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Dislodgement and thymineless elimination of N-group plasmids

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SUMMARY

Thymineless strains of *Escherichia coli* C600 were constructred harbouring both an R factor of the N incompatibility group (R46 or R447b) and a compatible plasmid (P*lac*-R447b of the A–C group or the Iα plasmid R62), which contained a segment of N group DNA. Selection was made for the transferred plasmid and dislodgement phenomena were manifest either as loss of an entire plasmid or as deletions of a region of plasmid DNA. Even after the two R factors had become established as separate replicons, the N group R factor but not the other plasmid exhibited instability.

Thymine starvation of strain C600 *thy* (R447b/R62) increased the elimination rate of the N group plasmid R447b but no elimination of R62 was observed. However, thymine starvation of strain C600 *thy* (R46/P*lac*-R447b) not only increased the rate of elimination of R46 but also increased the rate of loss of P*lac*-R447b. There was no detectable increase in nuclease activity in unstarved R46/P*lac*-R447b strains and it is concluded that dislodgement of R46 from these strains is not due to induction of the nuclease that has been proposed to be responsible for the elimination of N group plasmids during thymine starvation.

Two variants of *Plac*-R447b were isolated. These did not dislodge R46 from unstarved R46/P*lac*-R447b strains and were not lost during thymine starvation even though thymineless elimination of R46 occurred at normal frequency.

INTRODUCTION

Plasmids that are capable of stable coexistence (compatible plasmids) may show a type of interaction that has been called dislodgement (Coetzee, Datta & Hedges, 1972; Guerry, Falkow & Datta, 1974). Dislodgement occurs on entry of one plasmid into a cell in which a second is already established and may result in the elimination of the resident plasmid, loss of some of the phenotypic characteristics displayed by the resident plasmid, or recombination between the two plasmids

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(Coetzee, Jacob & Hedges, 1975). The class of susceptible plasmids include both laboratory constructs (for example P*lac*-R447b) that carry genetic material derived from an N plasmid, and naturally occurring plasmids (for example R62) known to contain DNA sequences homologous to N plasmids. Thus, all plasmids susceptible to dislodgement may owe this characteristic to possession of a segment of DNA derived from or related to an N plasmid. Coetzee *et al.* (1975) have suggested that an N plasmid, on entering a newly infected cell, produces a deoxyribonuclease that is specific for any N group DNA of the resident plasmid: once the incoming plasmid has established itself, production or expression of the nuclease would be suppressed.

Cells harbouring N group plasmids appear to produce a nuclease that is inducible by thymine starvation (Tweats, Pinney & Smith, 1974), which may be responsible for the thymineless elimination of N group plasmids (Pinney & Smith, 1971, 1972). Only N group R plasmids have been shown to be eliminated by thymine starvation (Birks & Pinney, 1975) and it has been suggested that the nuclease which is induced by thymine starvation, may be the dislodgement effector (Coetzee *et al.* 1975). Consequently we have investigated the effects of thymine starvation on the plasmids of strains harbouring two R factors, one an N group plasmid which is eliminated by thymine starvation, and the other a compatible plasmid containing a sequence of N group DNA.

METHODS

(i) *Strains and plasmids*

The initial mating, dislodgement and thymine starvation experiments were carried out using a thymine-requiring mutant of *Escherichia coli* strain C600 *thr leu thi supE44* (Bachmann, 1972) as the recipient. R factor donors in these experiments were strains of *E. coli* J62 *pro his trp* or J53 *pro met* (Bachmann, 1972) that harboured the necessary plasmid. *E. coli* strain N1072 *lop-8 sup*$^-$ and phage T4*am*H39X (Gellert & Bullock, 1970) were kindly supplied by Dr M. Gellert, Strain N1072 was made thymine requiring (Tweats *et al.* 1974) and was used in experiments to test for nuclease activity. The plasmids used in this work are listed in Table 1.

(ii) *Conjugation experiments*

Donor and recipient cultures were grown in Oxoid No. 2 nutrient broth (code CM67) + 60 μg/ml thymine. 0·5 ml of an exponential donor culture containing about 2×10^8 bacteria/ml was mixed with 1·0 ml of an overnight culture of the recipient (viable count about 1×10^9/ml) and 1·0 ml fresh broth. After 40 min mating, the cultures were washed with the basal salts solution of Davis & Mingioli (1950) (DM base), resuspended and diluted in DM base and plated on DM medium containing amino acids required by the recipient and an antibiotic to which the transferred R factor conferred resistance. Clones that grew after 3 days incubation were streaked onto DM medium containing, separately, all the antibiotics to which the plasmids conferred resistance and onto MacConkey agar (Oxoid Ltd, code CM7) + 60 μg/ml thymine to check for lactose fermentation.

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(iii) *Thymine starvation experiments*

Overnight cultures were grown in antibiotic-free DM medium, diluted 1 in 10 into the same medium and incubated until the viable count of these exponential cultures were approximately 2×10^8/ml. Cells were washed and resuspended in DM base and then diluted 1 in 50 into prewarmed DM medium containing glucose and all required amino acids, but no thymine. Samples from these thymine-starved cultures were diluted in nutrient broth and plated on MacConkey agar + 60 μg/ml thymine. After overnight incubation, the resulting colonies were replica plated onto DM medium containing the necessary antibiotics to check for retention of each resistance phenotype, and onto DM medium with lactose as sole carbon source to check for lactose fermentation.

RESULTS

(i) *Experimental rationale*

R46 and R447b are N group plasmids that are eliminated by thymine starvation (Pinney & Smith, 1972 and this paper), R62 is an R factor of group Iα which has about 20 % of its nucleotide sequence homologous with the N group plasmid N3 (Guerry *et al.* 1974). P*lac*-$\overline{\text{R447b}}$ is a hybrid plasmid of the A–C compatibility group, the DNA of which contains 4·7 % of its nucleotide sequence derived from R447b (Coetzee, 1974, Coetzee *et al.* 1975). It was intended to construct stable doubles of (P*lac*-$\overline{\text{R447b}}$/R46) and R62/R447b) in *Escherichia coli* strain C600 *thy*, starve the host of thymine and test the surviving clones for both elimination of the N group R factor and manifestations of dislodgement effects on the other plasmid. It was not possible to test an (R46/R62) double because of the complete overlap of resistance phenotypes, and since the N group DNA region of P*lac*-$\overline{\text{R447b}}$ specifying kanamycin resistance is derived from R447b these two replicons might be so unstable in the same host, due to recombination between their homologous regions of DNA, as to vitiate the experiment.

(ii) *Construction of strains harbouring two R factors*

Plasmids were transferred from donor strains of *E. coli* J62 or J53 into strains of C600 *thy* that already harboured the other plasmid. Antibiotic selection (Table 1) was made for the transferred plasmid only. Twenty out of twenty (20/20) exconjugants from crosses in which P*lac*-$\overline{\text{R447b}}$ had been transferred into strain C600 *thy* (R46) exhibited all phenotypic traits of both R factors; there was, therefore, no dislodgement of the resident N group plasmid. However, when R46 was transferred into strain C600 *thy* (P*lac*-$\overline{\text{R447b}}$), 17/20 exconjugants harboured both R factors, and in the other three clones the resident plasmid had been dislodged. Crosses between strains harbouring R62 and R447b showed there was little dislodgment of R62 (1/20) when R447b was tranferred into strain C600 *thy* (R62), but that the majority (15/20) of C600 *thy* (R447b) clones lost the resident plasmid on entry of R62.

Isolated colonies from streak-outs of exconjugant clones that exhibited all phenotypic traits of both R factors were restreaked to test for plasmid stability. For example, isolated clones of putative C600 *thy* (R46/P*lac*-$\overline{\text{R447b}}$) exconjugants

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that had been selected on kanamycin for P*lac*-R447b transfer and were now growing on tetracycline and therefore appeared to have retained the R46 plasmid (Table 1) were restreaked. Surprisingly it was found that 9/10 were now sensitive to kanamycin. Similar instability was exhibited by all other R factor combinations tested (Table 2).

Table 1. *Plasmids used in this work*

Designation	Incompatibility group	Resistance and other determinants	Selection	Reference
R46	N	Am Sm Su^L Tc	Tc 10 μg/ml	Datta & Hedges (1971)
P*lac*-R447b	A–C	Km Su^H Lac^+	Km 10 μg/m	Coetzee (1974)
R447b	N	Am Km	Km 10 μg/ ml	Hedges *et al.* (1973)
R62	Iα	Am Sm Su^H Tc	Tc 10 μg/ml	Guerry *et al.* (1974)

Am = ampicillin; Sm = streptomycin; Su = sulphonamides; Tc = tetracycline; Km = kanamycin; Lac^+ = lactose fermentation. The superscripts L and H for Su resistance refer to the phenotypes of Su-resistant colonies: isolated colonies of strain C600 *thy* (R46) = (Su^L) growing on Davis and Mingioli medium + Su were much smaller than those on control plates containing no antibiotic. Colonies of strains C600 *thy* (P*lac*-R447b) and C600 *thy* (R62)(= Su^H) were the same size on Su-containing medium as on control plates. These phenotypes were stable and served as additional checks for plasmid retention. The Su^H phenotype was dominant to Su^L in strains containing two plasmids.

Table 2. *Instability of strains carrying two plasmids of different incompatibility groups*

Strain	Antibiotic on which selected clones were growing	Clonal phenotype
C600 *thy* (R46/P*lac*-R447b)	Tc	9/10 Km^s; 1/10 all resistances
	Km	4/10 $Am^sSm^sTc^s$; 6/10 all resistances
C600 *thy* (P*lac*-R447b/R46)	Tc	8/10 Km^s; 1/10 $Lac^-Km^sSu^L$; 1/10 all resistances
	Km	1/10 $Am^sSm^sTc^s$; 9/10 all resistances
C600 *thy* (R62/R447b)	Tc	6/8 Km^s; 2/8 Am^sKm^s
	Km	6/8 Am^sSm^s; 2/8 Sm^s
C600 *thy* (R447b/R62)	Tc	2/5 Sm^sSu^s; 3/5 all resistances
	Km	3/5 Sm^sSu^s; 2/5 all resistances

Isolated exconjugant clones growing on either Tc or Km to select for the transferred plasmid (Table 1) were streaked to check for retention of all phenotypic traits. Clones that exhibited all expected characteristics of the two R factors were then restreaked from both Tc and Km plates. Superscript 's' denotes antibiotic sensitivity; Lac^- = lactose non-fermenting; 'all resistances' = clones that exhibited all expected phenotypic traits.

Cell lines that retained the antibiotic resistance patterns of both plasmids after two streak-outs were stored on nutrient agar slopes at 4°. Overnight subcultures from these slopes in nutrient broth were plated on MacConkey agar and the resulting colonies were tested for drug resistance by replica plating. None of the double R factor-containing C600 *thy* strains, namely (R46/P*lac*R447b), (P*lac*-R447b/R46), (R62/R447b) or (R447b/R62) was found to be stable. For example, when cultured for the first time, 47/294 (16 %) of colonies from strain C600 *thy*

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(R46/P*lac*-R447b) had lost R46. Ten days later the slope was tested again and it was found that the proportion of R46$^-$ clones in this subculture had risen to 110/139 (79 %). These results show that dislodgement effects continue long after replication of the incoming plasmid, under conditions that are subsequent to many rounds of replication of both plasmids.

(iii) *Thymine starvation and R factor elimination*

In an attempt to overcome these instability problems, strains C600 *thy* (R46/P*lac*-R447b) and C600 *thy* (R447b/R62) were first streaked onto MacConkey agar containing both tetracycline and kanamycin, i.e. selection was made for both plasmid on the same plate. An isolated clone, which grew after over-night incubation, was then used to inoculate drug-free DM medium. Exponential cultures were starved of thymine for 3 h after which time strain C600 *thy* is undergoing its maximal rate of thymineless death, and R factor elimination is normally at its peak in singly R$^+$ strains (Pinney & Smith 1971). The results (Table 3) show that even after this selection pressure had been applied for both R factors in strain C600 *thy* (R46/P*lac*-R447b), R46 was still unstable in cells subsequently grown in drug-free media. 58·3 % of clones from the exponential culture were tetracycline sensitive, and this increased to 66·8 % after thymine starvation for 3 h. All (32/32) tetracycline-sensitive clones tested were also sensitive to ampicillin and to streptomycin and it is presumed that this instability reflects a loss of the whole R46 plasmid. P*lac*-R447b was much more stable in strain C600 *thy* (R46/P*lac*-R447b). Indeed, only one clone out of 853 tested (0·12 %) that had not been starved of thymine had lost all the phenotypic traits of P*lac*-R447b. Seven other clones (0·82 %) had lost only kanamycin resistance and it is assumed that this reflects excision of the N group region of DNA that codes for kanamycin resistance in P*lac*-R447b (Coetzee *et al.* 1975). The frequency of occurrence of C600 *thy* (R46/P*lac*-R447b) clones that had lost kanamycin resistance alone doubled after thymine starvation, which is in line with a broadly similar increase in the proportion of kanamycin-sensitive colonies found after thymine starvation of the singly R$^+$ C600 *thy* (P*lac*-R447b) strain (Table 3). However, with the doubly R$^+$ C600 *thy* (R46/P*lac*-R447b) strain there was a significant increase ($\chi^2 = 21{\cdot}2$; $p = < 0{\cdot}01$) from 0·12 % before thymine starvation to 2·8 % after thymine starvation of clones that had lost all P*lac*-R447b traits (Table 3). The frequency of loss of the whole P*lac*-R447b plasmid was therefore increased over 20-fold by thymine starvation of strain C600 *thy* (R46/P*lac*-R447b). In contrast with these results, the singly R$^+$ C600 *thy* (P*lac*-R447b) strain did not exhibit complete loss of the P*lac*-R447b plasmid at any time (Table 3).

Similar experiments (results not shown) with strain C600 *thy* (R447b/R62) showed that R447b was unstable in cultures grown in drug-free media: 12·3 % of clones examined had lost kanamycin resistance. This was increased to a frequency 89·3 % after thymine starvation. R62 was stable in this double (< 0·2 % sensitive segregants) and the proportion of clones that had lost tetracycline resistance was not increased by thymine starvation.

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Table 3. *Plasmid instability and elimination in strains of* E. coli *C600* thy *before and after thymine starvation*

Strain	Time of thymine starvation (hours)	Clonal phenotype tested	No. of clones tested	No. of clones with particular phenotype	% of clones with particular phenotype	Further characteristics of clones
C600 *thy* (R46)	0	Tc^S	703	0	< 0·14	
	3	Tc^S	298	72	24·2	20/20 lost all resistances
C600 *thy* (P*lac*-$\overline{\text{R447b}}$)	0	Km^S	604	6	1·1	6/6 Km^S only
	3	Km^S	467	8	1·7	8/8 Km^S only
C600 *thy* (R46/P*lac*-$\overline{\text{R447b}}$)	0	Km^S	853	8	0·94	7/8 (0·82 %) Km^S only; 1/8 (0·12 %) Km^S Su^L and Lac^-
		Tc^S	853	493	58·3	16/16 lost all R46 resistances
	3	Km^S	530	25	4·6	10/25 (1·9 %) Km^S only; 15/25 (2·8 %) Km^S Su^L Lac^-
		Tc^S	530	354	66·8	16/16 lost all R46 resistances

Abbreviations as in Table 2.

(iv) *Selection of plasmids insusceptible to dislodgement*

In another attempt to overcome instability, the double R factor-containing strains C600 *thy* (R46/P*lac*-R447b) and C600 *thy* (R62/R447b) were reconstructed, but this time selection of exconjugants were made on plates selective for both the transferred and the resident R factor (i.e. containing both tetracycline and kanamycin). Twenty exconjugant clones were streaked from each mating and all were found to express the phenotypic characteristics of both R factors. Therefore, as would be expected, no dislodgement effects were seen under conditions where selection pressure was applied for both the resident and the incoming plasmid. However, after two serial subcultures on drug-free media, only 2/20 of the putative C600 *thy* (R46/P*lac*-R447b) clones and 0/20 of the C600 *thy* (R62/R447b) clones retained the resistance traits of both R factors. The two stable C600 *thy* (R46/P*lac*-R447b) doubles transferred R46 independently, but after selection for P*lac*-R447b a high incidence (19/20 and 12/20) of tested clones showed cotransfer of unselected R46 markers. However, recipients that had received R46 in this way once again transferred it independently and it is concluded that the two plasmids exist as separate replicons in the initial stable C600 *thy* (R46/P*lac*-R447b) isolates. Examination of cells from overnight drug-free cultures of these two clones showed that the incidence of occurrence of colonies that lacked the R46 phenotype was less than 0·2 %, compared with greater than 50 % in the C600 *thy* (R46/P*lac*-R447b) strain reported in Table 3. One of the stable strains was tested for thymineless elimination and it was found that the frequency of elimination of R46 was 29·0 % after 3 h thymine starvation No elimination (< 0·16 %) of the entire P*lac*-R447b plasmid occurred, compared with a frequency of 2·8 % from the original C600 *thy* (R46/P*lac*-R447b) strain after thymine starvation (Table 3). Since R46 was still eliminated from this strain it would appear that its 'eliminating nuclease' is still produced. However, the P*lac*-R447b plasmid is now resistant to elimination and will be referred to as P*lac*-R447b^{R} ('R' for resistant). It was therefore tested to see if it was still susceptible to dislodgement. Two clones of strain C600 *thy* (R46/P*lac*-R447b^{R}) that had been cured of R46 were used as donors and recipients in reciprocal crosses with strain J62(R46). There was no dislodgement of either plasmid (Table 4) and when isolated colonies from streak-outs of exconjugant clones were restreaked all (10/10 in each case) were also stable (Table 4). The stability of these strains is in marked contrast to the instability of the original C600 *thy* (R46/P*lac*-R447b) and C600 *thy* (P*lac*-R447b/R46) isolates (Table 2), and cannot be due to loss of the entire N group region of DNA because P*lac*-R447b^{R} is still resistant to kanamycin. This suggests that the dislodgement interactions seen between R46 and P*lac*-R447b occur in response to a specific sequence of N group DNA present on the P*lac*-R447b plasmid, rather than as the result of a generalized interaction between the N group plasmid and the related base sequence on the other genome.

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Table 4. *Insusceptibility to dislodgement of two stable isolates of plasmid* Plac-*R447b*

Donor strain	Recipient strain	Initial selection	Resistance phenotype of exconjugant clones	Medium from which isolated colony restreaked	Resistance phenotype of restreaked colony
C600 *thy* (P*lac*-R447b^R) – 1	J62 (R46)	Km	20/20 Am Su^H Tc Sm Km Lac$^+$	Tc	10/10 all resistances
				Km	10/10 all resistances
C600 *thy* (P*lac*-R447b^R) – 2	J62 (R46)	Km	20/20 all resistances as above	Tc	10/10 all resistances
				Km	10/10 all resistances
J62 (R46)	C600 *thy* (P*lac*-R447b^R) – 1	Tc	20/20 all resistances as above	Tc	10/10 all resistances
				Km	10/10 all resistances
J62 (R46)	C600 *thy* (P*lac*-R447b^R) – 2	Tc	20/20 all resistances as above	Tc	10/10 all resistances
				Km	10/10 all resistances

DISCUSSION

This investigation was initiated to test the hypothesis that because dislodgement phenomena and thymineless elimination are confined to plasmids of the N incompatibility group they are related. It was proposed that if this were so, thymine starvation of a susceptible double should induce dislodgement. This occurred with the P*lac*-R447b/R46 double in that thymine starvation increased the rate of elimination of both plasmids. However, with strain C600 *thy* (R447b/R62) thymine starvation eliminated only the R447b plasmid. Thus it would seem that while R62 can promote dislodgement it does not possess an elimination effector recognition site. P*lac*-R447b on the other hand possesses an elimination effector site as well as being able to promote dislodgement.

It has been suggested (Coetzee, *et al.* 1975) that the nuclease induced by thymine starvation, which eliminates only N group plasmids (Birks & Pinney, 1975), may also be responsible for dislodgement. As we found that dislodgement effects continued long after the establishment of two plasmids as separate replicons, it would seem possible that this instability may be due to induction of the same nuclease. We tested this possibility using the system developed by Gellert & Bullock (1970). This demonstrates nuclease activity in a strain as a reduction in titre of the ligase-defective phage T4*am*H39X when compared with its plating efficiency on a wild-type strain. If a nuclease were induced in, for example, the C600 *thy* (R46/P*lac*-R447b) double, then the efficiency of plating (e.o.p.) of the phage on this strain should be reduced in comparison with the titre on the C600 *thy* R^- strain. No such reduction was observed in any strain tested (results not shown). However, these results could be misleading because strain C600 carries an amber suppressor mutation (Bachmann, 1972). Strains carrying R46 and P*lac*-R447b were therefore constructed in *E. coli* strain N1072 *lop 8 thy sup*$^-$. The presence of R46 in this strain during thymine starvation results in the induction of nuclease activity as evidenced by a reduction in propagation efficiency of T4*am*H39X when compared with its burst size on the thymine-starved N1072 *thy* R^- strain (Tweats *et al.* 1974). However, no reduction in e.o.p. of T4*am*H39X was found on strain N1072 *thy* containing either R46 or P*lac*-R447b singly or together (data not shown). These results suggest strongly that the dislodgement phenomena seen in unstarved R46/P*lac*-R447b and R447b/R62 strains are not due to the induction of the nuclease responsible for thymineless elimination.

Coetzee *et al.* (1975) observed that dislodgement interactions occurred only immediately after transfer of an N plasmid into a strain carrying a susceptible replicon, whereas, we now find that elimination and deletion events occur many generations after formation of the doubly R^+ strains. This raises the question whether there is any real difference between dislodgement and incompatibility. One finding that suggests the two phenomena are different is the isolation of P*lac*-R447b^R, which despite retaining the kanamycin resistance gene encoded by N group DNA has lost the ability to promote dislodgement of the N group plasmid R46. Thus it would seem that dislodgement is not due to the mere presence of a

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segment of N group DNA, but is conferred by some specific base sequence(s). As we were so easily able to isolate mutants of P*lac*-R447b that had lost susceptibility to dislodgement, it would seem that its 5 mega-dalton sequence of N group DNA carries a limited number, or even only one such dislodgement effector sequence.

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