Taxonomy and Epidemiology of Gram-negative Bacterial Plasmids Studied by DNA–DNA Filter Hybridization in Formamide

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Phylogenetic and epidemiological relatedness among transferable plasmids belonging to the IncC, IncM and IncH incompatibility groups has been studied by DNA–DNA filter hybridization. Hybridization was carried out on nitrocellulose microfilters, at low temperature, in formamide and under paraffin oil. The degree of hybridization among plasmids belonging to the IncC and IncM groups supported the conclusions drawn from genetic classification. Studies on relatedness among plasmids belonging to the IncH group allowed their classification into three phylogenetic sub-groups. Comparison of DNA sequences of three plasmids sharing the same genetic properties and isolated from different bacterial species suggested an epidemiological spread of the same plasmid.

INTRODUCTION

Plasmids responsible for the transfer of drug resistance and for metabolic markers have been divided into several classes on the basis of their ability to coexist stably with plasmids of other groups (Datta & Hedges, 1971; Hedges & Datta, 1971; Chabbert *et al.*, 1972; Coetzee, Datta & Hedges, 1972; Grindley, Grindley & Anderson, 1972; Le Minor *et al.*, 1976; Scavizzi, 1973). Molecular hybridization of nucleic acids has been used to establish phylogenetic relatedness between plasmid deoxyribonucleic acids, and in particular to determine whether any correlation exists with the classification obtained from genetic tests. Guerry & Falkow (1971), Falkow (1975), Grindley, Humphreys & Anderson (1973) and Smith *et al.* (1973) found that plasmids of the IncFI, IncFII, IncI1, IncI2, IncB and IncW groups showed considerable homology within their groups and that there was a much lower degree of homology between plasmids of different groups. An exception was observed within IncH plasmids by Anderson, Humphreys & Willshaw (1975) and Grindley *et al.* (1973).

In the present study we wished to determine whether it was possible to extend these findings to two other groups of transferable plasmids which are frequently encountered, namely IncC (or Inc6) and IncM (or Inc7). We also studied the phylogenetic relatedness among isolated IncH plasmids harbouring resistance and metabolic markers. Secondly, we wished to survey the stability of some transferable plasmids belonging to the IncC group, and their epidemiological spread through different Enterobacteriaceae.

These molecular relatedness studies of IncC, IncM and IncH plasmids were carried out by adapting the hybridization technique recently described for RNA–DNA hybridization by Kourilsky *et al.* (1974), in which hybridization was performed on nitrocellulose microfilters, at low temperature, in formamide and under paraffin oil.

METHODS

Plasmids and bacterial strains. The plasmids studied are listed in Table 1, together with their host strain, incompatibility group, antibiotic resistance and metabolic markers, transfer ability and the origin and year of isolation of the parental strain.

Strain BM21 (14R525 κ -12 *Escherichia coli*, prototroph, nalidixic acid resistant), obtained from E. S. Anderson, was used as the bacterial host for all plasmids except those of group IncH. Strain BM03 (c600 κ -12 [F⁻] *thr leu thi lac*, rifampicin resistant) was used as the bacterial host for the group IncH plasmids. Neither strain harboured plasmids [R⁻].

Media and buffer solutions. For the preparation of labelled plasmid DNA, M9 liquid medium (Clowes & Hayes, 1968) was used with glucose as sole carbon source; total unlabelled DNA was obtained from strains grown in Brain Heart Infusion (BHI, Difco). Buffer solutions were saline/EDTA (0.5 M-NaCl, 0.01 M-EDTA, pH 8.0), and 20 $\times$ SSC (3 M-NaCl, 0.3 M tri-sodium citrate) filtered through a Millipore membrane.

Materials. Lysozyme was from Sigma; sodium lauryl sulphate (SLS) from Serlabo; RNAase (bovine pancreas) from Calbiochem; formamide ('proanalysis' grade) and proteinase K from Merck; phenol from Carlo Erba. Nitrocellulose membranes were Sartorius SM11306, 25 mm diam. [*methyl*-³H]Thymidine, specific activity 42 Ci mmol⁻¹, was purchased from the Commissariat à l'Energie Atomique, France.

Isolation of labelled plasmid DNA by dye-buoyant density gradient centrifugation. Plasmid DNA was labelled by growing bacteria in M9 with 400 μ Ci of [³H]thymidine per 20 ml culture to a density of 5×10^8 organisms ml⁻¹. Plasmid DNA was then extracted as described by Ingram, Richmond & Sykes (1973) except that an additional centrifugation to equilibrium in ethidium bromide-caesium chloride was used to eliminate any chromosomal DNA. Ethidium bromide was extracted with isopropanol saturated by Tris/EDTA (10 mM-Tris, 1 mM-EDTA, pH 7.5) and the DNA solution was dialysed against at least 400 vol. Tris/EDTA. The DNA was sonicated for five periods of 30 s at 4 °C in a M.S.E. sonic oscillator at maximum power and concentrated by ethanol precipitation at -20 °C. This preparation gave plasmid DNA with specific activities ranging from 5×10^4 to 1×10^6 c.p.m. μ g⁻¹ and yielded DNA fragments estimated from electron microscopy to have molecular weights of about 6×10^5 .

Preparation of unlabelled 'total' cell DNA. Bacteria were grown at 37 °C in 10 ml BHI to a density of 5×10^8 organisms ml⁻¹. Cells were washed with saline/EDTA and the pellet was resuspended in 1 ml saline/EDTA containing 1 mg lysozyme and 0.5 mg RNAase (DNAase-free). Cells were lysed by adding 50 μ l 20% (w/v) sodium lauryl sulphate, and the lysate was treated overnight at room temperature with proteinase K (predigested for 2 h) at a final concentration of 100 μ g ml⁻¹ (Kourilsky, personal communication). DNA purification and deproteinization were carried out with two saline/EDTA/saturated phenol extractions followed by two chloroform/isoamyl alcohol (24:1, v/v) extractions. The final supernatant was precipitated overnight with 2 vol. cold 95% (v/v) ethanol at -20 °C. Pelleted DNA was resuspended in 1 ml 0.3 M-NaOH. RNA was hydrolysed and DNA was denatured after 3 h incubation at 37 °C. The denatured DNA solution was neutralized by adding 0.2 ml 2 M-NaH₂PO₄ and quenched in ice; 20 $\times$ SSC and water were then added to achieve a final concentration of 2 μ g ml⁻¹ in 4 $\times$ SSC. The diphenylamine procedure (Shatkin, 1969) was used for the titration of denatured DNA solution. This solution was immediately used for the preparation of DNA filters.

DNA-DNA hybridization

The procedure used was an adaptation of the techniques described by Kourilsky, Leidner & Tremblay (1971) and by Kourilsky *et al.* (1974). For each hybridization experiment four kinds of filters were prepared: filters loaded with chromosomal DNA without plasmid DNA (called R⁻ filters); filters with chromosomal DNA and homologous plasmid DNA (R⁺_{hom} filters); filters with chromosomal DNA and heterologous plasmid DNA (R⁺_{het} filters); and filters without any DNA (blank filters).

Preparation of DNA filters. The filters were presoaked in 4 $\times$ SSC for at least 10 min. Alkali-denatured DNA was fixed to nitrocellulose filters (0 to 100 μ g per 25 mm diam. filter) by slow filtration through the membrane (5 ml min⁻¹; Denhardt, 1966). DNA and blank filters were washed with 4 $\times$ SSC pH 7.0 followed by 0.5 $\times$ SSC pH 7.0, and then placed on blotting paper and dried. They were desiccated overnight over silica gel at room temperature, then placed in plastic Petri dishes in a vacuum desiccator for 2 h at 80 °C (Gillespie & Spiegelman, 1965). Dry filters could be stored in a vacuum desiccator at 4 °C for 1 month.

Preparation of the annealing labelled mixture. The labelled plasmid DNA, treated as described above, was resuspended in 1 vol. 0.1 $\times$ SSC, denatured by 0.1 vol. 1 M-NaOH for 15 min at 4 °C and then neutralized with 0.1 vol. 2 M-NaH₂PO₄; 20 $\times$ SSC and formamide were added to give final concentrations of 2 $\times$ SSC and 50% (v/v) formamide. The total volume was calculated to obtain 300 to 500 c.p.m. per 4 μ l in the final annealing mixture.

Table 1. *Plasmids and their origins*

Strain*	Plasmid no.*	Incom-patibility group	Phenotypic characters†	Origin	Reference
BM273	pIP55	IncC	Ap Gm Tm Cm Su Fi ⁻	<i>Klebsiella pneumoniae</i> 35, Paris 1969	Witchitz & Chabbert, 1971
BM299	pIP64	IncC	Ap Gm Tm Cm Su Fi ⁻	<i>Pseudomonas aeruginosa</i> , Paris 1969	Witchitz & Chabbert, 1972
BM4001	pIP149	IncC	Ap Gm Tm Cm Su Fi ⁻	<i>K. pneumoniae</i> , Paris 1972	Witchitz, 1977
BM4002	pIP150	IncC	Sm Gm Cm Su Fi ⁻	<i>K. pneumoniae</i> , Paris 1972	Witchitz, 1977
BM275	pIP16a	IncC	Ap Km Su Fi ⁻	<i>Providencia stuartii</i> , Paris 1966	Chabbert <i>et al.</i> , 1972
BM300	pIP153	IncC	Ap Sm Km Cm Tc Su Fi ⁻	<i>Vibrio cholerae</i> El tor, Algeria 1972	Rahal, Gerbaud & Chabbert, 1973
BM4004	pIP151	IncM	Sm Gm Tc Su Fi ⁻	<i>K. pneumoniae</i> , Paris 1972	Witchitz, 1977
BM279	pIP135	IncM	Sm Gm Tc Su Fi ⁻	<i>Enterobacter cloacae</i> , Paris 1971	Witchitz & Gerbaud, 1972
BM276	pIP69	IncM	Ap Km Tc Fi ⁻	<i>Salmonella paratyphi</i> B, Créteil 1969	Chabbert <i>et al.</i> , 1972
BM4005	pIP56a	‡	Ap Gm Tm Cm Su Fi ⁺	<i>Escherichia coli</i> , Paris 1969	Witchitz & Chabbert, 1972
BM251	pIP162	IncFI	Ap Sm Cm Tc Su Fi ⁺	<i>Salmonella typhi</i> , France 1973	Chabbert & Gerbaud, 1974
BM301	R1-19K ⁻	IncFII	Ap Sm Cm Su Fi ⁺	<i>S. paratyphi</i> B, England 1964	Grindley <i>et al.</i> , 1973
BM271	RSa	IncW	Sm Km Cm Su Fi ⁻	<i>Shigella</i> , Japan 1968	Hedges & Datta, 1971
BM269	pIP113	IncN	Tc Fi ⁻	<i>Salmonella panama</i> , Paris 1968	Chabbert <i>et al.</i> , 1972
BM261	pIP112	IncI1	Km Cib Fi ⁻	<i>S. panama</i> , Paris 1968	Chabbert <i>et al.</i> , 1972
BM436	TP123(T ⁻)	IncH1	Sm Cm Su Fi ⁻	<i>S. typhi</i> , Mexico 1972	Anderson <i>et al.</i> , 1975
BM439	TP116	IncH2	Sm Cm Su Fi ⁻	<i>S. typhi</i> , Spain 1969	Anderson <i>et al.</i> , 1975
BM4006	pIP165	IncH	Sm Cm Tc Su Fi ⁻	<i>S. typhi</i> , Mexico 1973	Chabbert & Gerbaud, 1974
BM4007	pIP166	IncH	Sm Cm Tc Su Fi ⁻	<i>S. typhi</i> , Vietnam 1973	Chabbert & Gerbaud, 1974
BM437	pIP522§	IncH	Sm Cm Su Fi ⁻	<i>S. typhi</i> , Vietnam 1973	Chabbert & Gerbaud, 1974
BM440	MIP233	IncH	Sac Fi ⁻	<i>Salmonella ohio</i> , London 1972	Le Minor <i>et al.</i> , 1976
BM4008	MIP235	IncH	Lac Sm Cm Su Fi ⁻	<i>Salmonella oranienburg</i> , Sao Paulo 1973	Buissiere, Coynault & Le Minor, 1977

* The nomenclature used for strains and plasmids follows Novick *et al.* (1976). Plasmid-containing strains BM273 to BM261 were derived from strain BM21 and strains BM436 to BM4008 from strain BM03.

† Symbols for phenotypic characters. Antibiotic resistances: Ap, ampicillin; Cm, chloramphenicol; Sm, streptomycin; Km, kanamycin; Gm, gentamicin; Tm, tobramycin; Tc, tetracycline; Su, sulphonamide. Fi⁺, inhibition of conjugational transfer mediated by the plasmid; Fi⁻, lack of inhibition of conjugational transfer. Lac, Sac, ability to metabolize lactose, saccharose. Cib, production of colicin Ib.

‡ Compatible with all reference plasmids.

§ pIP522 was pIP166/1 (Chabbert & Gerbaud, 1974), a segregant of pIP166.

DNA-DNA hybridization in paraffin. Microfilters (5 mm diam.) were punched out of the large loaded filters (eight microfilters per 25 mm diam. filter). After numbering the microfilters, 4 µl of the reaction mixture was placed on each one and they were immediately coated with paraffin oil and incubated overnight in paraffin oil at 37 °C.

Washing procedure. After incubation the filters were taken out of the oil bath and paraffin oil was removed by wiping them with blotting paper. They were washed together twice (45 and 10 min) in a 0.1 % SLS/50 % formamide/2 × SSC pH 7.0 bath with magnetic stirring (about 100 microfilters in a 500 ml beaker) to completely remove the paraffin oil, then washed twice in 50 % formamide/2 × SSC pH 7.0, and finally rapidly rinsed in 2 × SSC pH 7.0 before drying on blotting paper. The amount of ³H-labelled DNA bound to each microfilter was determined by a 10 min count in an Intertechnique liquid scintillation spectrometer.

RESULTS

Methodology

Hybridization conditions and controls. Non-specific binding of labelled plasmid DNA to filters was tested for each hybridization by using blank filters. In $2 \times \text{SSC}/50\%$ formamide, counts were not significantly different from the background of the scintillation spectrometer.

Leaking of DNA was tested by using labelled bacterial DNA bound to filters, incubated and washed as usual. Up to 95% was retained when solutions were adjusted to pH 7.0 (± 0.5) throughout the entire process; this percentage was obtained regardless of the amount of denatured DNA bound from 1 to 100 μg .

Figure 1 shows a DNA saturation curve for hybridization of a constant amount of labelled plasmid DNA with increasing amounts of homologous unlabelled DNA. From this, we determined the amount of total denatured DNA which had to be used in order to provide an excess of binding sites. Nitrocellulose filters (25 mm diam.) were loaded with 60 μg of total denatured DNA and reassociation was performed with 0.005 to 0.01 μg of labelled DNA in 4 μl of annealing mixture (at 37 °C, in $2 \times \text{SSC}/50\%$ formamide for 18 h). The saturation curves obtained (Fig. 1) indicated that hybridization was nearly exhaustive. Under these conditions, the efficiency of hybridization ranged from 85 to 95%. The binding of labelled plasmid DNA to R^- filters ranged from 5 to 15% of the total binding to R^+_{hom} filters when the additional purification of plasmid supercoiled DNA was performed.

Measurement of plasmid homology. The values reported in Tables 2, 3 and 4 indicate the degree of reassociation, α_M , in $2 \times \text{SSC}/50\%$ formamide between homologous labelled plasmid DNA and unlabelled filter-bound plasmid DNA. α_1 (percentage of homology) is defined as:

$$\alpha_1 = \frac{(\text{counts bound to } \text{R}^+_{\text{het}} \text{ filters}) - (\text{counts bound to } \text{R}^- \text{ filters})}{(\text{counts bound to } \text{R}^+_{\text{hom}} \text{ filters}) - (\text{counts bound to } \text{R}^- \text{ filters})} \times 100$$

The reported values of α_M ($\pm$ standard deviation) are the means of at least eight α_1 determinations obtained by hybridizing 4 μl from the same annealing mixture with eight (or more) filters of the three kinds used (R^+_{hom} , R^+_{het} and R^- filters).

Taxonomy of bacterial plasmids

Homology among plasmids from different incompatibility groups. Table 2 shows the results of hybridization between plasmids belonging to the same incompatibility group (IncC or IncM group) and between plasmids belonging to different incompatibility groups (IncC or IncM plasmids with IncW, IncN, IncI1, IncFI or IncFII plasmids). The percentage of reassociation among plasmids belonging to the same group ranged from 66 to 100% whereas it was generally less than 15% between plasmids of different groups.

Homology among plasmids of the IncH group. Transferable resistance plasmids pIP165, pIP166 and pIP522 and metabolic plasmid MIP235, all belonging to the incompatibility group IncH, showed a very high percentage of reassociation with either TP123 or TP116 (reference plasmids from E. S. Anderson) but never with both (Table 3). These plasmids could therefore be classified into two subgroups: IncH1 and IncH2 (Table 3). Plasmids from group IncH1 (pIP165, pIP166, pIP522) showed a very close homology with TP123 and a very poor one with TP116 and MIP235. A notable exception was plasmid MIP233 which showed very poor homology with both IncH1 and IncH2 plasmids despite a strong incompatibility with them (Le Minor *et al.*, 1976).

Study of pIP56a, a plasmid compatible with all reference plasmid groups. pIP56a is a naturally occurring plasmid compatible with all reference plasmids (Witchitz & Chabbert, 1972; Witchitz, 1977), showing a positive fertility inhibition and the same multiresistance sequence ApGmTmCmSu as the series of IncC plasmids described below. pIP56a plasmid

Fig. 1. Saturation curve of pIP55. Nitrocellulose filters (25 mm diam.) were loaded with increasing amounts of denatured total DNA. Microfilters (5 mm diam.) were punched and incubated with about 0.005 μ g of previously sonicated and denatured 3 H-labelled pIP55 plasmid DNA. Re-association was performed in $2\times$ SSC/50% formamide at 37 $^{\circ}$ C for 18 h, under paraffin oil. ●—●, reassociation between pIP55 and total DNA of strain BM273(pIP55); ●---●, reassociation between pIP55 and total DNA of strain BM21(R $^{-}$). Bars represent 95% sampling error confidence limit. In these experiments, a plateau was obtained with an efficiency of 95%.

Table 2. Homology among plasmids from different incompatibility groups

Nitrocellulose filters (25 mm diam.) were loaded with 60 μ g of total denatured DNA and re-association was performed as indicated in Fig. 1. The results indicate the percentage of reassociation of 3 H-labelled plasmid DNA with unlabelled plasmid DNA relative to the reassociation with both homologous plasmid DNA and with *E. coli* chromosomal DNA. These values, α_1 ($\pm$ standard deviation), were the mean of at least eight α_1 determinations where α_1 is defined as:

$$\alpha_1 = \frac{(\text{counts bound to } R_{\text{het}}^{+} \text{ filters}) - (\text{counts bound to } R^{-} \text{ filters})}{(\text{counts bound to } R_{\text{hom}}^{+} \text{ filters}) - (\text{counts bound to } R^{-} \text{ filters})} \times 100$$

Total unlabelled DNA from:		Percentage of reassociation with labelled plasmid DNA from:						
Incom- patibility group	Plasmid no.	pIP55	pIP150	pIP16a	pIP153	pIP151	pIP135	pIP69
IncC	pIP55	100 $\pm$ 4	89 $\pm$ 2	78 $\pm$ 2	74 $\pm$ 2	5 $\pm$ 1	—*	—
IncC	pIP150	77 $\pm$ 5	100 $\pm$ 3	79 $\pm$ 2	79 $\pm$ 2	—	—	—
IncC	pIP16a	82 $\pm$ 2	74 $\pm$ 2	100 $\pm$ 3	66 $\pm$ 1	—	—	—
IncC	pIP153	83 $\pm$ 3	76 $\pm$ 3	73 $\pm$ 1	100 $\pm$ 3	—	—	—
IncM	pIP151	10 $\pm$ 2	—	—	—	100 $\pm$ 2	100 $\pm$ 6	88 $\pm$ 2
IncM	pIP135	—	—	—	—	97 $\pm$ 3	100 $\pm$ 5	87 $\pm$ 2
IncM	pIP69	5 $\pm$ 1	—	—	—	80 $\pm$ 1	81 $\pm$ 5	100 $\pm$ 4
IncW	RSa	7 $\pm$ 3	—	—	—	5 $\pm$ 1	—	—
IncN	pIP113	0 $\pm$ 2	—	—	—	5 $\pm$ 1	—	—
IncI1	pIP112	0 $\pm$ 3	—	—	—	6 $\pm$ 1	—	—
IncFI	pIP162	10 $\pm$ 3	—	—	—	12 $\pm$ 1	—	—
IncFII	R1-19K $^{-}$	17 $\pm$ 2	—	—	—	13 $\pm$ 1	—	—

* — indicates that the experiment was not performed.

DNA showed significant hybridization both with Fi $^{+}$ plasmids and with the group IncC plasmids pIP55 and pIP150 (Table 4). Assuming the molecular weights of pIP55 and pIP56a to be 105 and 41 respectively (P. Bennett, personal communication), the pIP55–pIP56a crossed hybridization showed that the two plasmids shared common DNA sequences

Table 3. *Homology among plasmids of the IncH group*

The procedures and expression of results are the same as those described in the legend to Table 2.

Total unlabelled DNA from:			Percentage of reassociation with labelled plasmid DNA from:			
Sub-group	Plasmid no.	Phenotypic markers	TP123	TP116	MIP235	MIP233
IncH1	TP123*	Sm Cm Su	100 ± 3	14 ± 3	14 ± 4	0 ± 1
IncH1	pIP166	Sm Cm Tc Su	91 ± 3	5 ± 2	6 ± 3	1 ± 1
IncH1	pIP522	Sm Cm Su	81 ± 3	—	—	—
IncH1	pIP165	Sm Cm Tc Su	92 ± 3	5 ± 1	—	3 ± 1
IncH2	TP116*	Sm Cm Su	5 ± 1	100 ± 6	86 ± 3	5 ± 1
IncH2	MIP235	Lac Sm Cm Su	14 ± 1	93 ± 6	100 ± 3	6 ± 1
	MIP233	Sac	3 ± 2	7 ± 3	—	100 ± 5

* TP123 and TP116 are reference plasmids for H1 and H2 groups, respectively, obtained from E. S. Anderson.

Table 4. *Study of pIP56a: a plasmid compatible with all reference plasmid groups*

The procedures and expression of results are the same as those described in the legend to Table 2.

Total unlabelled DNA from:			Percentage of reassociation with labelled plasmid DNA from:	
Incompatibility group	Plasmid no.	Phenotypic markers	pIP56a	pIP55
?	pIP56a	Ap Gm Tm Cm Su Fi ⁺	100 ± 6	16 ± 1
IncC	pIP55	Ap Gm Tm Cm Su Fi ⁻	42 ± 5	100 ± 2
IncC	pIP150	Sm Gm Cm Su Fi ⁻	40 ± 4	77 ± 5
IncFI	pIP162	Ap Sm Cm Tc Su Fi ⁺	25 ± 1	10 ± 3
IncFII	R1-19K ⁻	Ap Sm Cm Su Fi ⁺	29 ± 1	17 ± 2

equivalent to 26 kilobases (as $105 \times 0.16 = 41 \times 0.42 = 17$ Mdal). By a similar calculation, DNA sequences common to pIP56a and Fi⁺ plasmids amounted to about 15 kilobases.

Epidemiological survey of bacterial plasmids

The degree of relatedness of plasmids pIP55, pIP64 and pIP149 was studied by the same hybridization technique. They were selected from a series of plasmids, described by Witchitz & Chabbert (1972), which shared the following genetic properties: (i) they belong to incompatibility group IncC; (ii) they coded for associated resistance to kanamycin (Km), gentamicin (Gm) and tobramycin (Tm) controlled by an aminoglycoside 2"-adenylyl-transferase (Witchitz, 1975); (iii) the Gm character was always associated with other characters Ap Cm Su coding, respectively, for ampicillin-carbenicillin, chloramphenicol and sulphonamide resistances.

The results of crossed hybridization between pIP55 from *Klebsiella pneumoniae* serotype 35, isolated in 1969, and pIP64 isolated 1 month later from *Pseudomonas aeruginosa* (Witchitz & Gerbaud, 1972) showed a very close homology between these two plasmids ($99 \pm 3\%$ in both crossed hybridization experiments). Percentages of reassociation observed between pIP55 or pIP64 ³H-labelled plasmid DNA and total unlabelled pIP149 DNA (from *K. pneumoniae* serotype 68, isolated 3 years later), amounted in both cases to $100 \pm 4\%$, whereas pIP149 ³H-labelled plasmid DNA showed 87 ± 1 and $88 \pm 1\%$ of reassociation, respectively, with pIP55 and pIP64. These results indicated that the whole pIP55 DNA sequence was present in pIP64 and pIP149 plasmid DNAs. The fact that a significant fraction of pIP149 was not found to be present in pIP55 and pIP64 DNAs suggested that pIP149 had acquired additional DNA sequences.

DISCUSSION

In addition to the various practical advantages which led us to choose the microfilter hybridization technique in formamide which is particularly useful and convenient to perform a large number of DNA hybridization assays, the very high efficiency of reassociation (85 to 95 %) indicated that the kinetics were optimal and that the hybridization reaction was exhaustive. Moreover the very low degree of radioactivity bound to filters loaded with chromosomal DNA [5 % when extensive purification of plasmid DNA was carried out (Fig. 1)] and the slopes obtained at the plateaus led us to conclude that the mismatching generally observed using formamide at low temperature was not too high in our conditions of hybridization.

The comparison of plasmid DNA sequences within and between incompatibility groups provided three main results.

(1) Hybridization within group IncC and IncM plasmids and reference plasmids of other groups correlate with the results obtained by Guerry & Falkow (1971), Grindley *et al.* (1973) and Falkow (1975), using other techniques. Plasmids from the same incompatibility group showed a high degree of homology, but there was little homology among plasmids from different groups. The high degree of homology among plasmids of the same group probably reflected phylogenetic relationships.

(2) The peculiarities of group IncH plasmids have already been described by Grindley *et al.* (1973) and Anderson *et al.* (1975). With the exception of MIP233, all the IncH plasmids which we studied could be classified by the molecular hybridization technique into the IncH1 or IncH2 subgroups, according to Anderson's definition. The results observed with MIP233, a plasmid strongly incompatible with all IncH plasmids (Le Minor *et al.*, 1976), might allow the characterization of a new phylogenetic sub-group IncH3. These findings reflected some diversity between the genetic structure of plasmids belonging to the IncH group.

(3) Plasmid pIP56a, carrying the ApGmTmCmSu resistance markers associated with an Fi^+ expression, was compatible with all reference plasmids. Since it had been isolated from *E. coli* in a peritoneal dialysis liquid infected with three other species of bacteria, each harbouring an IncC Fi^- ApGmTmCmSu plasmid (Witchitz & Chabbert, 1972), it might have resulted from recombination between an Fi^+ plasmid and an IncC Fi^- ApGmTmCmSu plasmid. The molecular hybridization studies showed a significant reassociation of pIP56a with both Fi^+ plasmids and group IncC plasmids, confirming the hypothesis that pIP56a was a natural composite plasmid resulting from interactions between several plasmids. However, incompatibility tests previously performed and the amount of homology observed did not allow assignment of pIP56a to either supposed parental incompatibility group (IncC, IncFI or IncFII).

A large number of exchange mechanisms described in molecular studies of plasmids (such as legitimate and illegitimate recombinations, transposition, associations and dissociations of plasmids) seem to indicate that plasmids might be considered as labile aggregates of genetic fragments. Nevertheless, genetic and physical studies of natural plasmids showed common and stable properties (incompatibility, fertility inhibition expression, transfer functions) which are generally correlated with a high degree of homology between DNA sequences. These observations allowed the classification of transferable plasmids into distinct phylogenetic entities and introduced the concept of 'plasmid species' (Falkow, 1975).

The appearance and the dissemination of the peculiar gentamicin resistance marker, always associated with ApGmTmCmSu, and the genetic properties of plasmids harbouring these resistance genes (Witchitz & Gerbaud, 1972; Witchitz, 1977), suggested an epidemiological spread and this is supported by the results of hybridization between these plasmids.

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