Genetic and Molecular Characterization of R Plasmids Incompatible with R387 (IncK)

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More than 70 conjugative R plasmids have been isolated from wild-type strains originating mainly in South-East Europe and identified as incompatible with the reference plasmid R387 of the incompatibility group K. These plasmids, governing different resistance patterns, have been characterized as P1 cotransducible species of between 50 and 60 megadaltons. In contrast to the genetic similarity between all these plasmids and the reference type R387, their DNA revealed different digestion patterns after *Eco*RI treatment, although a number of common fragments could be identified. The molecular and genetic properties of these plasmid species demonstrate a phylogenetic relatedness.

INTRODUCTION

Conjugative and non-conjugative plasmids need to be classified for epidemiological purposes. In the past few years a number of unrelated plasmid groups have been established based on their distinct genetic and molecular properties (Datta, 1975, 1979). Such groups comprise plasmids that have been shown to be mutually related, independent of their origin and the bacterial cell functions for which they code.

Using incompatibility and pili-specificity tests, molecular size determination, hybridization techniques and endonucleolytic cleavage analysis some of the plasmid groups such as FI, FII, C, P and H have been well-characterized, whereas others have not (Chabbert *et al.*, 1972; Datta & Hedges, 1973; Grindley *et al.*, 1973; Sharp *et al.*, 1973; Coetzee *et al.*, 1972; Hedges, 1974; Grinsted *et al.*, 1977; Burkardt *et al.*, 1978; Roussel & Chabbert, 1978; for review, see Datta, 1979). A number of the less well-characterized plasmid groups are represented only by a single or a few members (e.g. J, K) and investigations of plasmids from wild-type strains should provide additional members of these groups and thus enable comparative studies of the group-specific properties. Such investigations have been undertaken during the past few years and more than 70 R plasmids have been isolated from *Shigella flexneri*, *Shigella sonnei* and *Escherichia coli* strains that are incompatible with R387, the reference plasmid and only representative of the K group (Hedges & Datta, 1971).

In this paper we describe 10 plasmids representative of the many variants isolated. The results presented demonstrate that the plasmids, including the reference plasmid R387, share many genetic and molecular properties.

METHODS

Strains, plasmids and phages. Standard strains, plasmids and phages used are listed in Table 1. The plasmids studied, all incompatible with R387, were chosen to represent the different types characterized among more than 70 such plasmids isolated from wild-type strains originating in different countries and collected between 1975 and 1977. Details of these 10 plasmids and three deletion mutants are listed in Table 2.

Conjugation and transduction. The conjugation procedure was that described by Tschäpe (1973) and transduction using phage P1kc was done as described by Lennox (1955).

Table 1. *Strains, plasmids and phages*

Designation	Relevant characters*	Source or reference
<i>E. coli</i>		
W677	<i>thi thr leu lac</i> (λ)	Clowes & Hayes (1968)
J53	<i>met pro</i> (λ)	Bachmann (1972)
HfrH	<i>thi</i> , F^+	Clowes & Hayes (1968)
Plasmids		
R387	IncK, Fi^- , CmSm	Hedges & Datta (1971)
R40a	IncC, Fi^- , ApKmSu	Datta (1975)
R446b	IncM, Fi^- , TcSm	Datta (1975)
R64-11	IncI, Dps(lfm), TcSm	Datta & Hedges (1973)
RN3	IncN, Dps(Ike, PRD1), TcSmSu	Datta (1975)
RP1	IncP, Dps(PRR1), TcKmAp	Grinsted <i>et al.</i> (1977)
F'tet	IncFI, Fi^- , Dps(M13, fr), Tc	Tschäpe (1973)
R100-1drd	IncFII, Fi^- , CmSmSuTc, 60 Md	P. M. Bennett
R1-19K	IncFII, Fi^- , Km, 47 Md	P. M. Bennett
R6K	IncX, Fi^- , SmAp, 26 Md	Kontomichalou <i>et al.</i> (1970)
R388	IncW, Dps(PRD1), TpSu, 21.5 Md	Datta & Hedges (1972)
RSF2124	ColEI :: Ap, 7.4 Md	So <i>et al.</i> (1975)
RSF1010	SmSu, 5.5 Md	Guerry <i>et al.</i> (1974)
pMB8	Col-imm, 1.8 Md	Meyers <i>et al.</i> (1976)
Phages†		
M13	F-specific	C. Höhne
fr	F-specific	M. Hoffmann-Berling
Ifm	I-specific, plaque mutant of If2	Tschäpe (1975)
Ike	N-specific	Khatoon <i>et al.</i> (1972)
PRR1	P-specific	Olsen & Thomas (1973)
PRD1	N-, W- and P-specific	Olsen <i>et al.</i> (1974)

* Abbreviations according to Bachmann (1972) and Datta (1979). Cm, Chloramphenicol resistance; Sm, streptomycin resistance; Ap, ampicillin resistance; Km, kanamycin resistance; Tc, tetracycline resistance; Su, sulphonamide resistance; Tp, trimethoprim resistance; Fi, fertility inhibition; Inc, incompatibility; Dps, donor phage specificity; Md, megadalton.

† Phage suspensions contained 10^6 plaque-forming units ml⁻¹.

Fi character. Fertility inhibition by the plasmids under investigation was tested in HfrH. The plasmids were transferred by conjugation to *E. coli* HfrH and the ability of these R^+ transconjugants to produce F-pili was tested using the F-pili-dependent phage M13. At least 20 colonies per R^+ HfrH strain were shown to be either sensitive (Fi^-) or resistant (Fi^+).

Donor phage specificity. *Escherichia coli* J53 strains carrying the plasmids under investigation were tested for their sensitivity to the plasmid-specific phages M13, fr, Ifm, PRR1, PRD1 and Ike (Table 1) either by spotting the phages on to a lawn of R^+ J53 or by testing their ability to propagate the plasmid-specific phages (Lawn *et al.*, 1967). As donor phage indicator strains we used *E. coli* J53 carrying the plasmids F'tet, R64-11, R388, RP1 and RN3, respectively (Table 1).

Incompatibility (Inc). Incompatibility was tested according to Coetzee *et al.* (1972) with the following modification. The plasmids under investigation were transferred by conjugation from *E. coli* W677 to *E. coli* J53 carrying the reference plasmids of the appropriate incompatibility groups (see Datta, 1975, 1979), or the reference plasmids were transferred to J53 clones carrying the plasmids under investigation. Fifty clones from each cross were tested for the presence of plasmids by replica-plating on to selective media after several subcultures in the presence or absence of a selective drug. As far as possible all plasmids under investigation were tested against one another.

DNA isolation and gel electrophoresis. Covalent closed circular (CCC) DNA of the plasmids was isolated by Triton X-100 extraction according to Thompson *et al.* (1974) and Grinsted (unpublished results) and by sodium dodecyl sulphate-NaCl precipitation as described by Meyers *et al.* (1976). A sample (50 μ l) of ethanol-precipitated and resuspended DNA was cleaved by *Eco*RI (Zimet, Jena, G.D.R.) as described by Barth & Datta (1977) and was analysed together with an uncleaved sample (50 μ l) in a vertical slab gel (0.3 $\times$ 8 $\times$ 15 cm) using 0.7 % (w/v) agarose (Marine Colloids, U.S.A.). Electrophoresis was carried out at 30 mA for about 3 h and then the gel was stained with ethidium bromide (0.5 μ g ml⁻¹) for 30 min. The DNA bands could be observed under short-wave u.v. light using a UG5 filter (VEB Schott, Jena, G.D.R.).

Table 2. *Origin and resistance pattern of the representative plasmids*

Plasmid	Derivation or bacterial source	Country	Resistance pattern* in <i>E. coli</i> K12
pIE312	<i>Shigella sonnei</i>	Bulgaria	TcCmSmSuApKm
pIE3121	Deletion mutant of pIE312		Tc
pIE3122	Deletion mutant of pIE312		TcCm
pIE3123	Deletion mutant of pIE312		CmSmSuApKm
pIE313	<i>Shigella sonnei</i>	U.S.S.R.	TcCm
pIE316	<i>E. coli</i>	G.D.R.	Tc
pIE347	<i>E. coli</i>	Bulgaria	TcCmAp
pIE348	<i>Shigella sonnei</i>	G.D.R.	TcCm
pIE363	<i>Shigella flexneri</i>	Bulgaria†	TcCmSmSuAp
pIE378	<i>Shigella flexneri</i>	Bulgaria	Tc
pIE379	<i>Shigella sonnei</i>	U.S.S.R.	TcSu
pIE380	<i>Shigella flexneri</i>	Bulgaria	TcCmAp
pIE381	<i>Shigella sonnei</i>	U.S.S.R.	TcCmSmSu
R387‡	<i>Shigella flexneri</i>	Roumania	CmSm

* For abbreviations see Table 1.

† *Shigella flexneri* (serotype 2a, phage type 15A) strains with identical plasmids have been isolated from cases of dysentery in G.D.R.

‡ See Table 1.

Molecular weight determination. Molecular weights were determined assuming the relationship of DNA size to mobility established by Meyers *et al.* (1976). Plasmids R100-1drd, R1-19K, R6K, R388, RSF2124, RSF1010 and pMB8 were used as reference standards. The molecular weight standard for restriction fragments (*Eco*RI) was phage λ CI/857 DNA which gives fragments of 13.74, 4.74, 3.73, 3.48, 3.02 and 2.13 megadaltons (Md) (J. Grinstead, personal communication). Results were averaged from at least three independent experiments.

RESULTS

Genetic characterization

The 10 selected plasmids (and the three deletion mutants derived from pIE312 after Plkc transduction) are representative of a group of more than 70 plasmids isolated between 1975 and 1977. They seem to be particularly distributed among *Shigella* strains originating in Bulgaria, U.S.S.R. and, recently, in G.D.R. (Table 2). All are cotransducible by bacteriophage Plkc which means that the majority of extracellular plasmids are genetically and molecularly identical to the donor plasmids. They can be transferred by conjugation to different members of the enteric bacteria, e.g. *Salmonella*, *Klebsiella*, *Enterobacter*, *Retterella* and *Providencia*. In addition, all the plasmids were Fi^- , since all HfrH clones carrying them remained sensitive to the F-specific phages M13 and fr. Additionally, the plasmid-harboring *E. coli* J53 strains were insensitive to all plasmid-dependent phages (donor specific phages) such as M13, fr, Ike, Ifm, PRD1 and PRR1 either by the direct spotting test, or by propagation experiments.

In testing their incompatibility properties, all the plasmids showed a considerably reduced frequency of transfer (superinfection immunity) to *E. coli* J53 carrying R387 (Table 3), the reference plasmid of incompatibility group K, compared with transfer to plasmid-free *E. coli* J53 or to other J53 clones carrying reference plasmids of other incompatibility groups. A similar superinfection immunity was found among all plasmids tested against each other; some representative examples are given in Table 4. In Tables 3 and 4 results for R40a (IncC) and R446b (IncM) are included as representative examples that showed no superinfection immunity. In addition to this reduced frequency of transfer to *E. coli* J53 carrying the plasmid R387, a high instability of the 'doubles' between the reference type R387 and the plasmids under investigation was found (Table 3). Such an instability could not be detected among 'doubles' constructed between other reference plasmid species and the

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

Results given are from one representative experiment.

Super-infecting plasmid	10 ² × No. of superinfected colonies of J53 carrying the resident plasmid:*				Stability of 'doubles' with plasmid:*†		
	R387	R40a	R446b	None	R387	R40a	R446b
pIE312	4	800	850	380	NT	NT	NT
pIE3121	4	780	700	490	50/10	50/50	NT
pIE3122	3	860	620	590	50/5	50/50	50/50
pIE3123	2	400	450	350	NT	50/50	50/50
pIE313	5	550	570	430	50/5	50/50	50/50
pIE316	5	400	NT	350	50/8	50/50	NT
pIE347	1	330	680	300	50/12	50/50	50/50
pIE348	1	380	420	350	50/10	50/50	50/50
pIE363	4	250	250	320	NT	50/50	50/50
pIE378	8	380	NT	430	50/7	50/50	NT
pIE379	2	280	220	250	50/10	50/49	50/50
pIE380	3	450	400	440	50/8	50/50	50/50
pIE381	1	370	350	290	NT	50/50	NT
R387	NT	420	480	390	NT	50/50	50/50
R40a	560	NT	510	320	50/50	NT	50/50
R446b	830	760	NT	860	50/50	50/50	NT

NT, Not testable.

* Plasmids R40a (IncC reference) and R446b (IncM reference) are included as representative examples of all reference plasmids without any superinfection immunity and complete stability of 'doubles'.

† Fifty colonies were replica-plated twice to select for the superinfecting plasmid and then tested for the presence of the resident plasmid. The results indicate the ratio superinfecting plasmid/resident plasmid.

Table 4. *Incompatibility between the deletion mutants pIE3121 and pIE3123 and the plasmids investigated*

Super-infecting plasmid	10 ² × No. of superinfected colonies of J53 carrying the resident plasmid:			Stability of 'doubles' with plasmid:	
	pIE3121	pIE3123	None	pIE3121	pIE3123
pIE312	2	3	450	NT	NT
pIE3121	NT	1	280	NT	50/10
pIE3122	3	5	420	NT	50/12
pIE3123	2	NT	390	50/8	NT
pIE313	2	2	450	NT	50/5
pIE316	NT	3	330	NT	50/10
pIE347	1	1	360	NT	50/8
pIE348	2	2	430	NT	50/8
pIE363	4	4	600	NT	50/12
pIE378	NT	3	350	NT	50/5
pIE379	3	4	370	50/13	50/12
pIE380	3	2	460	NT	50/12
pIE381	1	2	410	NT	50/9
R387	2	NT	380	50/10	NT
R40a	350	310	340	50/50	50/50
R446b	490	540	540	NT	50/50

NT, Not testable.

plasmids under investigation. The same instability was detected, however, among 'doubles' constructed between the various plasmids under investigation; representative results are given in Table 4. Again, results for R40a and R446b are included as representative of compatible controls (Tables 3 and 4). In summary, these results support the inclusion of the plasmids studied in the K incompatibility group.

Table 5. Molecular size and the *EcoRI* digestion fragments of *K* plasmid DNA

Plasmid	Size* (Md)	No. of <i>EcoRI</i> sites	Sizes of fragments after <i>EcoRI</i> digestion (Md)	Sum of fragment sizes
pIE312	60	10	12·0, 12·0†, 10·2, 9·2, 6·5, 5·4, 2·4, 2·2, 2·0, 1·9	63·8
pIE3121	52	9	12·0, 10·2, 8·4, 6·5, 5·4, 3·9, 2·4, 2·0, 1·9	52·7
pIE3122	54	9	12·0, 10·2, 9·2, 8·4, 6·5, 5·4, 2·4, 2·0, 1·9	58·0
pIE3123	57	7	13·7, 12·0, 10·2, 9·2, 6·5, 5·4, 2·2	59·2
pIE313	55	7	12·0, 12·0†, 11·5, 8·4, 6·5, 2·4, 2·0	54·8
pIE316	59	7	13·7, 12·0, 9·2, 8·4, 6·5, 5·4, 2·0	57·2
pIE347	60	6	12·0, 12·0†, 10·2, 9·2, 7·8, 7·0	58·2
pIE348	55	8	12·0, 11·5, 9·2, 8·4, 6·5, 5·4, 2·0, 1·9	56·9
pIE363	60	ND		
pIE378	60	7	13·7, 11·5, 9·2, 8·4, 6·5, 5·4, 4·9	59·6
pIE379	54	7	12·0, 10·2, 9·2, 8·4, 7·4, 5·4, 4·1	56·7
pIE380	60	6	12·0, 12·0†, 10·2, 9·2, 7·8, 7·0	58·2
pIE381	60	8	12·0, 12·0†, 10·2, 8·4, 6·5, 5·4, 2·4, 2·0	58·9
R387	55‡	7	13·7, 12·0, 10·2, 8·4, 6·5, 2·4, 2·0	55·2

ND, Not determined.

* Results for CCC DNA (see Methods).

† The presence of two 12 Md fragments was concluded from the broadness of the band in the gel and from digestion studies of deletion mutants.

‡ According to Barth *et al.* (1978), the size is 53·0 Md.

Molecular characterization

DNA from *E. coli* J53 carrying the plasmids under investigation was extracted and characterized with respect to molecular weight and to the numbers and sizes of the *EcoRI* cleavage fragments (Table 5). A single DNA species was detected in each R⁺ J53 clone, the plasmids ranging in size from 52 to 60 Md. The molecular sizes of the deletion mutants pIE3121 (52 Md), pIE3122 (54 Md) and pIE3123 (57 Md) indicate that a region of about 11 Md comprises the resistance genes and that a region of about 40 to 50 Md is occupied by other genes, such as those for transfer, replication and incompatibility. If such a large sequence is also necessary for the transfer, replication, incompatibility and maintenance functions of the other *K* plasmids, and if the other *K* plasmids identified in this paper are phylogenetically inter-related, a considerable degree of homology would be expected. We therefore attempted to determine the number of *EcoRI* cleavage sites and the sizes of the fragments generated. As indicated in Table 5, the number of *EcoRI*-sensitive sites ranged between 6 and 10 and the size of the fragments from 13·7 to 1·9 Md. The precise number of the *EcoRI*-sensitive sites, however, remains uncertain since we were unable in every case to identify unambiguously the number and sizes of the fragments smaller than 3 Md. However, the sums of the sizes of fragments are approximately the same as the sizes determined for the CCC DNA molecules (Table 5).

A number of the DNA fragments are identical, within the limits of our methods, in the various plasmids under investigation (Table 5). These are the fragment of sizes 12 Md (identified in 12/13), 10·2 Md (8/13), 9·2 Md (9/13), 8·4 Md (9/13), 6·5 Md (10/13) and 5·4 Md (9/13). It is interesting to note that the plasmids pIE313 and pIE348 seem to be identical with respect to their molecular size, resistance pattern and genetic properties but differ in their *EcoRI* restriction pattern. Both plasmids originated in *Sh. sonnei* (Table 2) but in different countries.

The precise molecular size characterization of the different fragments and their presumed homology demonstrating a phylogenetic relatedness is under investigation.

DISCUSSION

From *Shigella sonnei*, *Sh. flexneri*, and *E. coli* wild-type strains originating mainly in South-East Europe more than 70 conjugative R plasmids have been isolated and identified as incompatible with one another and with R387, the reference plasmid of the incompatibility group K. These plasmids share many genetic and molecular properties and seem to constitute a closely related group. They are cotransducible by bacteriophage P1kc, are Fi^- , and do not show sensitivity to the plasmid-specific phages M13, fr, Ifm, Ike, PRR1 and PRD1 in *E. coli* K12 substrains. The sizes of these plasmids are between 50 and 60 Md and distinctive fragments of their DNA could be identified after digestion with *EcoRI*.

The precise number of *EcoRI*-sensitive sites in these K plasmids could not be established because smaller fragments were difficult to detect reproducibly in the gels. It should be pointed out, however, that in summing the size of fragments the total is near to that measured for the CCC DNA molecules (Table 5).

Some of the fragment sizes detected among the digested K plasmid DNA were common to most of the species investigated (Table 5). It is assumed that these fragments represent sequences coding for incompatibility, transfer or replication functions. This assumption is supported by the results obtained with the pIE312 deletion mutants (pIE3121, pIE3122 and pIE3123). The K-specific functions, e.g. incompatibility, replication and transfer, remain unaffected in these mutants and also the digestion pattern of their DNA remains almost unchanged with respect to the common fragments.

The molecular and genetic properties of the K plasmids described in this paper demonstrate a phylogenetic relatedness in spite of their different geographical and bacterial origin.

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