Mapping of RP4 Plasmid Using Deletion Mutants of pAS8 Hybrid (RP4-ColE1)

V.A. Sakanyan, L.Z. Yakubov, S.I. Alikhanian, and A.I. Stepanov
Institute of Genetics and Selection of Industrial Microorganisms, Dorozhnaya 8, Moscow 113545, USSR

Summary. We used the hybrid plasmid pAS8 in order to conduct the genetic analysis of RP4 plasmid. The presence of two replicons in the hybrid plasmid permitted to expand the spectrum of deletion mutants of RP4 isolated, which are capable to autonomous replication. The shortening of the hybrid plasmid was achieved by P22 transduction, by induction of deletion mutants using mitomycin C, as well as by selection of Tra- mutants on the basis of resistance of cells to P-specific phages. These techniques have lead to isolation of clones possessing different combinations of plasmid resistance determinants.

Comparison of phenotypic characteristics of deletion plasmids pAS9, pAS10, pAS11, pAS12 and pAS10-2 permitted to propose the map for pAS8 plasmid with the following sequence of markers: tra-kan-ColE1-amp-tet...

Heteroduplex analysis of deletion mutants of pAS8 permitted to construct a physical map and to elaborate in greater detail the functional map of RP4 plasmid. The correlation between the ability of mutants to replicate in polA (TS) strain at nonpermissive conditions and the length of the deleted segment permitted to map rep genes of RP4 on a region with coordinates 9.8–17.3 kb. A relationship between the manifestation of incompatibility of mutants with Inc P-1 plasmids and the length of deletions points out that inc genes are located on DNA region with coordinates 2.1–9.8 kb. The analysis of replication of deletion mutants and the manifestation of incompatibility just as of the data about the size of appropriate deletions permitted to make the conclusion about the functional and genetic independence of the replication control and incompatibility control in RP4 plasmid.

Introduction

The unique property of RP4 plasmid to be transferred by conjugation to a wide range of recipients belonging to gram-negative bacteria (Datta et al., 1971; Olsen and Shipley, 1973) necessitates their thorough genetic analysis, particularly that of genes responsible for the conjugation transfer and autonomous replication of plasmid. Feasibility of genetic analysis largely depends on availability of deletion mutants, permitting to map plasmid markers relatively to each other.

The availability of hybrid RP4-ColE1 constructed in vitro (Stepanov et al., 1976) and designated as pAS8 (Stepanov et al., 1977) was expected to facilitate isolation of different deletion mutants of RP4 plasmid, since pAS8 contains two replicons. Plasmid pAS8 retained the ability to be transferred by conjugation and the RP4 determinants of drug resistance. It means that EcoRI restriction site of RP4 (Jacob and Grinter, 1975) is not located in the genes responsible for the transfer or controlling drug resistance (Stepanov et al., 1976).

This paper describes isolation of nonconjugative deletion variants of pAS8 plasmid by P22 transduction, treatment of cells by mitomycin C, or selection of mutants resistant to P-specific phages. On the basis of results obtained by genetic and heteroduplex analysis of deletion mutants of pAS8 we mapped genetic determinants of RP4 plasmid.

Materials and Methods

Bacterial Strains, Phages and Plasmids are described in Table 1. PR1 and RP4 plasmids have been used in experiments, assuming that they are identical (Holloway and Richmond, 1973; Jacoby et al., 1976), the data for RP1 plasmid were extrapolated to RP4.

For offprints contact: V.A. Sakanyan
The mating mixture was incubated, depending on the actual conditions of the experiment, for 60-120 min at 37°C. The frequency of plasmid transfer was calculated as the ratio of number of transconjugants to the number of donor cells.

**Transduction.** P22 mediated transduction was performed as described by Clowes and Hayes (1968) at the multiplicity of infection equal to 10. P1 transduction was carried out as described by Rosner (1972) using the phage P1-cml, clr-100 at the multiplicity of infection equal to 0.1.

**Elimination of Plasmids by Mitomycin C.** Overnight cultures of *E. coli* K12 J53 containing plasmids RP4, RP1 or pAS8 were diluted 1:50 in the fresh L-broth and grown to 2 x 10^8 cells/ml. Cell suspension were then diluted to 10^5 cells/ml in L-broth containing 0.3 µg/ml mitomycin C. The treated cells were incubated with vigorous shaking in the dark for 40 hours at 37°C. The cells were plated on nutrient agar and the colonies were replicated on the antibiotic containing medium.

**Assay of Plasmid Instability.** Cultures growing exponentially in L-broth were diluted to 10^7 cells/ml and incubated for 72 h at 37°C. Colonies grown after the plating on agar were tested for antibiotic resistance.

**Isolation of tra^- Mutants.** Cells of *E. coli* K12 J53 (RP1) or *E. coli* K12 J53 (pAS8) were added to 2 ml of molten 1.5% agar and the mixture was poured onto the 1.5% agar layer. After drying the drops of P-specific phage were applied onto the surface. The phage-resistant colonies appeared after incubation for 2 days at 37°C. The obtained phage-resistant bacteria were twice purified by streaking on ampicillin-containing minimal medium and tested for their donor ability with *E. coli* K12 J62 as a recipient.

**Transformation** was performed by the procedure of Mandel and Higa (1970) and Cohen et al. (1972).

**Immunity of Colicines.** Cells were tested for immunity to colicines as described by Clowes and Hayes (1968).

**Studies of Plasmid Incompatibility.** The incompatibility of plasmids was determined by the standard technique (Hedges et al., 1974). Tra^- plasmids R702, R906, or mutants of RP1 sensitive to antibiotics were transferred by conjugation into *E. coli* J53 containing the mutant plasmid tested.

**Tra^- mutants of pAS8 were transferred by transformation into C600 carrying R906 as a resident plasmid. The loss of the resident plasmid marker was regarded as an evidence of incompatibility of the tested plasmid.**

**Test for polA-Dependent Plasmid Replication.** Culture of *E. coli* K12 K55 polA^- was carrying plasmid tested was plated onto the agar containing the corresponding antibiotic and grown at 30°C for 2 days. Colony was picked up and transferred into 8 ml of L-broth and incubated with shaking at 30°C for 1-2 h. The bacterial culture was divided into 2 portions and further incubated at 30°C and at 42°C with vigorous aeration for 4-5 h. Samples were plated onto agar plates. After 24 h at 30°C the colonies were replicated onto antibiotic-containing media.

**Isolation of Plasmid DNA.** Plasmid DNA was isolated from bacterial sarkosyl lysates as described by Clewell and Helinsky (1970). EthBr-CsCl density centrifugation was performed in an Ti-75 rotor at 56,000 rpm/min and 20°C for 24 h (Beckman L5-65 Ultracentrifuge).