MUTAGENESIS AND CLONING OF Hly DETERMINANTS OF PLASMID pAP20

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Plasmid pAP20 is an F-like conjugative plasmid, controlling synthesis of active α-hemolysin by *Escherichia coli* cells [1]. Being a drd-plasmid, it belongs to the FIV incompatibility group [2], which distinguishes it from other Hly plasmids.

In the investigation described below, in order to determine the localization of Hly determinants in the genome of plasmid pAP20 experiments were carried out to study induction by transposon Tn5 of plasmid mutations accompanied by loss by the bacterial cells of their ability to synthesize active α-hemolysin, and also to study molecular cloning of these determinants.

EXPERIMENTAL METHODS

Cells of *E. coli* AP106 trp his lac recA str^r, carrying plasmid pAP20 and its three transposon-containing variants pAP20::Tn5 were used. Transposon Tn5 was introduced into the genome of plasmid pAP20 by the standard method in "three-parent" crosses, in which the donors were *E. coli* AP115 met thi lac Nal^r cells, containing the test plasmid, the "intermediate" recipients were *E. coli* KS1324 cells, containing transposon Tn5 in their chromosome, and the final recipients were *E. coli* C600 thr leu thi Rif^+ cells. Transconjugates were selected on nutrient agar (NA) with the addition of kanamycin (100 μg/ml) and rifampicin (50 μg/ml). The resulting Km and Rif transconjugants were plated out on NA containing human erythrocytes. Transposon-containing Hly^+ plasmids were then transferred into *E. coli* AP106.

Plasmid DNA was isolated from lysates clarified with Triton X-100 [4] followed by gradient (CsCl–ethidium bromide) centrifugation. Restriction analysis of DNA of plasmid pAP20, pAP20::Tn5 Hly^+, and pAP20::Tn5 Hly^- was carried out with the use of restriction endonucleases EcoR I, Hind III, Sal I, and Bgl II and by horizontal slab electrophoresis in 0.8% agarose gel by the method in [2]. The hemolysis determinants were cloned with the use of Sal I fragments of plasmid DNA, cut out of the gels [6]. These fragments were ligated with DNA of the vector plasmid pBR325 in buffer containing 5 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, and 0.07 mM ATP (pH 7.5). Polynucleotide ligase T^4 was added in a dose of 10 units per 50 μl of mixture. The mixtures were incubated for 18 h at 12°C. Transformation of *E. coli* HB101 was carried out by the standard method, the transformants being selected on NA with ampicillin [3]. α-Hemolysin production was determined as described previously [2].

RESULTS

The experiments began with restriction analysis of DNA of the original plasmid pAP20 by means of restriction endonucleases EcoR I, Hind III, Sal I, and Bgl II. The results of this analysis are given in Table 1.

As Table 1 shows, DNA of plasmid pAP20 has 10 EcoR I sites, nine Hind III sites, eight Sal I sites, and nine Bgl II sites. The smallest number of fragments is thus formed on restriction of DNA of plasmid pAP20 by endonuclease Sal I.

In the next experiments transposon Tn5, selecting cells which contained the test plasmid with incorporated transposon Tn5, but which were both Hly^+ and Hly^-, was introduced into the genome of plasmid pAP20, contained in *E. coli* AP106. On the assumption that the phenotype of Hly^- bacteria is the result of induction of mutations of plasmid pAP20 by transposon Tn5, clones of *E. coli* AP106 cells containing mutant Hly^--plasmids pAP20 = 2::Tn5, pAP20 = 5::Tn5,

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TABLE 1. Restriction Fragments of Plasmid pAP20

Fragment	Size of restriction fragment (in MDa)			
	EcoRI	HindIII	SalI	BglII
f1	15,7	25,1	13,5	16,9
f2	9,1	11,9	12,2	13,8
f3	9,0	6,5	12,0	7,5
f4	7,5	6,1	9,8	7,3
f5	7,1	5,9	6,7	6,1
f6	3,3	1,9	5,5	5,6
f7	3,2	1,7	5,2	3,2
f8	3,1	1,2	1,7	2,7
f9	2,5	0,8		2,5
f10	1,9			
Mol. wt., MDa	64,2	61,4	66,6	65,9

TABLE 2. Sal I Fragments of Plasmid pAP20 and Its Transposon-Containing Variants

Fragment	Plasmid					
	pAP20	pAP20-7::Tn5 Hly$^+$	pAP20-2::Tn5 Hly$^-$	pAP20-5::Tn5 Hly$^-$	pAP20-6::Tn5 Hly$^-$	pAP20-3::Tn5 Hly$^-$
f1	13,5	13,5	13,5	13,5	13,5	13,5
f2	12,2	12,2	12,2	12,2	12,2	12,2
f3	12,0	11,0	9,8	10,0	9,8	10,0
f4	9,8	9,8	9,7	9,8	8,5	9,8
f5	6,7	6,7	6,7	6,7	7,3	6,7
f6	5,5	5,5	5,9	6,0	6,7	6,0
f7	5,2	5,2	5,5	5,5	5,5	5,5
f8	1,7	4,8	5,2	5,2	5,2	5,2
f9		1,7	1,7	1,7	1,7	1,7
Mol. wt., MDa	66,6	70,4	70,2	70,6	70,4	70,6

TABLE 3. Properties of Hybrid Plasmids Containing Hly Region of Plasmid pAP20

Hybrid plasmid	Vector plasmid	Incorporated fragment	Size of incorporated fragment, megadaltons	Conjugativeness	Sensitivity to phage
pIS1	pBR325	3f	12,0	—	—
pIS2	pBR325	3f	12,1	—	—
pIS3	pBR325	3f	11,7	—	—
pIS4	pBR325	3f	12,0	—	—
pIS5	pBR325	3f	12,0	—	—

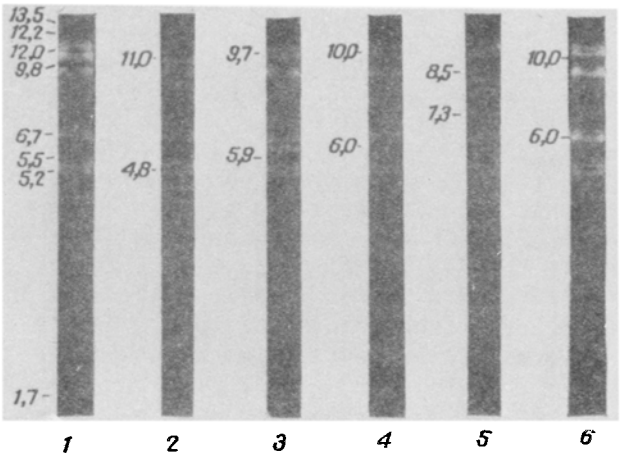

Fig. 1. Electrophoresis of restriction fragments of plasmid pAP20 and its transposon Tn5-containing variants. Tracks from left to right: 1) pAP20; 2) pAP20=7::Tn5 Hly$^+$; 3) pAP20=2::Tn5; 4) pAP20=5::Tn5; 5) pAP20=6::Tn5; 6) pAP20=3::Tn5.

pAP20=6::Tn5 and pAP20=3::Tn5, and also plasmid pAP20=7::Tn5 Hly$^+$, were selected for further investigation. Plasmid DNA was isolated from these cells, after which it was subjected to restriction analysis by endonuclease Sal I and compared with DNA of the original plasmid pAP20 and of plasmid pAP20=7::Tn5 Hly$^+$. The results of this analysis are given in Fig. 1 and Table 2.

Plasmid fragment f3, characteristic of the original plasmid pAP20, is absent in plasmids containing transposon Tn5. Meanwhile the transposon-containing plasmids, unlike the original plasmid, each contain nine Sal I sites, thanks to which their DNA is restricted to nine fragments, two of which are not found in DNA of the original plasmid.

Transposon Tn5, as we know, has a molecular weight of 3.4 megadaltons and possesses one recognition site of restriction endonuclease Sal I [5]. Therefore, when discussing the results of comparative restriction, we postulated that transposon Tn5 was incorporated into the Sal I fragment f3 of plasmid pAP20, and because it contains a Sal I restriction endonuclease recognition site, an additional Sal I fragment is formed during restriction, i.e., fragment f3 is restricted to two fragments, each of which differs from the original f3 fragment; however, the combined molecular weight of the two new fragments ought to be 15.4-16.0 megadaltons.

Comparison of the molecular weights of the newly formed fragments of DNA-transposon-containing variants of plasmid pAP20 confirms this hypothesis. For instance, the molecular weight of fragments f3 (11 megadaltons) and f8 (4.8 megadaltons) of plasmid pAP20=7::Tn5 is 15.8 megadaltons, that of fragments f4 (9.7 megadaltons) and f6 (6 megadaltons) of mutant plasmid pAP20=5::Tn5 is 16 megadaltons, that of fragments f4 (8.5) and f5 (7.3 megadaltons) of mutant plasmid pAP20 = 6::Tn5 is 15.8 megadaltons, and that of fragments f4 (9.7) and f6 (5.9) of mutant plasmid pAP20=3::Tn5 is 15.5 megadaltons. Thus the data obtained by comparative analysis of plasmid pAP20 and its transposon-containing variants supported the view that genes determining hemolytic activity of plasmid pAP20 are located in its Sal I fragment f3 with mol. wt. of 12 megadaltons.

To obtain further proof that determinants of α-hemolysin are located in the genome of plasmid AP20, the Sal I fragment f3 identified as the result of Tn5-mutagenesis was cloned in the composition of vector pBR325 by transformation of cells of strain *E. coli* HB101. Transformation experiments yielded *E. coli* HB101 transformants containing hybrid plasmids, designated pIS1, pIS2, pIS3, pIS4, and pIS5. In the next experiments DNA of the hybrid plasmids was isolated and their molecular dimensions determined in order to obtain the exact molecular dimensions of fragment f3 of plasmid pAP20 incorporated into them. The hemolytic properties and donor ability of cells containing the hybrid plasmids also were determined. The results of these experiments (Table 3) showed that the hybrid plasmids contain an Hly region. However, none of the hybrid plasmids determines either conjugativeness or sensitivity to donor-specific phage MS2.

It can be concluded from the results as a whole that the F-like drd-plasmid pAP20 contains Hly determinants controlling synthesis and secretion of active α-hemolysin by *E. coli* cells in the Sal I fragment f3, with a molecular size of 12 megadaltons. This fragment contains no determinants controlling genetic transfer of plasmid pAP20.

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