Relationship of Group P1 Plasmids Revealed by Heteroduplex Experiments: RP1, RP4, R68 and RK2 Are Identical

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The molecular relationships of the IncP1 plasmids RP1, RP4, R68 and RK2 were tested by electron microscopic examination of heteroduplexes. In several hybridization experiments molecules were detected which had a 7·8% portion of incomplete reannealing. This 'heterologous region' could be explained by the typical renaturation behaviour of the transposon Tn1. The identity of the Tn1 transposon present in RP1 and RP4 was proved by heteroduplex experiments with λ phage DNA containing this transposon. These results indicated that the plasmids RP1 and RP4 are identical. Additional heteroduplex experiments between plasmids R68.45 and RP8 and between R68.45 and RK2 were performed. R68.45, a derivative of R68, has a small DNA insertion and RP8 can be regarded as a large insertion mutant of RP4; both insertions were used as single-stranded hybridization markers. From the hybrid molecules formed, it was deduced that R68 and RK2 are identical with RP1 and RP4 as far as molecular structure is revealed by the technique used.

INTRODUCTION

A large amount of genetic and molecular biological work has been done with resistance plasmids of the P1 incompatibility group. These plasmids are characterized by their broad host-range among Gram-negative bacteria (Datta & Hedges, 1972; Beringer, 1974). Some of their derivatives can be used to mobilize chromosomal genes in different bacterial species (Stanisich & Holloway, 1971; Beringer, 1974; Towner & Vivian, 1976; Lacy & Leary, 1976; Haas & Holloway, 1976; Meade & Signer, 1977). In this study, the molecular relationships of the P1 incompatibility group plasmids RP1, RP4, RP8, R68, R68.45 and RK2 were tested.

Plasmids RP1 and RP8, isolated by Lowbury *et al.* (1969) and Black & Girdwood (1969), respectively, have been characterized by Grinsted *et al.* (1972) and Saunders & Grinsted (1972). RP4 was first described by Datta *et al.* (1971) and was thought to be identical with RP1 (Holloway & Richmond, 1973). R68 was first used in early studies for gene mobilization in *Pseudomonas aeruginosa* (Stanisich & Holloway, 1971) and probably originates from the same source as RP1 (Holloway & Richmond, 1973). R68.45 is a derivative of R68 that shows enhanced sex factor ability; it was isolated from *P. aeruginosa* (Haas & Holloway, 1976). RK2 was described by Ingram *et al.* (1973) and used for physical and genetic studies by Meyer *et al.* (1977); this plasmid was isolated in the same hospital as RP1 and R68 (M. Richmond, personal communication).

Because of their more or less common origin and very similar genetic and molecular data these plasmids are thought to be closely related, if not identical. This paper reports an electron microscopical heteroduplex investigation of RP1, RP4, R68 and RK2 which shows that they are identical. It supercedes an earlier publication (Burkardt *et al.*, 1977) in which it was reported that RP1 and RP4 were partially heterologous.

Table 1. *Plasmids and their properties*

Plasmid	Drug resistance markers*	Contour length (μm)†	Reference
RP1	Ap Km Tc	18.9 (1)	Lowbury <i>et al.</i> (1969)
RP4	Ap Km Tc	19.1 (1)	Datta <i>et al.</i> (1971)
RP8	Ap Km Tc	31.3 (1)	Black & Girdwood (1969)
R68	Ap Km Tc	19.1 (2)	Stanisich & Holloway (1971)
R68.45	Ap Km Tc	19.7 (3)	Haas & Holloway (1976)
RK2	Ap Km Tc	18.2 (4)	Ingram <i>et al.</i> (1973)

* Ap, Ampicillin; Km, kanamycin; Tc, tetracycline.

† Results from: (1) Burkardt *et al.* (1978); (2) Riess, unpublished; (3) Riess *et al.* (1978); (4) calculated from Meyer *et al.* (1977).

METHODS

Plasmids, strains and phages. The plasmids studied, their drug resistance markers and contour lengths are listed in Table 1. The *Escherichia coli* host strains were: NC22-1 for RP1 and RP4 (Burkardt *et al.*, 1978), NC22 for RP8 (Burkardt *et al.*, 1978), CSH51 for R68 and R68.45 and MV17 for RK2. *Escherichia coli* CSH51(R68) was constructed by R68 plasmid transfer from *Pseudomonas aeruginosa* PAO8(R68) to CSH51 (Miller, 1972) and *E. coli* CSH51(R68.45) by R68.45 plasmid transfer from *P. aeruginosa* PAO25(R68.45) to CSH51. Both *Pseudomonas* strains were a gift from B. W. Holloway, Clayton, Australia. *Escherichia coli* MV17(RK2) was obtained from D. R. Helinski, La Jolla, Calif., U.S.A., and the phage λ ::Tn1 from D. Botstein, Cambridge, Mass., U.S.A.

Plasmid isolation and treatment with restriction endonuclease EcoRI. The plasmid isolation procedure was described by Pühler *et al.* (1979). Plasmid DNA was digested with restriction endonuclease *EcoRI* (Boehringer) as described by Gautier *et al.* (1976).

Heteroduplex technique. DNA solutions containing 10 to 100 $\mu\text{g ml}^{-1}$ were normally used. The samples were dialysed against 10 mM-Tris/HCl buffer, pH 8, containing 1 mM- Na_2EDTA . The solutions of the two types of molecule to be tested were mixed so that the open circular molecules of both types were in a ratio of about 1:1. The relative proportions of open circular DNA, covalently closed circular DNA and DNA fragments had been determined previously. Samples of the mixture (3 μl) were added to 5 μl formamide (Fluka, Buchs, Switzerland; purified by recrystallization), buffered by the addition of 1 μl phosphate buffer (1 M, pH 7) and boiled in a water bath for 1.5 min to denature the double-stranded DNA.

For renaturation, 1 μl 2 M-sodium perchlorate was added and the sample was kept at 40 °C for 1 to 2 h. The final concentrations in the renaturation mixture were as follows: 50% (v/v) formamide, 0.1 M-phosphate buffer, 0.2 M-sodium perchlorate, 3 to 30 $\mu\text{g DNA ml}^{-1}$. Before spreading, the sample was diluted to lower the salt concentration; this improved the spreading of single-stranded DNA regions. Samples (2 μl) were added to 18 μl formamide (55%, purified) containing 0.45 M- Na_2EDTA . Spreading was carried out according to Kleinschmidt & Zahn (1959) taking the 20 μl sample plus 5 μl 0.5 M-phosphate buffer (pH 8.5) and 0.5 μl cytochrome *c* (5 mg ml^{-1} ; Sigma, Type V) as the hyperphase. The hypophase consisted of 30% formamide (Fluka, not purified), 1 μM - Na_2EDTA and 10 mM-Tris and was adjusted to pH 8.7 with a few drops of 1 M-HCl.

Parts of the hyperphase were picked up with parlodion-coated copper grids, positively stained with 10 μM -uranyl acetate in 90% (v/v) ethanol and rotary-shadowed at an angle of 6.5°.

Lengths were determined after optical projection of electron micrographs by measuring the molecule contours with a Brühl LM1 measurer (Brühl, Nürnberg, Germany).

RESULTS

Identity of plasmids RP1 and RP4

A heteroduplex experiment was carried out to determine whether RP1 and RP4 were different plasmids or only different designations for two identical molecules. Generally, electron microscopic preparations of the RP1/RP4 mixture showed molecules like the parental plasmids without special structures. However, Fig. 1 shows a region (arrowed), comprising 7.8% of the total length of the molecule, which is not totally annealed. Two experiments were carried out in order to map this region. (i) The distance between the *EcoRI* site and the region of incomplete annealing was determined. The restriction endo-

Fig. 1. Electron micrograph (a) and drawing (b) of a molecule found in an RP1/RP4 heteroduplex preparation. The arrow points to the region of incomplete annealing. (Double line, duplex DNA; thick line, reannealed duplex DNA within Tn1; single line, non-annealed DNA within Tn1.)

Fig. 2. Drawing from an electron micrograph of a molecule found in an RP1/RP4 heteroduplex preparation; the plasmid DNA had first been made linear by *EcoRI* digestion. The arrow points to the region of incomplete annealing. (Definitions of lines as in Fig. 1.)

nuclease *EcoRI* cuts both RP1 and RP4 at a single site (Grinsted *et al.*, 1977; Jacob & Grinter, 1975) giving rise to linear plasmid molecules of full length. In heteroduplex experiments with such linear RP1 and RP4 DNA, molecules were found which showed a typical region of incomplete annealing. The distance between this structure and one end of the molecules generated by *EcoRI* was 9.3% of the total length of the molecule (Fig. 2). No other single-stranded structures were detected. (ii) The second experiment was designed to determine the orientation of this region, i.e. to the right or left of the *EcoRI* site. An RP1/RP8 heteroduplex was therefore formed, the RP8 molecule being used to obtain an additional single-stranded marker, since it is known to be composed of an RP4 plasmid and a 12 μ m segment of extra DNA (Burkardt *et al.*, 1978). The RP1/RP8 heteroduplex molecule located the region of non-homology at a distance of 22% of the total length of the RP1 molecule from the insertion point of the additional RP8 DNA. Since the distance of the *EcoRI* cleavage site from the RP8 insertion point is known (Spitzbarth, 1978), the region of

Fig. 3. Special renaturation behaviour of transposon Tn1. In heteroduplex experiments the Tn1 transposon can hybridize in two ways: (i) by normal reannealing which leads to duplex molecules without single-stranded structures, or (ii) by looping-out of the transposon in a single strand, before duplex formation, due to its inverted repeats (Kleckner *et al.*, 1975). This DNA segment cannot completely anneal with its counterpart in a second renaturation step, despite their homology, because the DNA of the Tn1 loop is fixed by its double-stranded stem. This leads to heteroduplex molecules as shown in Figs. 1 and 2. Abbreviations: Tn1, Tn1 transposon; IR, inverted repeat in one strand; IR', inverted repeat in a complementary strand.

incomplete annealing was located at a point 9.3% of the total length from the *EcoRI* cleavage site in the direction of the RP8 insertion.

From these two experiments, the region of non-homology in relation to the single *EcoRI* cleavage site was unambiguously determined. Comparison of these results with the known genetic map of RP4 (Barth & Grinter, 1977) shows that this molecular segment coincides with the location of the transposon Tn1 which is present in RP1 and RP4 (Hedges & Jacob, 1974; Bennett *et al.*, 1977). The incomplete annealing in the RP1/RP4 heteroduplex experiments could therefore be explained as either a real heterology between RP1 and RP4 or due to the special renaturation behaviour of the transposon Tn1 (Fig. 3).

To distinguish between these two possibilities, plasmids RP1 and RP4 were hybridized with $\lambda::Tn1$ DNA. In both cases (Figs 4, 5) the transposon Tn1 from $\lambda::Tn1$ completely hybridized with the transposons in RP1 and RP4, indicating homology between RP1 and RP4 for the region in question. Thus RP1 and RP4 are identical and the region of incomplete annealing found in heteroduplexes between them is due to the special renaturation behaviour of the transposon Tn1. The heteroduplexes in Figs 1 and 2 could therefore be either a hybrid of an RP1 and an RP4 single strand or an RP1 homoduplex or an RP4 homoduplex, in each case the Tn1 transposon being looped out before annealing. In all three cases, the electron microscopic appearance would be the same.

Fig. 4. Drawing from an electron micrograph of an RP1/λ::Tn1 heteroduplex molecule. The region of homology (double line) is that between the arrows.

Fig. 5. Drawing from an electron micrograph of an RP4/λ::Tn1 heteroduplex molecule. The region of homology (double line) is that between the arrows.

Relationship of plasmids R68 and RP4

To test the relationship between R68 and RP4, heteroduplexes were formed between R68.45 and RP8. R68.45 is a derivative of R68 and RP8 is a derivative of RP4. Both derivatives have identical DNA to their parents except for a special DNA insertion. The insertion in R68.45 is a 0.6 μm fragment (Riess *et al.*, 1978), whereas RP8 is defined by a 12 μm insertion (Burkardt *et al.*, 1978). Because of their convenient single-stranded markers in the hybrid molecule, both mutants were used to identify heteroduplexes and locate any further single-stranded structures that may be present. Three features of the hybrid molecule are clearly visible in Fig. 6: (i) one double-stranded circle, 19 μm long, which corresponds to RP4 DNA; (ii) one large single-stranded loop, about 12 μm long, which represents the additional DNA of RP8 in comparison with RP4; and (iii) one small single-stranded 0.6 μm loop which shows the DNA insertion of R68.45 in comparison with R68. Thus the heteroduplex molecule showed only single-stranded regions which originated from the derivative plasmids indicating that the parental plasmids RP4 and R68 are identical.

Relationship of plasmids RK2 and R68

Plasmid RK2 is similar to RP4 in its restriction enzyme pattern (Meyer *et al.*, 1977) and originated from the same source (Ingram *et al.*, 1973), but it was uncertain whether or not RK2 is different from RP4. The identity of RK2 was checked by heteroduplex formation between R68.45 and RK2, the 0.6 μm DNA insertion of R68.45 being used as an indicator

Fig. 6. Drawing from an electron micrograph of an R68.45/RP8 heteroduplex molecule. The thin arrow indicates single-stranded R68.45 DNA and the thick arrow indicates single-stranded RP8 DNA. Single-stranded regions are shown as a single line.

Fig. 7. Drawing from an electron micrograph of an RK2/R68.45 heteroduplex molecule. The arrow indicates single-stranded R68.45 DNA (single line).

of heteroduplex formation (Fig. 7). The heteroduplex molecule was composed of a large double-stranded circle ($19\ \mu\text{m}$) which represented R68 DNA and a small single-stranded loop ($0.6\ \mu\text{m}$) which belonged to the R68.45 insertion. No additional single-stranded DNA was visible proving the identity of RK2 and R68.

DISCUSSION

The results of the heteroduplex experiments show that RP1, RP4, R68 and RK2 are identical. The experiments also confirmed the relationship of plasmids RP8 and R68.45 to RP4 and R68, respectively, revealed by earlier studies (Burkardt *et al.*, 1978; Riess *et al.*, 1978), namely that RP8 is equivalent to RP4 plus a $12\ \mu\text{m}$ DNA insertion and R68.45 is equivalent to R68 plus a $0.6\ \mu\text{m}$ DNA insertion.

Additionally, each one of these plasmid DNAs harbours an electron microscopically distinct single-stranded marker, typical of a transposon, which frequently appears during the renaturation process. Transposon DNA can loop out by its inverted repeats (Kleckner *et al.*, 1975), which easily hybridize within one strand because they are in close proximity. This intra-strand hybridization takes place very quickly (in 5 min or less). The looped-out molecular segment cannot then undergo a second, inter-strand annealing. Occasionally inter-strand annealing takes place before intra-strand annealing preventing looping-out of the transposon DNA. In this case, the molecules can hybridize completely if no DNA

heterologies are present, e.g. Figs 6 and 7. Both hybrid molecules exhibited only the single-stranded insertion markers of R68.45 and RP8, but some molecules were also found with a typical transposon region of incomplete annealing (not shown). This additional marker can be used to map the R68.45 insertion on the R68 plasmid. We found the 0.6 μ m insertion at 61.4% of the total length from the *Eco*RI site in the Tn1 direction (Riess *et al.*, 1979). The lack of any single-stranded structure in the Tn1 region in Figs 6 and 7 also proves the total DNA homology of the ampicillin gene region in RP8, R68.45 and RK2.

A misinterpretation of the typical Tn1 heteroduplex structure was the reason for our previous report that RP1 and RP4 were different (Burkardt *et al.*, 1977). Since the only difference between the plasmids is caused by the special renaturation behaviour of transposon Tn1, we now consider both plasmids to be identical as far as their molecular structures can be resolved by electron microscopic examination of heteroduplexes.

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