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pAP20 PLASMID CONTROLLING HEMOLYTIC ACTIVITY OF *Escherichia coli*

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UDC 579.842.11:579.252.5

KEY WORDS: hemolytic activity; pAP20 plasmid; *Escherichia coli*.

Most plasmids determining hemolytic (Hly) activity of *E. coli* have been found in bacteria of this species isolated from animals [5]. The F-like plasmids have been found to be separate from these Hly-plasmids and have been classified among Inc FIII-FIV and FVI groups [3]. Plasmid Hly pAP20 was identified for the first time in cells of a strain of *E. coli* isolated from man [1]. However, it has not been studied.

The aim of this investigation was to study the principal physicochemical, biological, and genetic properties of this plasmid.

EXPERIMENTAL METHOD

Strains of *E. coli* AP115 met thi lac Nal^r, AP106 trp his lac str and C600 thr leu thi lac str were used.

Hemolytic properties of bacteria with plasmid pAP20 were determined by seeding them on nutrient agar (NA) containing washed human erythrocytes, and incubating the seedings for 18 h at 37°C. α-Hemolysin production was determined by estimating hemolysis of a 2% erythrocyte suspension to which supernatant after centrifugation of 2.5-h broth cultures of bacteria containing plasmid pAP20 was added.

DNA of the test plasmid pAP20 was isolated by bacterial lysates, clarified with Triton X-100, followed by gradient (CsCl — ethidium bromide) centrifugation [4]. To determine the molecular weight of the plasmid, restriction analysis of its DNA was carried out with the aid of EcoRI enzyme and electrophoresis in 0.8% agarose gel. The buffer used for EcoRI enzyme contained 100 mM Tris-HCl buffer (pH 7.5) and 10 mM $MgSO_4$. The restriction reaction was stopped by heating the samples for 5 min at 65°C. The molecular weight of the plasmid was determined by adding together the molecular weights of its restriction fragments. *E. coli*-fragments of DNA of phage λ served as standards for molecular weight.

Transmissibility of the test plasmid was studied by the use of *E. coli* AP115, AP106, and C600 as recipients.

Compatibility of plasmid pAP20 was determined by Datta's scheme [2], using reference plasmids of all incompatibility (Inc) F groups. Data on surface exclusion were obtained by

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Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 97, No. 3, pp. 317-321, March, 1984. Original article submitted April 30, 1983.

 0007-4888/84/9703-0300$08.50

introducing plasmid pAP20 into plasmid-free recipient cells. Plasmids pAP20 and Hly212 were tagged with transposons Tn5 and Tn1 (respectively) by standard methods. The bacteria were crossed and their sensitivity to phage MS2 determined also by standard methods.

EXPERIMENTAL RESULTS

Cells of hemolytic strains of *E. coli* are known to synthesize several types of hemolysins, one of which (α-hemolysin) can diffuse into the medium and can be filtered. Analysis of the results of experiments to determine hemolytic activity of *E. coli* AP115 (pAP20) cells on agar with erythrocytes showed that they give colonies around which hemolytic zones about 2.3 mm in diameter are formed [6]. Supernatants of cultures of bacterial cells of this strain also induced hemolysis.

It was concluded from these findings that plasmid pAP20 determines α-hemolysin synthesis.

The results of restriction analysis showed that plasmid pAP20 contains 10 recognition sites for enzyme EcoRI, as a result of which during restriction 10 fragments are formed, with molecular weights of 15.85, 11.22, 6.31; 5.62; 3.47; 3.16; 3.02; 2.51; 2.24; and 1.99. Using data on molecular weight of EcoRI fragments of DNA from phage λ it was calculated that the molecular weight of plasmid pAP20 is $55.39 \cdot 10^6$ daltons.

Determination of phage-sensitivity of *E. coli* AP106 (pAP20) and AP115 (pAP20) cells showed that they are sensitive to F-specific phage MS2 which means that the pAP20 plasmid tested is an F-like plasmid of the drd type.

The study of transmissibility of plasmid pAP20 showed that it is transmitted from cells of some strains to cells of other strains of *E. coli* with a fairly high frequency, which in AP115 (pAP20) × AP106 crosses was $3.3 \cdot 10^{-2}$, in AP106 (pAP20) × AP115 crosses $4.0 \cdot 10^{-2}$, and in AP115 (pAP20) × C600 crosses it was $2.6 \cdot 10^{-1}$.

Having obtained data on the above-named properties of plasmid pAP20, in the next experiments the group of Inc F-like plasmids to which it belonged was determined. Since nine groups of Inc F-like plasmids are now known, compatibility (incompatibility) of this plasmid was determined with a reference plasmid of each group.

The results of experiments to study compatibility (incompatibility) of the pAP20 (Hly) plasmid with reference plasmids of groups Inc FI-FIX are given in Table 1.

It will be clear from Table 1 that in most crosses no significant surface exclusion was found. Transconjugants obtained from all conjugation crosses, depending on their plasmid content, can be placed in two classes. The first class consists of transconjugants obtained from crosses in which compatibility (incompatibility) of plasmid pAP20 with plasmid R124, the reference plasmid of the Inc FIV group, was determined. Most transconjugants obtained from crosses in which the introduced plasmid was pAP20 and the resident plasmid was R124 lost the introduced plasmid and completely preserved the resident plasmid. Conversely, transconjugants from crosses in which the introduced plasmid was R124 and the resident plasmid was pAP20 completely lost their resident plasmid. This result suggested that plasmid pAP20 is incompatible with plasmid R124, i.e., that plasmid pAP20 belongs to the Inc FIV group.

The second class consists of transconjugants obtained from the remaining crosses, in which compatibility (incompatibility) of plasmid pAP20 was studied with reference plasmids of other incompability F groups. These transconjugants contained introduced plasmid but partly lost their resident plasmid (from crosses in which reference plasmids of Inc FI, FIV, FV, FVII, and FVIII groups were used), or they completely preserved their resident plasmid but partly lost the introduced plasmid (from crosses in which reference plasmids of Inc FIII, FIV, FV, FVI, and FVIII groups were used). The results of a study of these transconjugants demonstrated neither compatibility nor incompatibility of the test plasmid with the reference plasmids. To study further characteristics of the transconjugants obtained from all crosses except that in which the introduced plasmid was R124 (Inc FIV) and the resident plasmid was pAP20, additional experiments were therefore carried out in which the degree of stability of coexistence of the two plasmids (pAP20 and one of the reference plasmids) was determined by clonal tests. In these tests transconjugants were cultured in nutrient broth (NB), after which seedings were taken from broth cultures of the transconjugants on NA, from which 20 colonies (clones) of each transconjugant were selected and their plasmid content analyzed. The results of the clonal test of transconjugants from the cross in which the introduced plasmid was pAP20 and the resident plasmid R124 showed that 99% of the clones tested had lost their introduced plas-

TABLE 1. Compatibility of Plasmid pAP20 (Hly) with Plasmids of Incompatibility F Groups (in *E. coli* AP115)

Plasmid		Selective marker	Frequency of transfer (per donor)	Surface exclusion index	Number of colonies of transconjugants (in %) whose cells contain		
introduced	resident				introduced plasmid	resident plasmid	both plasmids
pAP20 (Hly)	R386 (FI)	Hly	$1,0 \cdot 10^{-2}$	1,3	100	96	96
pAP20 (Hly)		Hly	$1,3 \cdot 10^{-2}$		100		
R386 (FI)	pAP20 (Hly)	Tc	$2,5 \cdot 10^{-4}$	4	100	90	90
R386 (FI)		Tc	$1,0 \cdot 10^{-3}$		100		
pAP20 (Hly)	R1—19 (FII)	Hly	$1,9 \cdot 10^{-2}$	0,7	100	93	93
pAP20 (Hly)		Hly	$1,3 \cdot 10^{-2}$		100	—	—
R1—19 (FII)	pAP20 (Hly)	Km	$2,5 \cdot 10^{-2}$	2,2	100	85	85
R1—19 (FII)		Km	$5,6 \cdot 10^{-1}$		100	—	—
pAP20 (Hly)	ColBR3 (FIII)	Hly	$1,1 \cdot 10^{-2}$	1,1	58	100	58
pAP20 (Hly)		Hly	$1,3 \cdot 10^{-2}$		100	—	—
ColBR3 (FIII)	pAP20 (Hly)	Cm	$5,6 \cdot 10^{-3}$	0,46	100	100	100
ColBR3 (FIII)		Cm	$2,6 \cdot 10^{-3}$		100	—	—
pAP20 (Hly)	R124 (FIV)	Hly	$2,0 \cdot 10^{-2}$	12	32	100	32
pAP20 (Hly)		Hly	$2,5 \cdot 10^{-1}$		100		
R124 (FIV)	pAP20 (Hly)	Tc	$2,9 \cdot 10^{-2}$	0,5	100	0	0
R124 (FIV)		Tc	$1,5 \cdot 10^{-2}$		100		
pAP20 (Hly)	Folac (FV)	Hly	$3,0 \cdot 10^{-4}$	43	75	100	75
pAP20 (Hly)		Hly	$1,3 \cdot 10^{-2}$		100		
Folac (FV)	pAP20 (Hly)	Lac	$3,1 \cdot 10^{-7}$	210	100	98	98
Folac (FV)		Lac	$6,8 \cdot 10^{-5}$		100		
pAP20 :: Tn5	pHly212 :: Tn1 (FVII)	Km	$1,4 \cdot 10^{-2}$	2,2	100	100	100
pAP20 : : Tn5		Km	$3,1 \cdot 10^{-2}$		100		
pHly212 : Tn1 (FVI)	pAP20 : : Tn5	Ap	$4,5 \cdot 10^{-5}$	1,2	40	100	40
pHly212 : : tn1 (FVI)		Ap	$5,7 \cdot 10^{-5}$		100		
pAP20 (Hly)	pAP38 : : Tn1 (FVII)	Hly	$1,6 \cdot 10^{-3}$	15	100	65	65
pAP20 (Hly)		Hly	$2,7 \cdot 10^{-2}$		100		
pAP38 : : Tn1 (FVII)	pAP20 (Hly)	Ap	$3,3 \cdot 10^{-8}$	63636	100	100	100
pAP38 : : Tn1 (FVII)		Ap	$2,1 \cdot 10^{-3}$		100		
pAP20 (Hly)	pAP43 : : Tn1 (FVIII)	Hly	$2,4 \cdot 10^{-3}$	11	100	63	63
pAP20 (Hly)		Hly	$2,7 \cdot 10^{-2}$		100		
pAP43 : : Tn1 (FVIII)	pAP20 Hly	Ap	$6,5 \cdot 10^{-5}$	12	98	100	98
pAP43 : : Tn1 (FVIII)		Ap	$7,8 \cdot 10^{-4}$		100		
pAP20 (Hly)	pAP42 : : Tn1 (FIX)	Hly	$4,3 \cdot 10^{-3}$	6,2	95	100	95
pAP20 (Hly)		Hly	$2,7 \cdot 10^{-2}$		100		
pAP42 : : Tn1 (FIX)	pAP20 (Hly)	Ap	$1,5 \cdot 10^{-3}$	20,6	100	100	100
pAP42 : : Tn1 (FIX)		Ap	$3,1 \cdot 10^{-2}$		100		

TABLE 2. Genetic Transfer from Diplasmid Donors *E. coli* AP115 and *E. coli* AP106

Cross	Selective marker	Frequency of transfer	Analysis of unselective transconjugant markers		
			marker	number of transconjugants tested	number of transconjugants containing marker studied
FI AP115 (R386)(pAP20)×AP106	Hly	$1,1\cdot10^{-2}$	Tc	15	0
	Tc	$2,8\cdot10^{-3}$	Hly	15	10
AP115 (pA20)(R386)×AP106	TC	$2,1\cdot10^{-3}$	Hly	20	7
	Hly	$2,3\cdot10^{-3}$	Tc	20	9
FII AP115 (R1—19)(pAP20)×AP106	Hly	$1,0\cdot10^{-1}$	Km	20	2
	Km	$1,6\cdot10^{-3}$	Hly	20	5
AP115 (pAP20)(R1—19)×AP106	Km	$5,6\cdot10^{-3}$	Hly	20	1
	Hly	$3,3\cdot10^{-2}$	Km	20	4
FIII AP115 (ColBR3)(pAP20)×AP106	Hly	$1,1\cdot10^{-3}$	Cm	16	1
	Cm	$1,2\cdot10^{-3}$	Hly	16	6
AP115 (pAP20)(ColBR3)×AP106	Cm	$2,2\cdot10^{-3}$	Hly	20	1
	Hly	$4,5\cdot10^{-2}$	Cm	20	5
FV AP115 (Folac)(pAP20)×AP106	Hly	$8,4\cdot10^{-4}$	Lac	20	0
	Lac	$8,4\cdot10^{-8}$	Hly	20	0
AP115 (pAP20)(Folac)×AP106	Lac	$6,5\cdot10^{-8}$	Hly	20	0
	Hly	$1,0\cdot10^{-3}$	Lac	20	1
FVI AP115 (pHly212 : : Tn1)	Km	$4,1\cdot10^{-4}$	Ap	20	20
(pAP20::Tn5)×AP106	Ap	$4,3\cdot10^{-4}$	Km	20	20
AP115 (pAP20 : :Tn5)	Ap	$1,1\cdot10^{-4}$	Km	20	4
(pHly212 : :Tn1)×AP106	Km	$2,3\cdot10^{-2}$	Ap	20	0
FVIII AP115 (pAP43 : : Tn1)	Hly	$6,9\cdot10^{-2}$	Ap	20	0
(pAP20)×AP106	Ap	$1,9\cdot10^{-2}$	Hly	20	0
AP115 (pAP20)(pAP43 : : Tn1)×AP106	Ap	$2,2\cdot10^{-4}$	Hly	20	5
	Hly	$1,9\cdot10^{-2}$	Ap	20	3
FIX AP115 (pAP42 : : Tn1)	Hly	$1,4\cdot10^{-2}$	Ap	20	2
(pAP20)×AP106	Ap	$4,5\cdot10^{-3}$	Hly	20	3
AP115 (pAP20)(pAP42) : : Tn1)×AP106	Ap	$1,0\cdot10^{-2}$	Hly	20	0
	Hly	$4,0\cdot10^{-2}$	Ap	20	4

mid. This means that plasmids pAP20 and R124 are in fact incompatible with each other. As regards the other clonal tests, they showed that cells of clonal cultures of nearly all transconjugants support both plasmids (pAP20 and one of the reference plasmids) in a stable state. The number of clones which lost one of their plasmids varied from 1 to 17%. The only exceptions are results of the study of plasmid content in cells of transconjugant clones from crosses in which compatibility of plasmid $_{p}$AP20 with plasmid $_{p}$AP38::Tn1 (group Inc FVII) was analyzed. Of 100 clones tested, cells of only one clone lost plasmid $_{p}$AP20, whereas cells of 77 clones lost plasmid $_{p}$AP38::Tn1. The results of this test indicate partial incompatibility of plasmid $_{p}$AP20 with plasmid $_{p}$AP38::Tn1, reference plasmid of the Inc FVII group.

To reach the final conclusion that the test plasmid pAP20 is compatible with reference plasmids of the Inc FI, FII, FIII, FV, FVI, FVIII, and FIX groups, experiments were carried out to study the character of transfer from diplasmid donors to recipients' cells.

Data in Table 2 show that separate transmission of plasmid pAP20 and one of the reference plasmids contained in the diplasmid donors takes place in all cases with different frequencies. This result is evidence of independent transfer of each plasmid, i.e., of absence of recombination between them, which is usually characteristic of incompatible plasmids. Consequently, plasmid pAP20 and reference plasmids of the Inc FI, FII, FIII, FV, FVIII and FIX groups are compatible with each other.

The general conclusion can be drawn from these findings that plasmid pAP20 is a F-like Hly plasmid of average molecular weight, which determines synthesis of α-hemolysin. It appears that the plasmid of the drd type belongs to the FIV group and, at the same time, is partially incompatible with plasmid $_{p}$AP38, belonging to incompatibility group Inc. FVII. Since it possesses these properties, plasmid $_{p}$AP20 differs from all other known Hly plasmids, and this makes it a useful model with which to study the genetics of incompatibility.

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