easily detected and discriminated by their FAB formulae (Table 1).

Nine prototypic plasmids of our collection were analysed by PstI-RFLP (Figure 3). RFLP analysis showed a great variability of restriction patterns with a discriminatory power comparable to that of the RST method. However, RFLP plasmid patterns cannot be compared easily among different laboratories, while sequence-based typing methods may support inter-laboratory exchanges for many different epidemiological purposes, including the detection of the spread of a common plasmid in different strains from different countries.

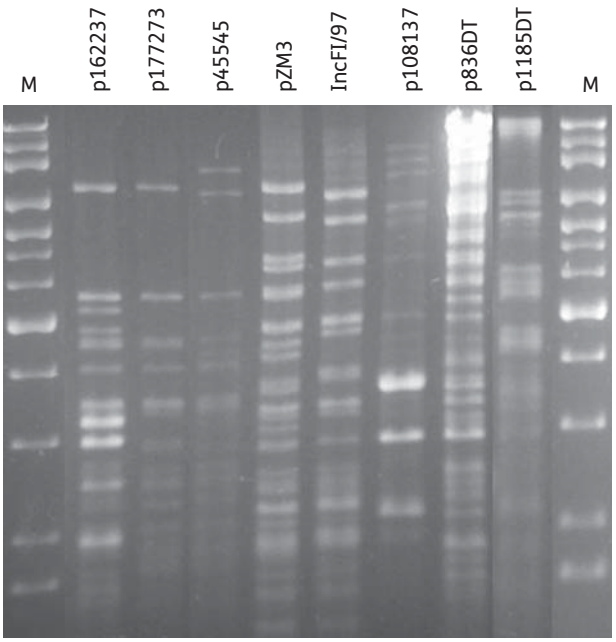

Figure 3. PstI digestion of the prototypic IncF plasmids. Lane M, 1 kb DNA extension ladder (Invitrogen).

Conclusions

The IncF plasmid family is clearly playing a major role in the dissemination of antimicrobial resistance in Enterobacteriaceae. Of 1224 plasmids successfully typed by PBRT, carrying ESBL or plasmid-mediated quinolone or aminoglycoside resistance genes, 331 were assigned to the IncF group (27%), demonstrating that the members of this plasmid family are prevalent in clinical, resistant isolates of Enterobacteriaceae.[11] Such prevalence cannot permit the recognition of similar plasmids simply on the basis of assignation to the IncF family. A further discrimination method is required to identify related plasmid scaffolds from IncF-unrelated plasmids. Furthermore, the simultaneous presence of more than one plasmid of this family, showing compatible phenotypes within the same bacterial cell, further complicates the interpretation of plasmid typing and content in the studied strains. When multiple IncF plasmids are present in the same cell, the application of this typing scheme should be performed on both donor and recipient strains, obtained by mating or transformation, to correctly assign the FAB formula to each single plasmid scaffold. RST may represent a subtyping scheme that is easier to apply than RFLP analysis, as it has already been described for multilocus sequence typing of plasmids belonging to the IncI1, IncHI2 and IncHI1 families.[34–36]

This typing scheme can be used to facilitate inter-laboratory comparative studies on IncF plasmids, to identify similar plasmids from bacteria from different sources and countries and to support molecular epidemiological surveillance of antimicrobial resistance, particulary in relation to IncF plasmids

associated with the spread of both virulence and resistance determinants.

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Transparency declarations

All authors have no conflicts of interest to declare relevant to this study.

Supplementary data

Figure S1 is available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

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