Characterization of Conjugative R Plasmids Belonging to the New Incompatibility Group IncU

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Five conjugative plasmids governing different antibiotic resistance patterns were identified in wild strains of enteric bacteria isolated in Czechoslovakia and the G.D.R. between 1976 and 1979. They have been characterized as members of the new incompatibility group IncU (reference plasmid RA3 from Japan). The molecular sizes of the IncU plasmids ranged between 18 and 37 megadaltons; their restriction fragment patterns indicated them to be distinct types.

INTRODUCTION

A wide variety of different plasmids belonging to many incompatibility (Inc) groups contribute to the development of antibiotic-resistant organisms, and the incidence of conjugative R plasmids has increased within the past few years (Richmond, 1975). By means of genetic tests (incompatibility and pili tests) and molecular characterization (size determination, hybridization and endonucleolytic cleavage) it has been shown that plasmids belonging to the same incompatibility group generally, but not always, have a high degree of DNA relatedness revealed by DNA reassociation and/or restriction enzyme analysis (Grindley *et al.*, 1973; Roussel & Chabbert, 1978; Chabbert *et al.*, 1979; Willshaw *et al.*, 1980; Tschäpe & Tietze, 1980; for review, see Datta, 1979). Therefore a phylogenetic relatedness of incompatible plasmids, in spite of their different geographical and bacterial origin, has been assumed (Datta, 1979). During the surveillance of R plasmids in enteric bacteria from hospital and other environments, we found, in different bacterial species, a number of conjugative plasmids that did not belong to any of the known incompatibility groups (see Datta, 1979; Novick *et al.*, 1976; Jacob *et al.*, 1977) and were incompatible with one another. However, they were found to be incompatible with the R plasmid RA3, from Japan, for which the group IncU had been tentatively introduced (see Sirgel *et al.*, 1981). These plasmids are described in this paper and characterized as members of this new group IncU.

METHODS

Strains, plasmids and phages. The *Escherichia coli* K12 standard strains, plasmids and phages used for the characterization of plasmids were listed by Tschäpe & Tietze (1980). *Escherichia coli* K12 CV601 (*thr leu thi lac rif^R*), rifampicin-resistant derivative of W677 (V. Chaloupecky, personal communication), and *Salmonella typhimurium* LT2 (prototroph) were also used as recipient strains. The reference plasmids for the incompatibility tests are described in Table 1. The plasmids characterized in this paper came from several different wild strains of enteric bacteria from various clinical and geographical sources (Table 2). They were collected between 1976 and 1979.

Genetic characterization. Plasmids were transferred by the conjugation procedure described by Tschäpe (1973). Fi character, pilus specificity and incompatibility properties were determined as described by Tschäpe & Tietze

Table 1. *Reference plasmids used for the incompatibility testing*

Plasmid	Relevant characters*			Reference
	Inc group	Drug resistance markers	Other properties	
R16	IncB	TcSmSuAp	Fi ⁻	Datta (1979)
pIE469	IncB	SuKm	Fi ⁻	Tschäpe <i>et al.</i> (1981)
pIP40a	IncC	KmApSu	Fi ⁻	Chabbert <i>et al.</i> (1972)
pIE468	IncC	SmTpSu	Fi ⁻	Tschäpe <i>et al.</i> (1981)
RA1	IncC [†]	TcSu	Fi ⁻	Datta (1979)
R711b	IncD	Km	Fi ⁺	Datta (1979)
pIA4643	IncE	CmSmSuTc	Fi ⁻	Arai (pers. comm.)
R386	IncF1	Tc	Fi ⁻ Dps(M13, fr)	Dennison (1972)
R1-19	IncFII	Km	Fi ⁻ Dps(M13, fr)	Datta (1975)
R124	IncFIV	Tc	Fi ⁺ Dps(M13, fr)	Datta (1975)
pIE509	IncFV	Km	Fi ⁺ Dps(fr)	Tschäpe (unpublished)
R27	IncH1	Tc	Fi ⁻	Datta (1975)
R726	IncH1	CmSmSu	Fi ⁻	Datta (pers. comm.)
R478	IncH2‡	TcCmKm	Fi ⁻	Hedges <i>et al.</i> (1975a)
pIE428	IncH2	CmSmSuGm(Tm)	Fi ⁻	Tschäpe <i>et al.</i> (1981)
R64-11	IncIa	TcSm	Fi ⁻ Dps(lfm)	Hedges & Datta (1973)
R621a	IncI γ	Tc	Fi ⁻ Dps(lfm)	Hedges & Datta (1973)
R391	IncJ	KmHg	Fi ⁻	Coetzee <i>et al.</i> (1972)
R387	IncK	CmSm	Fi ⁻	Hedges & Datta (1971)
pIE3121	IncK	Tc	Fi ⁻	Tschäpe & Tietze (1980)
R446b	IncM	TcSm	Fi ⁻	Datta (1975)
pIE373	IncM	ApKmSm	Fi ⁻	Tietze (1980)
R471	IncM§	Ap	Fi ⁻	Hedges <i>et al.</i> (1975a)
RN3	IncN	TcSmSu	Fi ⁻ Dps(Ike, PRD1)	Datta & Hedges (1971)
pIE492	IncN	CmSmSu	Fi ⁻ Dps(Ike, PRD1)	Tschäpe (unpublished)
RP1	IncP	TcKmAp	Fi ⁻ Dps(PRR1, PRD1)	Datta <i>et al.</i> (1971)
Rts1	IncT	KmTc	Fi ⁻	Datta (1975)
RA3	IncU	CmSmSu	Fi ⁻	Sirgel <i>et al.</i> (1981)
R783	IncV	CmSmSuAp	Fi ⁻	Datta (1979)
RSa	IncW	CmSmSuKm	Fi ⁻ Dps(PRD1)	Datta (1975)
R388	IncW	SuTp	Fi ⁻ Dps(PRD1)	Datta & Hedges (1972)
R6K	IncX	SmAp	Fi ⁻	Kontomichalou <i>et al.</i> (1970)
P1Cm	IncY	Cm	Fi ⁻	Hedges <i>et al.</i> (1975b)
R71a	Inc9	CmSmSuTcAp	Fi ⁻	Chabbert <i>et al.</i> (1972)

* The abbreviations used are those of Datta (1979). Drug resistance symbols: Ap, ampicillin; Cm, chloramphenicol; Gm(Tm), gentamicin/tobramycin; Km, kanamycin; Su, sulphonamide; Tc, tetracycline; Tp, trimethoprim. Fi, fertility inhibition; Inc, incompatibility; Dps, donor phage specificity.

† Previously designated as IncA (see Datta, 1979).

‡ Previously designated as IncS (see Datta, 1979).

§ Plasmids of IncL were reclassified as IncM members (Richards & Datta, 1979).

|| RA3 is the reference plasmid of IncU (see Sirgel *et al.*, 1981).

(1980). For incompatibility tests the reference plasmids listed in Table 1 were either transferred by conjugation from *E. coli* W677 (*thi thr leu*) to *E. coli* K12 J53 (*met pro*) clones carrying the plasmids described here, or *E. coli* K12 J53 strains carrying the reference plasmids were superinfected by the plasmids under investigation. Fifty colonies from each cross were replica-plated twice to select for the superinfecting plasmid and then tested for the presence of the resident plasmid. The pattern of the stability of the 'doubles' and the superinfection frequency were used to define the incompatibility properties of the plasmids.

Molecular characterization. The procedure for isolation and agarose gel electrophoresis of plasmid DNA was as described by Tschäpe & Tietze (1980). Digestion with restriction endonuclease *Bam*HI was carried out in TM buffer pH 7.5 (a solution containing 100 mM-Tris and 10 mM-MgCl₂). For *Pst*I digestion this buffer also contained 10 mM-2-mercaptoethanol, for *Eco*RI treatment both 10 mM-2-mercaptoethanol and 40 mM-NaCl, and for *Hind*III digestion 10 mM-2-mercaptoethanol and 70 mM-NaCl. The enzymes were gifts from Dr Hartmann (ZIMET, Jena, G.D.R.).

Table 2. Origin and properties of the plasmids investigated

Plasmid	Source		Resistance pattern* in <i>E. coli</i> K12	Transfer frequency† from:		
	Host and clinical origin	Location		Original host to CV601	CV601 to J53	J53 to LT2
pCV9‡	<i>Proteus morganii</i> (pyelonephritis)	Prague, C.S.S.R.	CmApKm	0.4	350	1.0
pIE420	<i>Escherichia coli</i> (pyelonephritis)	Osterwieck, G.D.R.	TcSuSmTp	3.0	450	3.0
pIE432	<i>Escherichia coli</i> (hospital sewage)	Wernigerode§, G.D.R.	TpSm	50	300	3.0
pIE447	<i>Salmonella dublin</i> (gastroenteritis)	Rostok, G.D.R.	Tc	3.6	550	4.5
pIE491	<i>Citrobacter freundii</i> (bacteriuria)	Wernigerode§, G.D.R.	TpSmSu	0.3	350	1.0

* For abbreviations see Table 1.

† The transfer frequency is expressed as the number of transconjugant colonies per 10⁵ donor organisms for an 8 h mating. The recipient strains *E. coli* CV601 (*thr leu thi lac rif*^R) (see Tschäpe *et al.*, 1981), *E. coli* K12 J53 (*met pro*) and *S. typhimurium* LT2 (prototroph) were chosen as representative examples.

‡ The plasmid pCV9 was isolated by Dr V. Chaloupecky, Prague, in 1976, and sent for incompatibility testing to the Institut für Experimentelle Epidemiologie, Wernigerode.

§ From the same hospital.

RESULTS AND DISCUSSION

Four conjugative R plasmids were isolated from clinical strains of *Proteus morganii*, *Escherichia coli*, *Citrobacter freundii*, and *Salmonella dublin*. These strains originated from different hospitals of the G.D.R. (Wernigerode, Osterwieck and Rostock) and Czechoslovak S.S.R. (Prague) and were found in various clinical cases (Table 2). A fifth plasmid was demonstrated in a strain of *E. coli* from hospital sewage.

Each of these plasmids (pCV9, pIE420, pIE432, pIE447 and pIE491) could be transferred by conjugation to *E. coli* K12 substrains and from them to other enteric bacteria. Although the initial transfer frequencies of the plasmids from the wild strains to *E. coli* K12 CV601 ranged from 0.4 to 50 transconjugant colonies per 10⁵ donor organisms, subsequent transfer between K12 strains occurred at similar frequency (300 to 550 transconjugant colonies per 10⁵ donor organisms) for all the plasmids (Table 2). As representative examples of the observed transfer frequencies, the data for transfer from *E. coli* K12 CV601 to *E. coli* K12 J53, and from *E. coli* K12 J53 to *S. typhimurium* LT2 were chosen. In *E. coli* Hfr(H), these plasmids did not repress the F-pilus production and are therefore designated Fi⁻.

Cultures of *E. coli* K12 J53 carrying the plasmids were not lysed by donor-specific phages M13, fr, Ifm, PRR1, PRD1, PR4 and Ike in surface spot tests and did not support propagation of these phages. With one exception, *E. coli* K12 strains carrying the plasmids listed in Table 2 did not show immunity to superinfection by the reference plasmids, and transconjugant clones stably maintained both resident and incoming plasmids. However, incompatibility was observed with the plasmid RA3, the reference plasmid for the new group IncU (see Sirgel *et al.*, 1981). Moreover, immunity to superinfection could be demonstrated between the plasmids pCV9, pIE420, pIE432, pIE447 and pIE491 (as far as suitable markers for selections were available).

Transconjugant clones carrying two plasmids ('doubles') lost the resident plasmid if selection for the incoming plasmid was imposed. Some representative examples of these results are given in Table 3. The results summarized in Table 3 demonstrate that the plasmids pIE420, pIE432, pIE447, pIE491 and pCV9 belong to the new incompatibility group IncU.

The molecular sizes of the IncU plasmids ranged between 18 and 37 megadaltons as determined by agarose gel electrophoresis (Table 4). The number and sizes of the fragments

Table 3. *Determination of incompatibility by testing superinfection immunity and the stability of 'doubles' in E. coli K12 J53*

Super-infecting plasmid	Transfer frequency to J53* with the resident plasmid:						Stability of 'doubles'† with plasmid:				
	pIE420	pIE432	pIE447	pIE491	pCV9	None	pIE420	pIE432	pIE447	pIE491	pCV9
pIE420	NT	3.5	3.0	3.5	0.5	350	NT	NT	NT	NT	10
pIE432	NT	NT	0.3	NT	0.3	280	NT	NT	20	NT	20
pIE447	NT	30	NT	2.5	55	360	NT	6	NT	10	1
pIE491	NT	4.5	5.0	NT	0.5	300	NT	NT	10	NT	15
pCV9	0.5	1.0	2.5	1.5	NT	510	13	1	10	15	NT
RA3	0.8	3.5	3.0	10	8	350	10	6	10	10	10
R16	320	350	370	310	370	360	50	50	NT	50	50
pIP40a	600	580	550	620	620	600	50	50	50	50	50
R711b	350	320	380	310	300	330	50	50	NT	50	50
pIA4643	110	110	130	140	100	150	50	50	50	50	50
R726	100	150	150	170	110	100	50	50	50	50	50
pIE428	850	820	820	750	790	800	50	50	50	50	50
R64	NT	560	460	550	500	530	NT	50	50	50	50
R391	550	520	570	510	NT	580	50	50	50	50	NT
R387	120	120	100	150	130	160	50	50	50	50	50
pIE373	380	360	350	300	300	350	50	50	50	50	50
RN3	NT	480	450	490	500	480	NT	50	50	50	50
RP1	300	280	310	270	300	320	50	50	50	50	50
Rts1	150	120	120	100	140	120	50	50	50	50	50
R783	340	370	390	300	310	360	50	50	50	50	50
RSa	400	420	410	440	450	420	50	50	50	50	50
R6K	80	75	100	75	100	110	50	50	50	50	50

NT, Not testable, because suitable markers for selection were not available.

* The transfer frequency is expressed as the number of transconjugant colonies per 10^5 donor organisms for a 2 h mating.

† Fifty transconjugants which carried the superinfecting plasmid were tested for the presence of the resident plasmid. The results show the number of clones which maintained both plasmids.

Table 4. *Molecular sizes and restriction patterns of the plasmids*

Plasmid	Size (Md)*	Sizes of restriction fragments (Md)†		
		<i>EcoRI</i>	<i>BamHI</i>	<i>PstI</i>
pCV9	25	—	(25)	(14.1), 6.4, 1.25, 1.20, 1.07, 1.00
pIE420	26	12.0, 5.4, 3.6, 3.5, 2.9, 1.15	(15.0), 3.4, 2.1, 2.1, 1.40	(19.7), 4.9, 1.40
pIE432	28	10.9, 7.0, 4.9, 2.8, 2.0, 1.50, 1.20	(28)	(28)
pIE447	18	(18)	(18)	—
pIE491	37	(15.5), 11.0, 7.4, 1.75, 1.40	(26.7), 4.7, 3.0, 1.30, 1.25	(22.2), 6.2, 2.4, 1.90, 1.80, 1.25, 1.20
RA3	29	12.7, 8.5, 3.4, 2.3, 1.70, 1.35	(23.5), 5.5	(22.7), 1.80, 1.70, 1.60

—, No sensitive sites identified.

* Molecular size of covalently closed circular (CCC) DNA in megadaltons (Md), determined by agarose gel electrophoresis, relative to CCC DNA of reference plasmids (Meyers *et al.*, 1976) in the range 1.8, 5.5, 7.4, 21.5, 26.0, 47.0, 60.0 Md (Tschäpe & Tietze, 1980).

† For molecular size standards, phage λ CI857 DNA digested with *EcoRI* or *HindIII* was used, which gives fragments of 13.74, 4.74, 3.73, 3.48, 3.02 and 2.13 (Thomas & Davies, 1975), and 15.6, 6.29, 4.30, 2.85, 1.46, 1.19 and 0.40 (Gottesman & Adhya, 1977), respectively. Values in parentheses were estimated indirectly by calculations based on the molecular size of CCC DNA.

generated by *EcoRI*, *BamHI* and *PstI* differed considerably and common restriction patterns could not be observed (Table 4).

The plasmids pIE420, pIE432 and pIE491 share some drug resistance markers, but do not show any similarity in their restriction fragment pattern. The plasmids pCV9 and pIE447

have single cleavage sites for *Bam*HI and either one or no *Eco*RI cleavage site, but they differ completely in their *Pst*I cleavage pattern. Therefore a simple derivation of the plasmids from one another, or from a common ancestor, is not supported by the evidence of their molecular sizes, restriction patterns and resistance determinants. The genetic and molecular results presented in this paper support the assumption that the five plasmids characterized here are distinct but related members of the new plasmid group IncU. However, this has to be demonstrated by analysing their polynucleotide sequence relationships.

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