Functions Antagonistic to Plasmid Formation by λN^- Chromosomes

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It was concluded in the preceding paper that λN^- cI^- genomes probably failed to form plasmids within infected *Escherichia coli* cells because they leakily express functions that act to destabilize the plasmid state. This prediction was investigated by examining the effect upon plasmid-forming ability of the loss of possible anti-plasmid functions. The loss of Ter function was found to allow long-term plasmid formation, although the efficiency of initial plasmid formation and the heritable stability without selection were low. The combined loss of the *int*, *red* and *gam* gene functions also promoted plasmid growth, although the absence of Ter^A was necessary. In contrast, the presence of Ter⁸⁰ (due to an *h*⁸⁰ substitution) did not prevent plasmid formation when the *int*, *red* and *gam* genes were absent, indicating that Ter⁸⁰ does not attack the closed-circular form of the λ chromosome. The combined loss of the *ter*, *int*, *red* and *gam* gene functions facilitated fully efficient inheritance of the λN^- cI^- plasmids in the absence of selection, although the efficiency of initial plasmid formation remained low. However, cells harbouring such plasmids suffered a decline in viability, indicating that the plasmids expressed a function (or more than one) that acts to debilitate the host cells – presumably an effect that is increased with this genotype because the modified λN^- cI^- plasmids are inherently more stable. The possible involvement of the λ *S* and *kil* functions in destabilization is discussed.

INTRODUCTION

Mutants of bacteriophage λ lacking *N* gene function are able to form plasmid replicons within infected *Escherichia coli* cells (Signer, 1969; Kleckner & Signer, 1977). However, they do so inefficiently and the plasmid state is very unstable and short-lived (Hadfield & Brammar, 1984). In the latter paper, we suggested that the cause of these defects in plasmid formation is the *N*-independent leaky expression of certain λ functions that act to destabilize the plasmid state. If so, then the elimination of such functions by mutation should enhance plasmid formation and stability.

Candidates for the role of antagonizing plasmid formation are the products of the *int*, *red* and *gam* genes, the *b*-region encoded endonucleases and the packaging functions. The *int*, *red* and *gam* gene products act to modify the structure of the monomeric closed-circular λ DNA, which is formed initially upon infection. Potential plasmid destabilization by the packaging system is mediated by the terminase function (Ter), through its binding-cutting action at the *cos* site and the initiation of DNA encapsidation. Endonucleases encoded by the *endo* and *ben* genes are able to cleave both λ and *E. coli* DNA.

The *endo*, *ben*, *int*, *red* and *gam* genes are all dispensable for lytic phage growth, and are located within a region of the λ genome that can readily be deleted or replaced. The terminase is encoded by two *ter* genes, *A* and *Nu1*, both of which are essential for lytic growth (Wang & Kaiser, 1973; Becker & Gold, 1978). Thus, Ter function can be eliminated by available amber mutations in gene *A*, which give a functional *A* protein in suppressing host cells (to enable lytic phage propagation) but eliminate Ter activity in non-suppressing cells.

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In this paper, we report the effects of eliminating the *int*, *red*, *gam* and *A* gene functions upon plasmid formation by λN^- phage genomes, and also of the presence of $\phi 80$ left-arm substitutions affecting the endonuclease and terminase genes. Our results show that the plasmid state can be stabilized by the use of appropriate mutant phage genotypes.

METHODS

Bacterial and phage strains. These are listed in Tables 1 and 2, respectively.

General techniques. Media, and methods other than those detailed below, were as described by Hadfield & Brammar (1984).

Analysis of the DNA species present in cells by agarose-gel electrophoresis. Cells from 1 ml overnight-grown liquid culture were pelleted in an Eppendorf microfuge and resuspended in 70 μ l 25% (w/v) sucrose, 50 mM-Tris/HCl pH 8.0, and then cooled on ice. Lysozyme (10 μ l of 10 mg ml⁻¹ in 0.25 M-Tris/HCl pH 8.0) was gently stirred in, and after 5 min on ice 20 μ l 0.25 M-EDTA pH 8.0 was added. After a further 10 min on ice, 60 μ l 2% (v/v) Triton X-100, 50 mM-Tris/HCl pH 8.0, 65 mM-EDTA was gently stirred in, and following a further 5 min on ice the tube was transferred to a 70 °C water bath for 5 min to ensure total lysis. The tubes were then spun at 15000 r.p.m. for 30 min and the centrifuge stopped without braking. The supernatant was decanted into another Eppendorf tube and gently extracted with 1 vol. phenol (redistilled, water-saturated and equilibrated with 10 mM-Tris/HCl pH 8.0 before use) plus 1 vol. chloroform. After spinning in a microfuge for 5 min, the upper phase was carefully removed with a Pasteur pipette, avoiding the protein debris at the interface. This was re-extracted with phenol/chloroform, the upper phase was transferred to a new tube, and 0.1 vol. sodium acetate pH 5.6 was added, followed by 2 vols absolute ethanol. After gentle mixing, the precipitated DNA (plus RNA) was pelleted by a 10 min spin in a microfuge. The tube was drained and the pellet washed with 70% (v/v) ethanol. The pellet was vacuum dried and resuspended in 100 μ l 10 mM-Tris/HCl, 1 mM-EDTA, pH 8.0.

Table 1. *E. coli* strains

Strain	Genotype	Reference or Construction
AA129	<i>sup^c recA56 lacP⁺ lacZ^ΔM15 (gal- atr⁺-bio-uvrB)^Δ trp^ΔLD102</i>	Anilionis (1977)
QR47	<i>supE</i>	Weil & Signer (1968)
Ymel	<i>supF</i>	Goldberg & Howe (1969)
C600	<i>supE tonA thr leu thi</i>	Appleyard (1954)
CH28	<i>supF tonA</i>	T5 selection of Ymel
CH51	<i>supE tonA</i>	T5 selection of QR47
CH131	<i>supE recA1 tonA</i>	Hadfield & Brammar (1984)
CH157	<i>supE tonA thr leu thi</i> (P2)	Lysogen of C600

Table 2. Phage λ strains

λ stock no. (CH#)	Genotype	Reference or cross
1	λ^+	—
6	λimm^{21}	Liedke-Kulke & Kaiser (1967)
7	λcI^{26}	Meselson (1964)
54	$\lambda imm^{21} Sam7$	W. J. B., unpublished
58	$\lambda h^{80} trp51 Nam7, am53$	Brammar <i>et al.</i> (1974)
138	$\lambda h^{80} trp50 Nam7, am53 cItsA12$	Franklin (1971)
159	$\lambda Aam32$	Wang & Kaiser (1973)
161	$\lambda Aam32 imm^{21} cI^-$	$\lambda 159 \times \lambda 229$: clear plaques on CH28
164	$\lambda h^A Aam32 trp50 Nam7, am53 cItsA12$	$\lambda 138 \times \lambda 161$: selected on CH157; purified on CH51
187	$\lambda h^A Aam32 trpBG2 Nam7, am53 cI^A KH54$	Hadfield (1980); Murray & Brammar (1973)
229	$\lambda h^{80} atr^A imm^{21} cI^-$	N. E. Murray (Edinburgh University), unpublished
230	$\lambda plac5 imm^{21} Pam80$	R. Pastrana (Edinburgh University), unpublished
253	$\lambda h^A trp50 Nam7, am53 cItsA12$	$\lambda 138 \times \lambda 6$: selected on CH157; purified on CH51
285	$\lambda h^A Aam32 trp51 Nam7, am53 cI^-$	$\lambda 58 \times \lambda 164$: selected on CH131
286	$\lambda h^A trp51 Nam7, am53 cI^-$	$\lambda 58 \times \lambda 253$: selected on CH131

Samples (10 μ l) of this preparation were mixed with an equal volume of 10% (w/v) Ficoll, 0.05% (w/v) xylene cyanol FF and loaded into a slot of a 1% (w/v) agarose gel (Tris/acetate buffer pH 8.2) for electrophoresis. Afterwards, the gel was stained by 15 min immersion in ethidium bromide (2.5 μ g ml⁻¹), then washed for the same period in distilled water. This after-staining avoided loss of the supercoiled plasmid DNA band during the run due to unwinding caused by the intercalating dye (see Bauer *et al.*, 1980).

Transfer of DNA from agarose gel to nitrocellulose filter, labelling of probes, hybridization and autoradiography. DNA was bidirectionally transferred from the agarose gel by the method of Smith & Summers (1980). DNA probes were labelled with ³²P by nick-translation, and the hybridization and autoradiography performed essentially as described by Jeffreys & Flavell (1977).

Preparation of phage DNA and isolation of restriction fragments. Phage DNA was prepared by Murray *et al.* (1977). DNA fragments resulting from digestion by restriction enzymes (Bethesda Research Laboratories) were separated at 4 °C in a horizontal gel of 0.8% low-gelling-temperature agarose (Miles) in Tris/acetate buffer (40 mM-Tris, 20 mM-sodium acetate, 1 mM-EDTA, pH 8.2) containing 10 μ g ethidium bromide ml⁻¹. Bands were cut out of the gel under UV illumination, placed in a 2 ml Eppendorf tube with 3 vols 8 M-sodium perchlorate (precleaned by passing through a glass-fibre filter) and heated for 5 min at 65 °C to melt the gel. Afterwards 1 mg Kieselgel H60 (Merck product 7736) was added for every 200 μ l, mixed and the mixture left for 10 min at room temperature. The DNA-bound Kieselgel was pelleted by a 2 min spin in a horizontal microfuge and the supernatant discarded. The pellet was washed by dispersing in 3 vols 6 M-sodium perchlorate (pretreated as above), repelleting and then draining off the supernatant. DNA was eluted from the Kieselgel by dispersing in 200 μ l TE (10 mM-Tris/HCl pH 8, 1 mM-EDTA) and pelleting the Kieselgel. The pellet was re-extracted with 100 μ l TE and the supernatants were pooled.

The DNA was cleaned by precipitation, by addition of 33 μ l 2 M-sodium acetate pH 5.6 and 667 μ l ethanol. After a 15 min spin in a microfuge, the DNA pellet was washed with 70% ethanol, vacuum dried and resuspended in 300 μ l TE. Residual carbohydrate contamination was removed by adding 300 μ l 2.5 M-potassium phosphate pH 8.0 and 300 μ l methoxyethanol, and mixing. After spinning for 5 min in a microfuge, the upper phase was removed to a new tube and gently mixed with an equal volume of ethanol. After a 5 min spin in the microfuge, the DNA resided on the surface of a droplet of phosphate buffer at the bottom of the tube. This droplet was removed with a pipette and the rest of the solution drained off, leaving the DNA in the tube. The DNA was then washed with 70% ethanol and any further phosphate droplets were removed in the same way. After a third 70% ethanol wash (no droplets should be present or else a further wash is necessary), the DNA was drained free of ethanol, vacuum dried and then resuspended in 50 μ l TE.

RESULTS

Choice and construction of phages

The choice of λN^- transducing phages was governed by several factors. First was the requirement to have phages of the same overall genetic composition, barring those differences that were specifically required for individual experiments. Secondly, the *red⁻ gam⁻* phage employed must also be able to propagate adequately by lytic growth. Efficient lytic growth on a *rec⁺* host (also *sup⁺* due to the need to suppress amber mutations) by such a phage requires the presence of a *chi* site (Enquist & Skalka, 1973; McMilin *et al.*, 1974; Henderson & Weil, 1975). It was also required to be able to make *Aam* mutants. Use of *h⁸⁰* provided a convenient counter-selectable marker for the introduction of *h^A*, particularly so when *Aam* was to be introduced, since the *N* gene also contained amber mutations. *h⁸⁰* is also of interest, as well as a genetical tool, since it encodes the packaging functions of the related lambdoid phage $\phi 80$. Phages λ and $\phi 80$ package their DNA in a similar manner, and their terminases perform the same functions. The $\phi 80$ and λ terminases are encoded by equivalent genes and are composed of similar subunits (Sumner-Smith & Becker, 1981; Sumner-Smith & Gold, 1981). The terminal sequences of the two phages are the same and the terminases are able to cross-react (Murray *et al.*, 1975). Other leakily expressed products of genes contained within the *h^A* and *h⁸⁰* regions (extending from *cos_L* to *att*) may affect plasmid formation differently. Thus, the λ -encoded endonucleases may differ in activity from similar $\phi 80$ endonucleases. Differences due to gene content may also occur. Although the gene content of λ and $\phi 80$ is similar (Yoderain, 1977), the $\phi 80$ genome is 8% smaller than that of λ , indicating the possible absence of some genes that are not essential for growth.

The λN^- transducing phages chosen were $\lambda h^{80} trp51 Nam7.am53 cI^-$ and $\lambda h^{80} trp50 Nam7.am53 cI::At2$. Both phages contain the *trpED* genes, the leader sequence, the attenuator and the

tryptophan-free (TRP^-) and tryptophan-containing (TRP^+) plates. The maintenance stability value was given by the percentage ratio of the number of colonies that grew on TRP^- plates over those on TRP^+ plates. The plasmids were well maintained, again indicating good viability of this genotype. A stability value of 91% was observed for singly-infected cells, and similar values for multiply-infected cells, showing that the number of input phages did not influence plasmid maintenance (Table 3b). When transduced cells were grown up in TRP^- liquid medium, the resulting cultures showed no evidence of cell lysis. This contrasts with the selected λN^- *vir* transductants previously examined, of which selected cultures contained a proportion of lysed cells (Hadfield & Brammar, 1984). Presumably, leaky expression of the lysis proteins by the λh^{80} *trp50* (*int red gam*) $^\Delta$ *Nam7,am53 cIts* plasmids was low enough to be tolerated by the host cells.

The presence of intracellular λ genomes was examined by making lawns of transduced cells and spot-testing. All 20 isolates tested showed the same responses: (1) they allowed the growth of λimm^{21} ; (2) λimm^{21} *Pam80* produced occasional plaques, indicating either *P* product complementation or marker rescue of a functional P^+ allele from intracellular λ genomes; (3) λimm^{21} *Sam7* also grew, giving a confluent spot, presumably due to complementation involving activation of the plasmids' p_R promoter by the *Q* gene product of the superinfecting phage; (4) λcI^- grew, indicating that the transduced cells were not fully anti-immune; (5) λcI^+ grew to form a turbid spot, showing that there was too little *cro* protein within the carrier cells to inhibit *cI*-repressor synthesis.

Thus, although λh^{80} *trp50* (*int red gam*) $^\Delta$ *Nam7,am53 cIts* had greatly improved plasmid characteristics, it still had the flaws of lower than maximal initial plasmid formation and maintenance in non-selecting conditions. Lack of anti-immunity of the carrier cells may also indicate less than maximal plasmid copy number. Such defects might be due to an inhibitory function, such as the action of *Ter*.

Effects of the loss of the int, red and gam genes on λh^+ N^- plasmid formation

Plasmid formation by λh^+ *trp50* (*int red gam*) $^\Delta$ *Nam7,am53 cIts* was compared with that by λh^+ *trp51 int* $^\Delta$ *Nam7,am53 cI^-* as the non-deletion control. The *trp50* variant transduced exceedingly rarely when infecting singly, producing very tiny colonies (about 0.2 mm in diameter) that remained small and could not be sub-cultured. The results otherwise showed complete failure to transduce in the plasmid mode by either genotype (Table 3c, d). From these results it would seem that the success of λh^{80} *trp50* (*int red gam*) $^\Delta$ *Nam7,am53 cIts* can be attributed to the combined presence of the h^{80} left-arm substitution and the *int-red-gam* deletion. This suggests that the $\phi 80$ terminase, unlike that of λ , is unable to attack and destabilize the monomeric closed-circular plasmid form of the λ chromosome.

Effects of the loss of Ter function on λh^+ N^- plasmid formation

The effects of *Ter* function on λN^- plasmid formation were examined by contrasting plasmid-forming ability when the enzyme was present and absent. Initially this was done with the *red* and *gam* genes present. Thus, λh^+ *Aam32 trp51 int* $^\Delta$ *Nam7,am53 cI^-* was examined for *Ter^-* plasmid formation, with the λh^+ *A⁺ trp51 int* $^\Delta$ *Nam7,am53 cI^-* variant tested previously (Table 3c) serving as the *Ter^+* control. With active *Ter^+* present the phage completely failed to transduce in the plasmid mode. In contrast, the *Ter^-*-defective *Aam* variant transduced 5–6% of the infected cells when infecting singly and more when infecting multiply, reaching a maximum of 14.5% at an m.o.i. of 6 (Table 3e). These transduced cells continued to grow, forming progressively larger colonies, indicating the continued viability of the established plasmids. The *ter^-* plasmids were also quite well maintained during non-selecting growth, at a level of around 10% in the stability test. From these results it is clear that *Ter^+* has a highly effective anti-plasmid activity that can prevent λN^- plasmid formation, both when the DNA is circular monomeric (as with λh^+ *trp50 Nam7,am53 cIts*) and when it is concatenated (as apparent with λh^+ *trp51 Nam7,am53 cI^-*).

Absence of *Ter* activity is therefore essential for successful plasmid formation by λh^+ N^- genomes. This supports the view that the benignity of h^{80} to plasmid formation in the absence of

Table 3. *Effects of red and gam, of host range and of Ter function on the establishment and stability of λ plasmids*

Strain AA129 (*sup^o recA trpED^A*) was transduced to *Trp⁺* with (a) λh^{80} *trp51 int^A Nam7am53 c1⁻*, (b) λh^{80} *trp50 (int red gam)^A Nam7am53 c1tsAt2*, (c) λh^A *trp51 int^A Nam7am53 c1⁻*, (d) λh^A *trp50 (int red gam)^A Nam7am53 c1tsAt2*, (e) λh^A *Aam32 trp51 int^A Nam7am53 c1⁻*, (f) λh^A *Aam32 trp50 (int red gam)^A Nam7am53 c1tsAt2*. Both the frequency of transduction and the maintenance stability figures are mean values for four independent measurements at each m.o.i. Variation between the independent measurements on either side of the mean was within one-tenth of the mean value for figures over 5% and within one-fifth of the mean for figures under 5%.

M.o.i.	Frequency of initial transduction (%)	Maintenance stability (%)	Frequency of initial transduction (%)	Maintenance stability (%)
(a) $\lambda red^+ gam^+ h^{80}$ (<i>Ter⁺</i>)			(b) $\lambda red^- gam^- h^{80}$ (<i>Ter⁺</i>)	
10 ⁻⁴	< 10 ⁻²	ND	22.2	91.1
0.1	< 10 ⁻²	ND	22.5	90.3
1	2.1	ND*	25.7	92.0
2	5.1	ND*	40.6	97.7
6	4.5	ND*	100.0	98.0
10	3.3	ND*	54.0	92.5
20	2.5	ND*	50.1	92.4
(c) $\lambda red^+ gam^+ h^A$ (<i>Ter⁺</i>)			(d) $\lambda red^- gam^- h^A$ (<i>Ter⁺</i>)	
10 ⁻⁴	< 0.5 $\times$ 10 ⁻⁵	ND	4.5 $\times$ 10 ⁻⁵	ND*
0.1	< 0.5 $\times$ 10 ⁻⁵	ND	4.3 $\times$ 10 ⁻⁵	ND*
1	< 0.5 $\times$ 10 ⁻⁶	ND	9.4 $\times$ 10 ⁻⁶	ND*
2	< 0.5 $\times$ 10 ⁻⁶	ND	< 0.5 $\times$ 10 ⁻⁶	ND
6	< 0.5 $\times$ 10 ⁻⁶	ND	< 0.5 $\times$ 10 ⁻⁶	ND
10	< 0.5 $\times$ 10 ⁻⁶	ND	< 0.5 $\times$ 10 ⁻⁶	ND
20	< 0.5 $\times$ 10 ⁻⁶	ND	< 0.5 $\times$ 10 ⁻⁶	ND
(e) $\lambda red^+ gam^+ h^A$ <i>Aam32</i> (<i>Ter⁻</i>)			(f) $\lambda red^- gam^- h^A$ <i>Aam32</i> (<i>Ter⁻</i>)	
10 ⁻⁴	6.1	7.1	5.3	133.5
0.1	5.5	7.2	5.4	133.3
1	5.7	7.9	5.3	134.5
2	8.2	9.2	8.3	135.0
6	14.5	10.8	10.1	135.0
10	13.5	11.4	9.7	134.0
20	13.5	4.9	5.6	130.7

ND, Not done due to failure to obtain transductants; ND*, not done due to failure of transductants to subculture.

the *int*, *red* and *gam* genes, but not in their presence, is due to failure of *Ter*⁸⁰ to attack monomeric circular forms of the λ genome. Although absence of *Ter* function led to improved plasmid-forming ability, the inability to transduce with complete efficiency and to be maintained completely within the cells during growth without selection indicates that other defective characteristics remain, as is known to be the case. Several of the transduced isolates were spot-tested for the presence of intracellular λ genomes and for anti-immunity, as described for the λh^{80} *trp50 Nam7am53 c1ts* transductants. Intracellular λ genomes were detected, but the cells were not anti-immune. Thus, plasmid numbers were not apparently being controlled by *cro* protein autorepression.

Effects on λh^A N⁻ plasmid formation of the combined loss of Ter and the int-red-gam gene functions

The combination of a *ter* deficiency with an *int-red-gam* deletion eliminates two proven sources of λ plasmid instability. The question remains as to whether these functions are the only ones that destabilize the plasmid. Analysis of a λN^- phage carrying both of these alterations was undertaken to reveal if other functions remain to prevent non-defective plasmid formation and growth. Thus the phage λh^A *Aam32 trp50 (int red gam)^A Nam7am53 c1ts* was tested for plasmid formation, in comparison with the similar λh^A *A⁺ trp50 (int red gam)^A Nam7am53 c1ts* variant tested previously (Table 3d), which was totally defective, and the λh^A *Aam32 trp51 int^A*

Nam7,am53 cI⁻ variant (Table 3e) that showed modest plasmid-forming ability. Initial plasmid formation by λh^+ *Aam32 trp50 Nam7,am53 cIts* was at a similar frequency to that found with the λh^+ *Aam32 trp51 Nam7,am53 cI⁻* variant (Table 3f). The transduced colonies kept growing, and selected liquid cultures showed no evident cell lysis. However, the colonies were small: approximately 0.2 mm in diameter after 24 h incubation at 37 °C, compared with about 2.0 mm for uninfected cells or cells transduced by other λ plasmids.

Plasmid maintenance stability values showed a change with the λh^+ *Aam32 trp50 (int red gam)⁺ Nam7,am53 cIts* genotype. Values of over 130% were found, independent of the initial m.o.i. Since the same numbers of cells were plated on both the TRP⁻ and TRP⁺ plates for these stability tests, it must be interpreted that fewer were able to grow on the non-selecting plates after growth in non-selecting liquid medium. Thus selection for growth with tryptophan supplied endogenously by the enzymes encoded on the plasmids evidently enabled greater survival of the transduced cells than when the tryptophan was supplied externally.

The colonies obtained after growth in TRP⁺ broth, on both the TRP⁻ and TRP⁺ plates, were of two distinguishable sizes: either ~2 mm or ~0.2 mm in diameter after 24 h incubation at 37 °C. When colonies were picked from TRP⁻ plates on to fresh TRP⁺ plates, three types of growth response were obtained. The larger colonies either continued to grow well or grew only slightly. The small colonies either grew slightly or failed to grow at all. When those colonies that grew were picked again on to TRP⁺ plates a further decrease in ability to grow was observed. This seems to indicate that the viability of the plasmid-carrying cells progressively declines. However, contrarily, this measurable decline in individual colony viability did not coincide with a demise of the transduced population as a whole, since liquid cultures of the plasmid-carrier cells could be grown up to normal stationary phase density in both selecting and non-selecting medium. The phenomenon that the viability of transduced cells is greater under plasmid-selective conditions than in non-selecting conditions cannot be explained from the data available here. However, it does seem that the λh^+ *Aam32 trp50 (int red gam)⁺ Nam7,am53 cIts* plasmids express a protein that debilitates the host cells in some way, without affecting the plasmids themselves. Spot-tests on transduced cells gave the same response as described earlier for the λh^{80} *trp50 Nam7,am53 cIts* variant. Again failure of the cells to display anti-immunity indicated that the intracellular λ genomes were probably not present at a maximal copy number controlled by *cro* protein autorepression.

Confirmation that the modified λN^- genomes transduce by forming plasmids

The host-phage system employed in this study was designed so that transduction could only occur via plasmid-forming λ genomes (Hadfield & Brammar, 1984). The lack of complete heritable stability of a transducing marker, as found in two cases, accords with its being plasmid-borne. Verification of the plasmid nature of the transducing λ genomes was sought by analysis of the DNA species within transduced cells.

Total DNA was prepared from transduced cells and compared with that from uninfected cells by agarose-gel electrophoresis. Only one DNA band was obtained from the uninfected cells, whereas a second band was also obtained from the transduced cells (Fig. 2a). The *int-red-gam* deficient phage genomes each formed bands of equivalent mobility to a closed-circular λ genome. However, in the cells transduced by λh^+ *Aam32 trp51 Nam7,am53 cI⁻* this additional band ran slightly faster, more towards the linear configuration, presumably as a consequence of the *red⁺ gam⁺* phenotype. The DNAs were prepared from equal volumes of overnight-grown cells. Although cell numbers were similar, the cells gave rise to different amounts of plasmid DNA, dependent upon genotype: λh^{80} *trp50 Nam7,am53 cIts* > λh^+ *Aam32 trp51 Nam7,am53 cI⁻* > λh^+ *Aam32 trp50 Nam7,am53 cIts*. Presumably this reflects the health of the plasmid and the plasmid-host relationship.

Confirmation that these bands result from extrachromosomal λ genomes was obtained by hybridizing one copy of a bitransfer of this gel with a ³²P-labelled λ fragment probe containing a segment of the DNA present in each of the transducing λ *trp N⁻* genomes but having no homology with cryptic λ prophage sequences present in the host cell's chromosome (Kaiser & Murray,

Fig. 2. Characterization of plasmid DNA. (a) Agarose-gel electrophoresis of the DNA from lysed *sup⁺ recA trpED^Δ* cells (AA129) transduced to *Trp⁺* growth by *λtrp N⁻* phages. (1) Non-transduced host control (grown in *TRP⁺* medium); (2) transduced by *λh⁸⁰ trp50 Nam7.am53 cItsAt2*; (3) transduced by *λh^Δ Aam32 trp51 Nam7.am53 cIt*; (4) transduced by *λh^Δ trp50 Nam7.am53 cItsAt2*; (5) closed-circular size marker, plasmid RP4 (55 kb); (6) *λ*-sized closed-circular size marker, cosmid clone cm.a47 (about 49 kb; Pirrotta *et al.*, 1983); (7) linear *λ* DNA extracted from phage particles (48.5 kb; Sanger *et al.*, 1982).

(b) Autoradiograph of one filter copy of a bitransfer of the above gel after hybridization with a ³²P-labelled *λ* fragment probe having no homology with the host bacterial chromosome but present in the *λ* transducing phage genomes. Because the *λ* genome has extensive homology with the *E. coli* K12 chromosome, the *λ* fragment probe used had to be carefully selected. Thus, restriction fragments of *λ* DNA were separated by agarose-gel electrophoresis, Southern transferred to a nitrocellulose filter and then hybridized with ³²P nick-translated AA129 host bacterial DNA. Those *λ* fragments having no homology with the bacterial DNA probe failed to give signals after autoradiography. One such fragment, generated by *Eco*RI cleavage at 65.6% *λ*⁺ and *Bam*HI cleavage at 71.0% *λ*⁺, was used as the probe for autoradiograph (b). It hybridized to each of the *λ* plasmid bands and the linear *λ* DNA control.

(c) Autoradiograph of the second copy filter after hybridization with a ³²P-labelled *trpE* fragment probe. This fragment (2.0 kb) was cleaved from *λtrpBG2* (*λ*CH187) phage DNA by restriction at the *Hind*III site in *trpD* (Hopkins *et al.*, 1976) and the *Bam*HI site in the *λ* DNA at 71.0% *λ*⁺. The *trpBG2* substitution ends very close to this *Bam*HI site, so that no homology with *λ* is detectable by hybridization. The *trpE* fragment probe only hybridized with the *trp*-transducing *λ* plasmid bands in autoradiograph (c).

1979). Autoradiography revealed hybridization to the additional bands in the transduced cells and not to the host chromosomal DNA (Fig. 2b). Because the probe fragment used crosses the *att-gam* deletion breakpoint associated with *trp50*, signals with this genotype are weaker. This is only noticeable with the smaller amount of *λh^Δ Aam32 trp50 Nam7.am53 cIts* DNA, since the signal from *λh⁸⁰ trp50 Nam7.am53 cIts* is strong enough to saturate the X-ray film response. The

second filter copy was hybridized with a *trpE* fragment probe, which comprised a segment of the *trp* operon present in the infecting λN^- transducing phage genomes, but wholly absent in the *trpLD102* deletion present in the host cells' chromosomes. Autoradiography showed hybridization to the λ plasmid bands, and none to the chromosomal DNA, nor the linear phage λ DNA (Fig. 2c). This degree of hybridization is sufficient to detect homologous sequences present in the *E. coli* chromosomal DNA, indicating that the transducing genes are probably present only on the plasmids and have not been transferred to the bacterial chromosomes.

DISCUSSION

The elimination of certain gene functions promoted plasmid formation by λN^- *cI*⁻ genomes. The genes involved must have been expressed, and their products acted in some way counter to the plasmid state, for their loss to have caused an improvement in plasmid-forming ability. However, because there are no other promoters, the only way that these genes could have been expressed in N^- conditions is for some of the transcripts initiated from *p*_L and *p*_R to have leaked through the serially arranged Rho-dependent terminators *t*_{L1}-*t*_{L2} and *t*_{R1}-*t*_{R2}, respectively. These results are therefore in accordance with our finding that substantial leaky transcription of the whole λ genome occurs in N^- conditions (Hadfield & Brammar, 1984). They also corroborate the proposal that the products of some genes so expressed act to destroy the plasmid form of the λN^- chromosome initially established upon infection of the host bacterial cell, and thereby make plasmid growth only transient.

The *Ter* function of λ was found to be a powerful anti-plasmid agent, since a further elimination of *Ter*^A activity alone was sufficient to facilitate long-term plasmid formation by λN^- *cI*⁻ genomes. Since the *red* and *gam* genes were present in this case, the intracellular forms of the $\lambda ter^- N^- cI^-$ plasmids were presumably not closed-circular, but to some degree polymeric and rolling-circular.

When the *red* and *gam* genes were also removed (the *int* gene was absent in both cases), newly formed plasmids were trapped in the monomeric closed-circular configuration within the *recA* host cells. Consequently the inheritance of the plasmids in the absence of selection was substantially increased, such that no plasmid-free segregants could be found. The efficiency of initial plasmid formation was not altered, remaining at the 5% level. It would seem therefore that if the λ plasmids are kept in the closed-circular monomeric form, their ability to propagate through subsequent generations is substantially enhanced – presumably because monomers are produced at the maximum possible copy number. However, some further defect must still remain on the $\lambda ter^- int^- red^- gam^- N^- cI^-$ chromosomes, which prevents them from establishing as plasmids with complete efficiency when they infect potential host cells.

$\lambda N^- cI^-$ plasmids carrying the *h*⁸⁰ left-arm substitution failed to form plasmids in the presence of the *red* and *gam* genes (*int* was absent), but did succeed when the *int*, *red* and *gam* genes were all absent. This suggests that for some reason, unlike *Ter*^A, *Ter*⁸⁰ does not attack closed-circular λ DNA monomers and by its binding-cutting action prevent them from growing as plasmids. In contrast, the polymeric and rolling-circular forms of the genome are susceptible to attack by *Ter*⁸⁰.

The failure of the $\lambda ter^- int^- red^- gam^- N^- cI^-$ genomes to establish as plasmids with complete efficiency could be due to the presence of a further gene which codes for still another function with an anti-plasmid activity. In this respect, one previously considered class of genes encoding potential anti-plasmid functions has not been accounted for on these modified λ chromosomes. These are the λ -encoded endonucleases. However, none of the phages used here contained the known endonuclease genes, *endo*^A and *ben*^A, since they were initially *h*⁸⁰, and the *h*⁻ variants were made by crossing through the region of homology to the left of *endo*^A and *ben*^A. However, analogous *endo*⁸⁰ and *ben*⁸⁰ genes could be present in all of these variants. Presumably, such endonuclease genes would be transcribed, just like the other genes, although if they were translated and processed into active proteins, they would indiscriminately cleave both plasmid and chromosomal DNA, and, because of the effect of the latter, be a very efficient cause

of lethality. This suggests that the activities of these endonucleases must be prevented in some way; otherwise λ plasmid formation (and also lytic growth) would not be possible. Indeed, the nature of the remaining plasmid defect does not suggest that it is caused by leaky endonuclease expression. Consequently, the low efficiency of initial plasmid formation is probably due to the effect of some other function.

Host bacterial cells harbouring the $\lambda ter^- int^- red^- gam^- N^- cI^-$ plasmids suffered a decline in viability. This defect could result from the leaky expression of one or more functions by these λ plasmids that act to debilitate the host cells. The effect of such functions would presumably have become more evident now, and not before because these plasmids are more inherently stable due to their modified genotype. Because the λ genome has been extensively characterized, it is possible to predict that certain genes might code for functions harmful to the host cells. Thus, an examination of the λ genotype (Echols & Murialdo, 1978; Szybalski & Szybalski, 1979) revealed two such genes, *S* and *kil*.

The *S* gene product is involved in cell lysis; its role appears to be to alter the structure of the cytoplasmic membrane so that the lysozymes (products of the *R* and *Rz* genes) can gain access to the cell wall. Lack of *S* function in a lytic infection therefore keeps the cell intact and DNA synthesis continues beyond the normal lysis period, leading to over-production of the number of phage particles. Since the cytoplasmic membrane houses many important metabolic functions, any attack upon it may impair the growth of the cell. The *S* product directly attacks the membrane by hydrolysing phospholipids, leading to the cessation of respiration, alteration of permeability of the cytoplasmic membrane and reduced DNA synthesis (Adhya *et al.*, 1971; Reader & Siminovitch, 1971 *a, b*). The effects of p*S* are not manifest until very late in a normal lytic infection (after about 50 min), indicating a tolerance to initial low levels of the protein. This correlates with our previous observations (Hadfield & Brammar, 1984) that the majority of $\lambda N^- r2, r1, r3$ plasmid-containing cells could tolerate the high-level leaky expression of the lysis genes. Thus, expression of p*S* may be incapacitating to the cell, but not necessarily lethal, unless in very high amounts.

Induction of $\lambda N^+ O^- P^-$ lysogens results in killing of the host cells. Such killing cannot be due to replication of the prophage, and has been ascribed to the synthesis of a lethal product by the prophage. The gene (*kil*) that codes for this product has been mapped between the *gam* and *cIII* genes (Greer, 1975 *a*). The *kil* product (Kil) can be purified as a predominant polypeptide from λ -infected cells (Greer & Ausubel, 1979). The killing of induced $\lambda N^+ O^- P^-$ lysogenic cells is actually characterized by a gradual decline in colony-forming ability, resulting ultimately in the survival of only 1 in 10^4 cells. Such a decrease in cell viability would seem to be similar to that noted above with the λ plasmid-carrying cells. How the killing function is mediated is not yet understood, although its action causes cell filamentation (Greer, 1975 *a, b*), which is normally associated with reduced DNA replication (reviewed by Witkin, 1976); yet it does not inhibit DNA synthesis, nor promote the degradation of DNA. Gross RNA and protein synthesis do not seem to be affected either. Certain observations suggest that Kil could have a nuclease activity, in apparent contradiction to the lack of DNA degradation. Other properties of Kil-affected cells suggest that the protein acts on the cell envelope (Greer, 1975 *a, b*).

The activities of the *kil* and *S* proteins could account for some of the defective characteristics of the $\lambda ter^- int^- red^- gam^- N^- cI^-$ plasmids. Clearly the decline in viability of the plasmid-carrying cells could be due to leaky expression of the *kil* and *S* gene products. Whether the expression of such anti-host functions could also cause the low efficiency of initial plasmid formation is not clear. The infected cells that failed to become transduced survived the infection and could grow in non-selecting medium, so the failure of plasmid transduction was not due to lethality. However, it is feasible that plasmid-encoded functions could arrest the growth of a host cell in selecting conditions, and thereby prevent successful plasmid transduction in the majority of cases. An examination of the effects of the loss of *kil* and *S* gene functions should clarify the extent of their contributions to λ plasmid defectiveness.

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