Molecular comparison of the IncX plasmids allows division into IncX1 and IncX2 subgroups

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We have investigated molecular relationships and evolution of plasmids classified genetically to incompatibility (Inc) group X, in particular by comparison of plasmids from the pre-antibiotic era (PAE) with contemporary R-plasmids. On the basis of restriction analysis, R6K, the best-described and 'prototype' plasmid of the IncX group, exhibited little similarity with the other plasmids in this Inc group. Other contemporary IncX R-plasmids exhibited a substantial degree of interrelationship, and were also related to PAE IncX plasmids. When the origin of plasmid replication of R6K was used as a replicon probe, R6K was the only plasmid tested which exhibited homology. Other contemporary and PAE IncX plasmids exhibited homology with the origin of plasmid R485. These data suggest that the IncX group should be subdivided. R485 may be regarded as representative of the major subgroup present before and after the advent of antibiotic selection pressure. Plasmids of this subgroup, IncX1, possess an internal region which yields five characteristic *EcoRV* fragments. R6K may be regarded as representative of subgroup IncX2, of which it is presently the sole well-described member. The antibiotic resistances encoded by contemporary IncX R-plasmids are due to insertion of identifiable transposons in progenitor plasmids identical to the R485 subgroup of PAE IncX plasmids.

Introduction

The conjugative plasmid R6K, first detected in *Escherichia coli*, was found to be a multicopy R-plasmid of 40 kb (Kontomichalou *et al.*, 1970) and was assigned to a new incompatibility group, IncX (Hedges *et al.*, 1973). The two best-studied representatives of this group, as defined genetically (Datta, 1979), are R6K and R485. Physical-genetic maps of these plasmids are presented in Fig. 1, which shows that there are few if any common features. The two plasmids encode structurally similar pili, but R485 also encodes a second unique pilus type. The plasmids are incompatible in that stable 'doubles' cannot be formed, although their (bidirectional) segregation is atypically slow (Bradley, 1982). IncX plasmids have been implicated in the acquisition and dissemination of transferable drug resistance in pathogenic enterobacteria, for example in animal strains of *Salmonella*

typhimurium (Threlfall *et al.*, 1986). IncX plasmids have also been shown to participate in co-integration events with virulence-associated plasmids in *Salmonella* (Platt *et al.*, 1988).

The replication of R6K is well characterized and a variety of small autonomously-replicating derivatives have been constructed (Filutowicz *et al.*, 1985). It contains three origins of replication which function *in vitro*, designated α , β and γ (see Fig. 1). A structural gene for the replication-initiation protein has been characterized (Crosa, 1980; Kolter & Helinski, 1978). Studies with R485 showed that *in vivo* the incompatibility reaction between this plasmid and R6K depended on interaction between a functional R485 replicon (see Fig. 1) and an active R6K β -origin (Stalker & Helinski, 1985). Other R6K replication functions were not involved in its incompatibility with R485. No extensive DNA homology was found between the replicons of R485 and R6K over long nucleotide stretches (Stalker & Helinski, 1985). Couturier *et al.* (1988) found no homology between a cloned probe for the γ origin of replication of R6K (which is inactive *in vivo*) and other IncX plasmids studied. Two IncX plasmids hybridized with a replicon probe from IncQ plasmids. The origin of replication of R485 has been cloned (Stalker & Helinski,

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Abbreviation: PAE, pre-antibiotic era.

Table 1. *Plasmids and DNA probes*

(a) <i>Plasmids</i>					
Plasmid	Year of isolation	Original plasmid host	Antibiotic resistances	Size (kbp)	Reference
<i>Contemporary IncX plasmids</i>					
R6K	1969	<i>Escherichia coli</i>	ApSm	40	Kontomichalou <i>et al.</i> (1970)
R485	1971	<i>Proteus morganii</i>	Su	56	Hedges <i>et al.</i> (1973)
TP227	1975	<i>Salmonella newport</i>	Ap	44	Bradley (1980)
TP228	1975	<i>Salmonella newport</i>	KmNmSmSpSuTcHg	72	Bradley (1980)
TP231	1976	<i>Shigella dysenteriae</i>	ApCmTc	56	Bradley (1980)
pHH1187	1977	<i>Escherichia coli</i>	SmSpSuTp	62	Datta <i>et al.</i> (1980)
pHH1372	1979	<i>Klebsiella</i> K16	ApSmSpSuTp	62	Hughes <i>et al.</i> (1981)
pXAK	1980	<i>Salmonella typhimurium</i>	ApKm	56	Threlfall <i>et al.</i> (1986)
<i>PAE IncX plasmids</i>					
pMUR25	1926	<i>Salmonella choleraesuis</i>	—	39	Datta & Hughes (1983)
pMUR155	1939	<i>Salmonella typhimurium</i>	—	37	
pMUR440	1938	<i>Salmonella moscow</i>	—	37	
pMUR509	1936	<i>Salmonella typhi</i>	—	38	
pMUR510	1938	<i>Salmonella typhi</i>	—	38	
pMUR581	1935	<i>Salmonella typhi</i>	—	44	
pMUR583	1932	<i>Klebsiella ozaenae</i>	—	44	
pMUR586	1940	<i>Klebsiella</i> sp.	—	44	
(b) <i>DNA probes</i>					
Probe	Restriction fragment	Clone	Origin element	Reference	
IncX _{R485}	<i>Eco</i> RI-TaqI/0.59	pDS49	R485	Stalker & Helinski (1985)	
IncX _{R485}	<i>Eco</i> RI/2.75	pDS20	R485	Stalker & Helinski (1985)	
IncX _{R6Kα,β origins}	<i>Hae</i> II/2.3	pMU609	R6K	A. J. Pittard & A. Praszkie, pers. comm.	
IncX _{R6K origin}	<i>Hind</i> III/0.9	pULB2405	R6K	Couturier <i>et al.</i> (1988)	
IncQ	<i>Eco</i> RI/0.357	pULB2424	R1162	Couturier <i>et al.</i> (1988)	
<i>tnpA</i>	<i>Bam</i> HI- <i>Hae</i> II/1.0	pUB2403	Tn3	Ward & Grinsted (1987)	
<i>tnp</i>	<i>Bam</i> HI/0.9	Co1E1::Tn7	Tn7	Barth <i>et al.</i> (1978)	
<i>tnpA/R</i>	<i>Eco</i> RI/1.3	pUB2401	Tn21	Ward & Grinsted (1987)	

R6K (38 kbp)

R485 (56 kbp)

Fig. 1. Physical-genetic maps of archetypal IncX plasmids. Maps were redrawn from the data of Kolter & Helinski (1978) and Stalker & Helinski (1985) and aligned from the single *Bam*HI site, which is the only apparent conserved feature in these plasmids. Restriction sites are indicated above the maps as follows: B, *Bam*HI; E, *Eco*RI; H, *Hind*III. Replication origins are indicated by arrowheads. Genes encoding antibiotic resistance are shown as open boxes, and the gene encoding the *trans*-activator of R485 replication is shown by a stippled open box. The scale in kbp is shown below the maps, and at the bottom of the figure.

1985) but its molecular relatedness among IncX plasmids has not been documented.

Pathogenic enterobacteria (*Salmonella*, *Shigella*, *Escherichia*, *Klebsiella* and *Proteus* spp.) from human infections of wide geographical distribution were collected by E. D. G. Murray between 1917 and 1954, the pre-antibiotic era (PAE), and preserved in sealed glass tubes on agar slants. When opened in 1980, about 70% of these PAE isolates were found to be still viable. They represent bacterial genera, of which contemporary examples frequently contain plasmids encoding multiple antibiotic resistance. In a study of PAE plasmids in the Murray Collection, Hughes & Datta (1983) made a genetic analysis of transferable plasmids among PAE *Enterobacteriaceae*, showing that conjugative plasmids of the same Inc groups existed in enterobacteria before and after the medical use of antibiotics. Several other molecular genetic studies (e.g. Green, 1984; Jones & Stanley, 1992; Sedgewick *et al.*, 1989) have employed Murray PAE strains to elucidate the evolution of plasmid and other genes.

The objective of this study was to characterize

Fig. 2. *EcoRV* fingerprint of contemporary and PAE *IncX* plasmids. The molecular size markers used were derived from an *EcoRI*/*HindIII* digest of phage λ (tracks 1, 5, 9, 15 and 18). The other tracks contained: track 2, pHH1187; 3, R6K; 4, TP227; 6, TP228; 7, TP231; 8, R485; 10, R300B; 11, pMUR509, R300B; 12, pMUR583, R300B; 13, pMUR25, R300B; 14, pMUR155, R300B; 16, pMUR440, pHH1310a; 17, pHH1310a. Plasmids R300B (Sm^rSu^r) and pHH1310a (Ap^r) appear together with individual PAE plasmids because of their use as co-mobilized indicators of self-transfer by the PAE plasmids (Datta & Hughes, 1983).

molecular relationships among *IncX* plasmids, and to compare contemporary *IncX* R-plasmids with PAE *IncX* plasmids from the Murray collection. We have sought thereby to provide a molecular phylogenetic classification of this plasmid group.

Methods

Bacteria, media and culture conditions. *IncX* plasmids used in this study, their isolation dates and original host are listed in Table 1. PAE plasmids were analysed in *E. coli* K12 J53, a recipient strain to which they had been transferred using the plasmids R300B or pHH1310a as co-mobilized marker plasmids (Datta & Hughes, 1983). All strains were stored on nutrient agar slopes at room temperature and grown on nutrient agar/broth at 37 °C for 18 h. Antibiotics (Sigma) were added to media at the following concentrations ($\mu\text{g ml}^{-1}$) for plasmid selection: ampicillin, Ap (100); chloramphenicol, Cm (25); tetracycline, Tc (10).

Plasmid analysis and DNA hybridization. Analysis of plasmid DNA was done by the method of Kado & Liu (1981), and plasmids were visualised by electrophoresis on horizontal 0.7% (w/v) agarose gels. Agarose gels were vacuum-blotted (Pharmacia-LKB Vacu-Gene apparatus) onto Hybond N nylon membrane (Amersham) for hybridization experiments. Plasmid DNA for restriction enzyme analysis or for preparation of probe fragments was extracted from 500 ml nutrient broth cultures, using cleared lysates and CsCl/ethidium

bromide density gradient purification (Sambrook *et al.*, 1989). DNA probe fragments were excised after digestion with enzymes listed in Table 1. Whole plasmid DNA or restriction fragments were separated on 1% low-melting-point agarose (Gibco-BRL), prior to labelling with digoxigenin-11-dUTP (Boehringer-Mannheim) by random-primed synthesis in agarose. Membrane filters blotted from agarose gels (see above) were hybridized at 68 °C in $5\times\text{SSC}$, and thereafter stringently washed (final wash in $0.1\times\text{SSC}$ at 55 °C). [$1\times\text{SSC}$ is 0.15 M-NaCl, 0.015 M-sodium citrate, pH 7.0]. For certain experiments, washing conditions of reduced stringency ($2\times\text{SSC}$ at 55 °C) were employed. Filters were colour-developed according to Boehringer-Mannheim's instructions. DNA probe fragments used in the study are listed in Table 1.

Results

Common features of a major group of IncX plasmids

CsCl-purified DNA was digested with the restriction enzymes *EcoRV*, *Bam*HI, *Bgl*II, *Eco*RI, *Pst*I and *Sal*I. In all digests, R6K showed very few or no bands in common with any other *IncX* plasmids (for *EcoRV*, see Fig. 2; other data not shown) and on this basis it was judged to be distantly related. For other *IncX* plasmids, *EcoRV* digests, which yielded a wide range of fragment sizes, were used for general comparative analysis (see

Fig. 3. Diagrammatic representation of pMUR25 plasmid homology relationships. *EcoRV* digests of representative IncX plasmids were transferred to nylon membrane and probed with labelled pMUR25 DNA. The membrane filter which is represented was washed under conditions of high stringency. Solid lines represent fragments which showed homology to this probe; dotted lines denote non-homologous fragments. The restriction fragments due to R300B and pHH1310a have been removed. The tracks contained: 1, R6K; 2, TP228; 3, R485; 4, pMUR25; 5, pMUR440. Molecular size markers are indicated on the left. Conserved 'core-region' fragments of the IncX1 subgroup are arrowed on the right.

Fig. 2). One obvious feature of *EcoRV* digests was that none of the eight *EcoRV* fragments of R6K (Fig. 2, track 3) was conserved among other IncX plasmids. On the other hand, common fragments of 2.1, 0.94, 0.84, 0.82 and 0.63 kbp were found in all plasmids except R6K. These five *EcoRV* fragments exhibited features of a conserved region specific to a plasmid subgroup. With respect to the PAE IncX plasmids there were identical digest patterns for pMUR509 (Fig. 2, track 11) and pMUR510 (not shown), and also for pMUR583 (Fig. 2, track 12), pMUR581 and pMUR586 (not shown), although these plasmids were isolated from *Salmonella typhi*, *Klebsiella ozaenae* and *Klebsiella* sp. at different dates. Plasmids pMUR583, pMUR25 and pMUR155 showed only minor *EcoRV* site variations (Fig. 2, tracks 12, 13 and 14). Greater variation was seen among the contemporary IncX R-plasmids, but there were clearly discernible common regions in R485 and pHH1187

(eight common *EcoRV* fragments in Fig. 2, tracks 8 and 2), and in pHH1372 (eight common fragments, data not shown). Similarly, plasmids TP227, TP228 and TP231 shared at least ten common *EcoRV* fragments with each other (Fig. 2, tracks 4, 6 and 7) and with pXAK (data not shown). There were two sites for *PstI* in most of the PAE plasmids, but pMUR581, pMUR583, pMUR586 and pMUR25 contained a third site. Four, five or six *PstI* sites were found in all contemporary IncX R-plasmids (data not shown).

Homology relationships with pMUR25

Total plasmid DNA from the earliest-isolated PAE plasmid, pMUR25, was labelled and used to evaluate homology relationships among all IncX plasmids. Fig. 3 presents a diagrammatic representation of the results of such experiments. The previously discussed five common *EcoRV* fragments of contemporary and PAE plasmids all showed homology with pMUR25 as shown by the arrows on the right of Fig. 3. Six to eight other *EcoRV* fragments in contemporary plasmids and virtually all *EcoRV* fragments of the PAE plasmids showed homology. Addition of the molecular sizes of the five conserved homologous fragments found in both PAE and contemporary plasmids suggested that they might represent a 'core-region' of about 6 kbp. This was not present in R6K (Fig. 3, track 1), in which only an *EcoRV* fragment of 2.2 kbp exhibited homology with pMUR25. The bands not hybridizing in the contemporary plasmids were presumed to have arisen from recombinational events including transposition of mobile DNA during acquisition of antibiotic-resistance genes.

Evidence for an R485 Inc Rep homology group

Data obtained from hybridization experiments with replicon probes are presented in Table 2. Seven out of eight contemporary R-plasmids and all eight PAE plasmids exhibited homology with the replication origin of R485. When the R485 origin was used to probe *EcoRI* digests (Fig. 4a) of representative plasmids from each group, all except R6K (track 3) exhibited homology (Fig. 4b). It is noteworthy that this 0.59 kbp R485 origin probe detected two homologous *EcoRV* fragments in all these plasmids (in pMUR155, track 13, these were comigrating). A 2.75 kbp *EcoRI* fragment of R485 encoding a *trans*-activator of replication (Stalker &

Fig. 4. Two groups of IncX replicons. (a) *EcoRI* digest of contemporary and PAE IncX plasmids. The tracks contained: track 1; phage λ *EcoRI*/*HindIII* digest; 2, pHH1187; 3, R6K; 4, TP227; 5, TP228; 6, TP231; 7, R485; 8, λ *EcoRI*/*HindIII*; 9, R300B; 10, pMUR509 R300B; 11, pMUR583 R300B; 12, pMUR25 R300B; 13, pMUR155 R300B; 14, pMUR440 pHH1310a; 15, pHH1310a; λ *EcoRI*/*HindIII*. (b) Southern blot of (a), hybridized with the X_{R485} replicon probe.

Table 2. DNA probe homologies among IncX plasmids

The five Inc/Rep and three transposase probes are listed at the top; contemporary R-plasmids and PAE plasmids are listed down the Table. Results of stringent hybridization experiments (see Methods) are indicated as follows: +, strong homology; - no homology; p, partial (weak) homology.

	X _{R6K}	X _{R485}	Q	Tn3	Tn7	Tn21
R6K	+	-	-	+	-	-
R485	-	+	p	-	-	-
TP227	-	+	-	+	-	-
TP228	-	+	-	-	-	+
TP231	-	+	-	+	-	+
pHH1187	-	+	p	-	+	-
pHH1372	-	+	-	+	+	-
pXAK	-	+	-	+	-	-
pMUR25	-	+	-	-	-	-
pMUR155	-	+	-	-	-	-
pMUR440	-	+	-	-	-	-
pMUR509	-	+	-	-	-	-
pMUR510	-	+	-	-	-	-
pMUR581	-	+	-	-	-	-
pMUR583	-	+	-	-	-	-
pMUR586	-	+	-	-	-	-

Helinski, 1985) reacted uniquely with these plasmids and not with R6K (data not shown). Conversely, even under conditions of reduced stringency (see Methods), R6K was the only plasmid which reacted with either of two IncX replicon probes representing the γ or the α and β origins of R6K. It should be noted that this lack of homology with the β origin of R6K has not been reported previously.

Plasmids R485 and pHH1187 showed weak partial (about 20%) homology with an IncQ probe, but no other plasmids did so. As a whole, the results presented above are consistent with the existence of major (R485-type) and minor (R6K-type) groups of IncX plasmids, and with the fact that no representatives of the minor (R6K) group were found among PAE plasmids.

Transposon homology

The presence or absence of transposable elements was determined by DNA hybridization and is shown in Table 2. No transposon homology was found in any PAE IncX plasmid. Of the contemporary plasmids, Tn3 homology was found in all plasmids carrying ampicillin-resistance markers (i.e. R6K, TP227, TP231, pHH1372 and pXAK). Homology with Tn7 was found in plasmids pHH1187 and pHH1372, which carry a trimethoprim-resistance marker. Homology with Tn21 was found in plasmids TP228 and TP231. R485, although it carries a sulphonamide-resistance marker, did not show homology with any tested transposase probe (Table 2).

Discussion

From the digest patterns of IncX plasmids shown in Figs 2 and 3 it can be seen that a conserved region exists in one subgroup of these plasmids. A common evolutionary origin is therefore indicated for contemporary and PAE IncX plasmids other than R6K. The 'core-region' characterized is composed of five small and easily resolved *EcoRV* fragments.

In contemporary IncX plasmids, insertion of readily identifiable transposons was demonstrated. Thus in the IncX group, complex R-plasmids have been produced by sequential addition of transposons, such as of Tn3 and Tn21 in the case of TP228. Evolutionarily-related or identical (pMUR583-pMUR581-pMUR586 type) conjugative plasmids, were evidently present in the *Enterobacteriaceae* (see Table 1) before the advent of selection pressures due to the widespread use of antibiotics. The PAE plasmids have few *PstI* sites, presumably reflecting their adaptation to *Enterobacteriaceae* such as *Providencia*. Their contemporary relatives on the other hand have up to six *PstI* sites, imported by transposons such as Tn3. Thus, the evidence of this study confirms that PAE plasmids readily became vehicles for the spread of antibiotic-resistance transposons.

The molecular fingerprint homology analysis reported here indicates that although R6K is indeed incompatible with other members of the IncX group of PAE and contemporary origin, its molecular evolution is distinct. We therefore consider that R6K exemplifies an incompatibility sub-group which is of minor prevalence. By way of precedent, the IncHI group plasmids have been divided into Inc HI1, HI2 and HI3 on the basis of relative homology relationships (Grindley *et al.*, 1973; Roussel & Chabbert, 1968). We propose that in a similar manner, the R485 subgroup of Inc group X should be termed IncX1, since it is currently more prevalent, and we have presented evidence of its existence prior to the isolation of R6K. The R6K subgroup should then be termed IncX2. IncX plasmids have been described by Prager *et al.* (1988), whose *EcoRV* fingerprints, although not complete, suggest that they may be members of IncX2. To our knowledge these are the only other IncX2 plasmid candidates so far isolated.

IncX plasmids could still be generally identified by classical genetic incompatibility with R6K or R485. Stalker & Helinski (1985) have speculated that the two-way incompatibility between the R6K β origin and the R485 origin reflects structural competition for a putative cellular replication or partition component. Homology relationships between the R6K β origin, which is that responsible for incompatibility, and other IncX plasmids had not been previously reported.

Members of the IncX1 subgroup proposed would be

defined by possession of the core region in *EcoRV* fingerprints, and/or by homology with an R485 replicon probe. Some evidence was found in this study that further subgroups exist within IncX1. For example many other, larger, restriction fragments were found in common between the three 'TP' plasmids and the related PAE plasmids pMUR25, pMUR155 and pMUR440 (see Fig. 2). Similarly R485, the two 'pHH' plasmids, and three PAE plasmids exhibited conserved restriction fragments other than those from the core region. At present, new members of the IncX2 group should be defined by homology with the R6K Inc/Rep probe. In view of the detailed analysis of R6K replication (Scott, 1984), the extent and distribution of this subgroup deserves further study.

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