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Studies with RP1 deletion plasmids: Incompatibility properties, plasmid curing and the spontaneous loss of resistance

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Abstract. Four deletion plasmids, pHH301, pHH302, pHH303 and pHH401, obtained from RP1 DNA-transformed bacterial clones, were shown to be incompatible with three P plasmids in *Escherichia coli* K12 strains. Kinetic experiments and colony tests were used to verify the position of these R plasmids. *Pseudomonas aeruginosa* and *E. coli* strains, harbouring deletion plasmids, could be cured by using two mutagens, acriflavine and mitomycin C, which affect a percentage of the cell population. The deletion plasmid-positive strains could also be induced at an elevated temperature to spontaneously loose their plasmids.

Keywords. RP1 deletion plasmids; incompatibility; P group; plasmid curing.

Introduction

Strains of *Pseudomonas aeruginosa* and *Escherichia coli* were transformed (Cosley and Oishi, 1973) with sheared pieces of RP1 DNA (Haque, 1979). Transformed clones contained a number of R plasmids, four of them were conjugally transferable and were deletion mutants of RP1. These were pHH301, pHH302, pHH303 and pHH401. These deletion products were both physically and genetically smaller than RP1. RP1 is an R-plasmid isolated from a strain of *P. aeruginosa* (Lowbury *et al*., 1969), having sex characteristics and high promiscuity and conferring resistance to carbenicillin, tetracycline, kanamycin and neomycin. The four deletion plasmids differed from the parent in their antibiotic-resistant characteristics.

Plasmid incompatibility is the inability of two different plasmids to coexist stably in the same host cell in the absence of a continued selection pressure (Novick *et al*., 1976). Incompatibility and entry exclusion are often used to determine if plasmids are related (Datta, 1975; Datta and Barth, 1976; Uhlin and Nordström 1975). Plasmid incompatibility was suggested to be due to competition for either a replicational site or a segregational site (Jacob *et al*., 1963). Alternatively it could occur due to repression of the initiation of replication (Pritchard *et al*., 1969).

Since the use of acriflavine dyes to cure an F factor from a bacterium (Hirota, 1960), numerous mitogens were used for eliminating plasmids from harbouring

strains. The elimination of plasmids by mitogens is apparently not due to a specific effect of drugs on the extrachromosomal DNA. These compounds affect cell growth and also help plasmid elimination by degrading DNA. The maximum effects were observed only if replicative processes were impaired (Barker and Hardman, 1978). Likewise elevated temperature affects plasmids elimination by similar processes. In this paper we show that the deletion plasmids, like the parent plasmid RP1, belong to P incompatibility group. The cells could be cured of the harbouring plasmids by mitogens and elevated temperature, resulting in the loss of antibiotic-resistance.

Materials and methods

Plasmids and bacterial strains employed

Table 1 lists the P plasmids, deletion plasmids and bacterial strains employed in this study.

Table 1. Plasmids and bacterial strains used.

Plasmid	Resistance pattern[a]	Harbouring strains[b]	Reference
RP1	Cb^{r}Tc^{r}Nm^{r}Km^r	J53/J62	Lowbury *et al.* (1969)
R702	Sm^{r}Tc^{r}Km^{r}Su^r	J53/J62	Hedges (1975)
R751	Tp^r	J53/J62	Jobanputra and Datta (1974)
R906	Sm^{r}Cb^{r}Su^r	J53/J62	Hedges *et al.* (1974)
pHH301	Tc^r	J62	Haque (1979)
pHH302	Cb^r	J62	Haque (1979)
pHH303	Cb^{r}Tc^r	J62	Haque (1979)
pHH401	Cb^{r}Tc^r	J62	Haque (1979)
Bacteria		**Essential characteristic[a]**	**Reference**
P. aeruginosa strains	1822	Harbours RP1	Lowbury *et al.* (1969)
	HH2060	pro$^-$met$^-$auxotroph of 6750	Haque (1979)
	HH2101	HH2060 harbouring pHH301	Haque (1979)
	HH2102	HH2060 harbouring pHH302	Haque (1979)
	HH2103	HH2060 harbouring pHH303	Haque (1979)
E. coli strains	J53	pro$^-$met$^-$	
	J62	lac$^-$pro$^-$his$^-$trp$^-$	Clowes and Hayes (1968)
	HH1501	J53 harbouring pHH401	Haque (1979)

[a] Abbreviations used Cb, carbenicilin; Tc, tetracycline; Nm, neomycin; Km, kanamycin; Sm, streptomycin; Su, sulphanamide; Tp, trimethoprim; met, methionine; pro, proline; lac, lactose; his, histidine; trp, tryptophan.

[b] The test plasmids were harboured by both J53 and J62 strains and their chrornosomally nalidixic acid-resistant substrains. The deletion plasmids were harboured by J62 strains and their chromosomally nalidixic acid-resistant substrain only.

Media and growth conditions

The minimal medium was medium E of Vogel and Bonner (1956), supplemented with glucose (0·2%) and the required amino acids (25 μg of the L-epimer per ml). The complete medium was the LB medium of Bertani (1951), plus medium E supplemented with glucose (0·2%). LA was the LB medium containing 1·5% agar. Nutrient broth, nutrient agar and minimal agar as specified (Clowes and Hayes, 1968) were also used, along with L-broth and L-agar (Lennox, 1955) which were used as enriched media. Growth was determined at 37° C.

Measurement of entry exclusion

The frequency of transfer of P plasmids from related strains of *E. coli* K12 to R^+ strains of the reciprocal hosts was determined at 37° C after 16 h mating conjugation (Datta *et al*., 1971). The mating mixtures were plated on LA that contained nalidixic acid (30 μg per ml), carbenicillin (50 μg per ml), tetracycline (50 μg per ml), trimethoprim (20 μg per ml), streptomycin (50 μg per ml) and kanamycin (30 μg per ml) in various combinations. Selection was for the chromosomal marker (nalidixic acid-resistance) and for a resistance on the incoming plasmid. The relative frequency of transfer by conjugation to *E. coli* K12 strains with or without a second P plasmid gave a measure of entry exclusion (Meynell *et al*., 1968).

Measurement of incompatibility

The colony test was employed to obtain the degree of incompatibility (Burman and Nordström, 1971; Uhlin and Nordström, 1975). To prepare transconjugant clones, two techniques, antibiotic selection for the entering plasmid and double-antibiotic selection for both plasmids, were employed (Taylor and Grant, 1977). The kinetics of incompatibility between pHH303 and R702, for example, were measured with cells from clones isolated by double-antibiotic selection (Datta and Barth, 1976; Taylor and Grant, 1977; Uhlin and Nordström, 1975).

Plasmid curing

Two methods, employing acriftavine (Barth *et al*., 1976) and mitomycin C (Chakrabarty, 1972), with some modifications described below, were used.

Spontaneous loss of resistance

The method of Cornelius *et al*. (1976), with modifications, was employed.

Results

Phenotypic characters determined by deletion plasmids

Three deletion plasmids, pHH301, pHH302 and pHH303, were obtained from RP1-transformed strains of *P. aeruginosa*. The fourth, pHH401, was isolated from a transformed *E. coli* strain. These plasmids, like RP1, could be conjugally transferred to various bacterial species, *e.g*., *Salmonellatyphimurium* , *Proteus mirabilis* and *P. putida*. These plasmids were transferred to a new host J62 (table 1) to prove that the resistance was actually located on plasmids. The strains harbouring

the four deletions were resistant to fewer antibiotics than the strains harbouring RP1 (table 1). It has been shown that strains behaving phenotypically as antibiotic-resistant cells possessed, along with the chromosomal DNA, covalently-closed circular DNA molecules of characteristic molecular weights and monomeric sizes (unpublished observations).

Quantitative studies of incompatibility

The plasmid R702 was introduced into strain J62–pHH303, and clones carrying both plasmids were selected on LA plates containing carbenicillin and streptomycin. Colonies were picked and transferred to LB media without antibiotics. During subsequent cultivation, both plasmids were lost exponentially and with almost equal frequency; 7% per cell doubling time (figure 1 and table 2). Some segregation had already occurred in the colony. This is more readily observed in the results from colony tests presented in table 2, which also show that the two plasmids were lost with equal frequency. Table 3 also shows that similar results were obtained irrespective of direction of transfer. Figure 1 and tables 2 and 3 show the same pattern of incompatibility behaviour between the three deletion plasmids pHH301, pHH302 and pHH40l and R702.

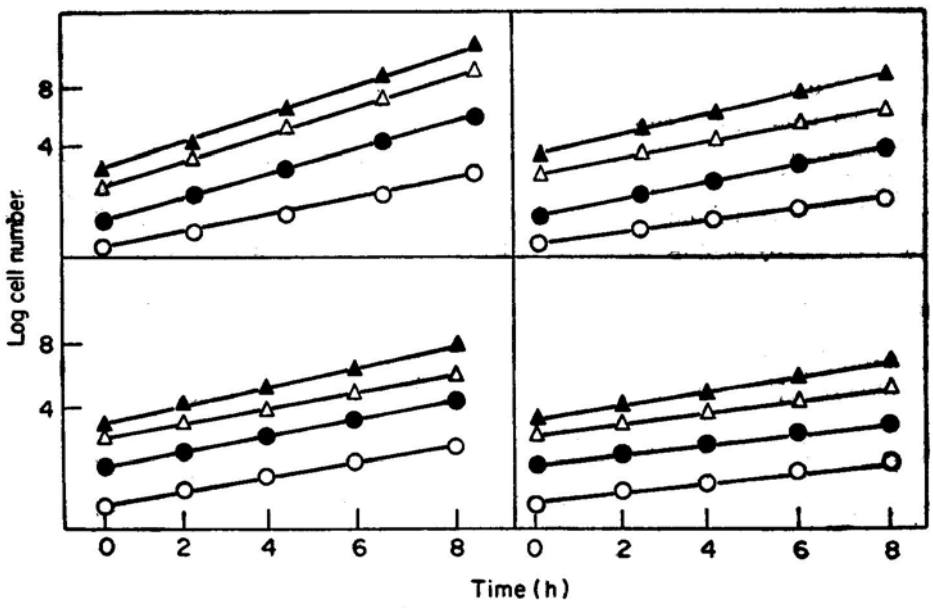

Figure 1. Segregation of deletion plasmids and R702 in cells carrying both plasmids. Clones of strain J62 containing deletion plasmids and R702 were selected on LA plates by double-antibiotic. selection and grown in drug-free LB medium at 37° C. Samples were withdrawn and tested for the number of total viable cells, ▲; deletion plasmid cells, ○; R702 cells, ●; cells; with both plasmids, Δ.

(*a*) J62–pHH301/R702, (*b*) J62–pHH302/R702, (*c*) J62–pHH303/R702 and (*d*) J62–pHH401/R702.

Table 2. Quantitative incompatibility test of the deletion plasmids and the test. Plasmid R702.

Incoming plasmid	Resident plasmid	Frequency of loss[a] (% per cell doubling)	
		Deletion plasmid	R702
R702	pHH301	9	8
R702	pHH302	6	7
R702	pHH303	10	8
R702	pHH401	8	9

[a] Calculated from figure 1.

Table 3. Colony test for incompatibility between the deletion and test plasmids.[a]

Incoming plasmid	Resident plasmid	% Daughter colonies containing		
		Deletion plasmid	Test plasmid	Both
R702	pHH301	40	34	26
pHH301	R702	45	37	18
R702	pHH302	46	35	19
pHH302	R702	41	36	13
R702	pHH303	81	3	16
pHH303	R702	61	27	22
R702	pHH401	41	42	17
pHH401	R702	44	33	23
R751	pHH301	45	32	23
pHH301	R751	52	28	20
R751	pHH302	43	31	26
pHH302	R751	48	36	16
R751	pHH303	76	8	16
pHH303	R751	68	22	10
R751	pHH401	48	28	24
pHH401	R751	40	37	23
R906	pHH301	38	50	12
pHH301	R906	42	36	22
R906	pHH302	47	40	13
pHH302	R906	46	34	20
R906	pHH303	51	37	12
pHH303	R906	39	40	21
R906	pHH401	47	41	12
pHH401	R906	43	40	17

[a] Deletion Plasmids were carried by J62 and test plasmids were carried by J53.

Incompatibility of two other plasmids

R751 and R906, both belonging to P incompatibility group, and the four deletion plasmids were studied. Our results (table 3), involving R751 and R906, reveal and confirm the same pattern of behaviour as described with R702 above. Identical incompatibility patterns were obtained when resistance to streptomycin and tetracycline were used as the markers (figure 2 and table 4).

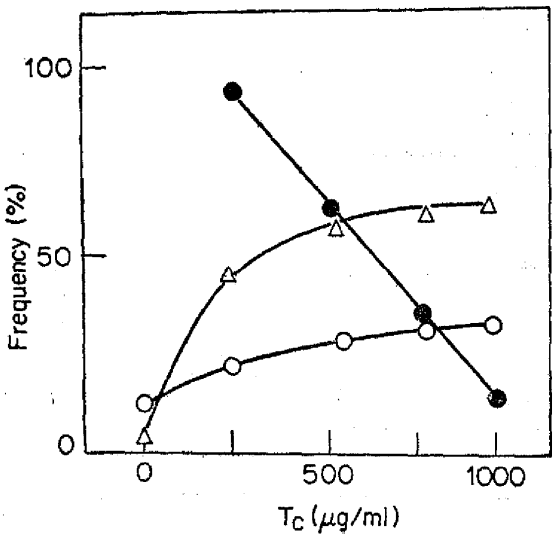

Figure 2. Transfer of test plasmid to strain J62-pHH303.

Selection for recipient cells, carrying both plasmids, was made on LA plates containing nalidixic acid, streptomycin (50 μg per ml) and different amounts of tetracycline R702 cells, ● ; pHH303 cells ○ ; cells with both plasmids, Δ.

A complicating factor in the results presented above is the fact that the segregational event is underestimated as plasmids are transferred during the experiments. The effect of transfer, however, was small since the rate of transfer of pHH303 for example, was about 1% per generation if the recipient contained the test plasmid R702. This was shown, for example, by growing strains J53–R702 and J62–pHH303 for at least 8 doublings, diluting the cultures to 10^4 cells per ml, mixing the two cultures and incubating the mixture at 37° C. Samples were taken at intervals for viable counts, for growth in the presence of Tc and Sm and also in the presence of both antibiotics. Essentially the same result was obtained if the deletion plasmid pHH401 was transferred to a recipient carrying R702 as if the donor carried the deletion (table 4).

Loss of plasmids from the cells

Treatment of the plasmid-carrying cells with either of the compounds resulted in a certain population of the cells losing the plasmids as judged by the loss of anti-

Table 4. Conjugal transfer of P plasmids.

Donor	Recipient	Transfer frequency[a]
J53–R702	J62	7×10^{-3}
J53–R702	J62–pHH301	2×10^{-3}
J53–R702	J62–pHH302	4×10^{-3}
J53–R702	J62–pHH303	6×10^{-3}
J53–pHH401	J62–pHH401	7×10^{-4}
J62–pHH301	J53	1×10^{-3}
J62–pHH302	J53	3×10^{-3}
J62–pHH303	J53	5×10^{-3}
J62–pHH401	J53	7×10^{-3}
J62–pHH301	J53–R702	3×10^{-3}
J62–pHH302	J53–R702	7×10^{-3}
J62–pHH303	J53–R702	5×10^{-3}
J62–pHH401	J53–R702	4×10^{-3}

[a]Frequencies of recipients carrying donor plasmids per input donor. Determined from 16 h mating at 37° C in LB media.

biotic substance. Table 5 shows that at concentration of the mutagens greater than 10 μg/ml, about 15–20% of the cells were cured. Using appropriate markers (carbenicillin and tetracycline) similar results were obtained with strains HH2101, HH2102 and HH1501. Simultaneous loss of resistance to carbenicillin and tetracycline in strains carrying plasmids with multiple resistance markers showed that mitomycin C did not act as a mutagen but brought about loss of the plasmids.

Spontaneous loss of antibiotic resistance

Spontaneous loss of carbenicillin resistance in HH2103, grown at 32° C, occurred on an average in 1·6% of the clones (table 6). The carbenicillin-sensitive clones were also sensitive to tetracycline. On the other hand, cultures grown at 42° C, possessed, on an average, 53% clones which became sensitive to carbenicillin during their growth. This demonstrates that strain HH2103, harbouring pHH303, while growing for 20 generations at 42° C, tended to spontaneously loose its plasmid and 53% of its daughter cells failed to harbour the plasmid during their growth and division. Similarly after 20 generations at 42°C, HH2101 possessed 47% clones which lacked resistance to carbenicilin while HH2102 possessed 51 % clones which were sensitive to carbenicillin, HH1501, harbouring pHH401, was affected the least by growth at 42° C. This strain, when grown for 20 generations, lost the plasmid to an extent of 1% when grown at 32°C and to about 17 20% when grown at 42°C. At 42°C, the deletion plasmids, pHH301, pHH302 and pHH303, harboured by J53 strains tended to spontaneously segregate out much less frequently than when harboured by HH2060. On an average about 20% of the harbouring J53 cells turned sensitive to either carbenicillin or tetracycline or to both after growth at the elevated temperature.

Table 5. Curing of carbenicillin.degradation from *P. aeruginosa* HH2103 by mitogens.

	Colonies examined		Cb⁻
	Total	Cb⁻[b]	(%)
(A) With acriflavine			
Acriflavine[a] (μg ml^{-1})			
0	196	2	1·04
5	208	11	5·3
10	212	34	16·0
20	216	44	19·0
30	152	24	15·7
(B) With mitomycin C			
Mitomycin (μg ml^{-1})			
0	210	2	0·95
2·5	205	7	3·4
5·0	204	36	17·6
10·0	135	24	17·7
20·0	100	16	16·0

[a] Cultures irradiated for 1 min and growing in the presence of given amounts of mitogens were examined.

[b] For scoring Cb⁻ phenotype, single colonies obtained after mitogen treatment were applied to nutrient-agar plates with Cb. The colonies which failed to grow on these plates were taken as Cb⁻ clones.

* Values obtained with 12 independent cultures of the same clone (6 cultures at 32° C, 6 cultures at 42° C). Cb, carbenicillin.

P. aeruginosa HH2103 (harbouring the plasmid pHH303) grown in nutrient broth was washed and suspended in Tris buffer (0·02 M, pH 8·0) at a density of about 10^4 cells per ml. The cells were transferred to L-both containing graded dose (0, 2·5, 5·0, 10 and 25 μg) of either acriflavine or mitomycin C and incubated overnight. The cells were pretreated with graded doses of UV before transferring to acriflavine medium. Appropriately diluted cultures were applied to L-agar plates and the colonies replicated on to nutrient agar plates containing 25 μg/ml carbenicillin to score for sensitive clones.

Discussion

The deletion plasmids, derived from RP1 DNA-transformed bacterial clones, are strongly incompatible with P group plasmids, R702, R751 and R906. Compatibility between a pair of plasmids is an ability on the part of the host to support the replication of both plasmids. When pHH303 and R702 were introduced together in *E. coli* K12 they were unstable. This was the situation with the other deletion

Table 6. Spontaneous loss of carbenicillin degradation character in *P. aeruginosa* HH2103 after 20 generations of growth*.

32° C				42° C			
No. of colonies tested	Cb⁻ colonies		average	No. of colonies tested	Cb⁻ colonies		average
	No.	%			No.	%	
136	2	1·3		122	76	62	
81	1	1·2		134	78	55	
186	3	1·5	1·6	102	49	48	53
121	2	1·6		86	48	56	
71	1	1·4		98	56	57	
86	1	1·2		78	32	41	

* Values obtained with 12 independent cultures of the same clone (6 cultures at 32° C, 6 cultures at 42° C). Cb, Carbenicillin.

P. aeruginosa HH2103 (harbouring the plasmid pHH303) was grown in fortified minimal medium containing carbenicillin (20 μg/ml) for 18 h at 32° C. The culture was subsequently grown for 2 generations in the same medium in the absence of the antibiotic. After appropriate dilution the cells were plated on minimal agar and one clone was used to make several cultures to be grown at 32° and 42° C. After 20 generations samples from each culture were replica-plated on L-agar and L-agar containing carbenicillin.

plasmids as well. In the experiments described above R702, as well as R751 and R906, were lost more frequently than the deletion plasmids. It seems, therefore, that deletion plasmids are higher than R702, R751 and R906 in the 'hierarchy' of P plasmids, but this position can be reversed by relatively trivial and uncontrolled changes in the conditions of experiments (Datta and Barth, 1976).

Treatment of *P. aeruginosa* and *E. coli* strains, containing the deletion plasmids, with either acriflavine or mitomycin C, renders a certain percentage of these strains sensitive to either carbenicillin or tetracycline or to both. The data presented above, demonstrating the curing effect of acriflavine on plasmid-positive strains, are consistent with results concerning the exclusion effect of mitomycin C.

All the strains, harbouring the deletion plasmids when grown at an elevated temperature, showed characteristic segregation of R^- clones from the R^+ colonies. While in *P. aeruginosa* the deletions were able to segregate out spontaneously much more easily, these plasmids in *E. coli* K12 strains were less able to do so. Attempts at detecting the breakdown products of plasmids into various determinants have been unsuccessful and it could be speculated that the deletion plasmids possess defects in their *tra* operons which make them susceptible to segregation at the elevated temperature more easily in *P. aeruginosa* than in *E. coli* K12 strains.

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