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Interaction between the plasmid R6K and *Escherichia coli* with defective DNA polymerase I

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SUMMARY

Initial experiments demonstrated that the plasmid R6K cannot be transferred to or maintained readily in the *E. coli* DNA polymerase I deficient strain JG138 *polA1*. Results with *E. coli* MM386 *polA12* (R6K), which has a temperature sensitive polymerase I enzyme, showed cell division becomes abnormal when the polymerase I enzyme of the host bacteria is inactivated at the restrictive temperature. Under conditions of polymerase I deficiency, R6K replication, as measured by monitoring R-factor-mediated β-lactamase activity, also becomes abnormal with the loss of multiple R6K copies per cell and the apparent maintenance of a single R-factor copy per cell.

1. INTRODUCTION

The DNA polymerase I protein encoded by the *Escherichia coli polA* gene possesses at least three enzyme activities, a 5′-3′ polymerase, a 3′-5′ exonuclease and a 5′-3′ exonuclease (as summarized by Setlow & Kornberg, 1972). The latter activity appears to be essential for normal growth and viability of *E. coli* K12 (Konrad & Lehman, 1974).

Although the polymerizing activity of DNA polymerase I participates in the replication of the bacterial chromosome and most large conjugative plasmids, other polymerases can readily substitute for this activity in strains that lack the 5′-3′ polymerase and the 3′-5′ exonuclease functions of the polymerase I complex (Tait & Smith, 1974). However, the absence of these two activities of polymerase I prevents the replication of many small non-conjugative plasmids, which form multiple copies of plasmid DNA per cell in *polA*$^+$ strains (Kingsbury & Helinski, 1970; Grindley & Kelley, 1976).

The R-factor R6K confers resistance to ampicillin up to concentrations of 400 μg/ml and to streptomycin (Datta & Kontomichalou, 1965; Kontomichalou, Mitani & Clowes, 1970; Helinski, 1973). R6K, unlike most conjugative plasmids, replicates via catenated replicative intermediates (Kupersztoch & Helinski, 1973) to form multiple R-factor copies per cell (Kontomichalou, Mitani & Clowes, 1970).

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Exponential phase R6K$^+$ host cells contain 13 plasmid copies, which rise to 38 copies at the onset of stationary phase, when replication of the main chromosome ceases. Penicillinase (β-lactamase) assays of cultures in exponential and stationary phase show a correlation between the estimated number of R-factor copies and β-lactamase activity per cell (Kontomichalou *et al.* 1970; Macrina, Weatherly & Curtiss, 1974).

Therefore as R6K is a conjugative plasmid, but forms multiple copies of R-factor DNA per *E. coli* cell, it was of interest to determine whether R6K replication was affected by DNA polymerase I deficiency. This paper reports that R6K cannot be transferred to, or be maintained readily in the *E. coli* polymerase I deficient strain JG138 *polA1*. Investigations with a temperature-sensitive *E. coli* DNA polymerase I mutant, MM386, have shown that although R6K appears to require DNA polymerase I to form multiple copies during the growth cycle, this R-factor can be maintained as a small number of copies per cell in MM386 for many generations at the restrictive temperature.

A preliminary report of some of this work has been published (Tweats & Smith, 1975).

2. MATERIALS AND METHODS

Bacterial strains

The *E. coli* bacterial strains used in this investigation are listed in Table 1. The polymerase deficient strains and strain MM300 were kind gifts from Dr M. Monk and J6-2 *rif* was kindly given by Dr N. Datta.

R6K (Kontomichalou *et al.* 1970), is an fi$^-$ IncX R-factor (Hedges *et al.* 1973) otherwise known as R-TEM, and confers high-level resistance to ampicillin and streptomycin (Datta & Kontomichalou, 1965). R1 is an fi$^+$ Inc FII R-factor, otherwise known as R-7268 (Hedges & Datta, 1972; Datta & Kontomichalou, 1965) and confers resistance to ampicillin, chloramphenicol, streptomycin and sulphonamide. This latter R-factor was originally isolated in Professor E. S. Anderson's laboratory.

Media

Media and plating procedures were as described by Tweats, Pinney & Smith (1974).

Mating

R-factor transfer was carried out as described by Smith (1969) at various temperatures as stated in the text, using 10 μg of chloramphenicol per ml to select for R1 and 400 μg of ampicillin per ml to select for R6K. Should the β-lactamase gene have transposed into the chromosome, such strains would fail to grow because the single gene copy in the chromosome only gives resistance to 100 μg/ml ampicillin at the most. Transfer of the complete R-factor was in each case verified by checking that all the other respective antibiotic resistances were present in the transconjugants.

A separate protocol was used for the transfer of R6K from MM386 (R6K) to

J6-2 *rif*. MM386 (R6K) was grown on MacConkey agar containing 300 μg benzylpencillin per ml and incubated at 30 °C for 40 h. A single colony isolate was used to inoculate Oxoid nutrient broth no. 2, which was then incubated overnight at 30 °C. J6-2 *rif* was grown overnight in Oxoid no. 2 broth containing 25 μg rifampicin per ml to maintain its level of rifampicin resistance. This culture was washed

Table 1. Escherichia coli *strains*

Strain	Genotype*	Reference on source
MM300	*ilv thyA rha lacZ str*	Monk & Kinross (1972)
JG138	*thyA rha lacZ polA12 str*	Monk, Peacy & Gross (1971)
MM386	*rha lacZ polA12 str*	Monk & Kinross (1972)
58-161/sp†	*met*	Meynell & Datta (1966)
J6-2	*pro his trp lac str*	Clowes & Rowley (1954)
J6-2 *rif*	*pro his trp lac str rif*	N. Datta

* Symbols for genetic markers as listed by Taylor & Trotter (1972).
† 58-161/sp behaves as F^- but retains some F^+ characters (Meynell & Datta, 1966).

twice in Davis and Mingioli basal salts medium (Davis & Mingioli, 1950) to remove rifampicin before resuspending in one third of its original volume in nutrient broth. Mating was carried out as follows:

(*a*) 1·0 ml of J6-2 *rif* suspension and 4·5 ml of nutrient broth were added to 1·0 ml of MM386 (R6K) culture. The mixture was incubated for 5 h at 30 °C before removing samples which were then washed by centrifugation and resuspended to one third original volume with broth. Dilutions were plated onto nutrient agar containing 25 μg of rifampicin per ml and 80 μg of benzylpencillin per ml. These plates were incubated overnight at 30 °C.

(*b*) 0·1 ml of overnight MM386 (R6K) culture was subcultured into 4·5 ml of nutrient broth and grown for 27 h at 42 °C. Mating was then carried out at 30 °C for 5 h as in (*a*) using 1·0 ml of this culture as donor.

Assay of β-lactamase activity

R-factor β-lactamase activity was assayed by use of the hydroxylamine technique described by Dale & Smith (1971). Routinely benzylpencillin (kindly donated by Glaxo Laboratories Limited) was used as the substrate at a concentration of 1·8 mg/ml in each reaction mixture. The assay was carried out at 30 °C. Enzyme activities were expressed as micromoles of substrate hydrolysed/min/mg of protein, i.e. standard international units (in) per mg of protein (Thompson, 1962). In practice milliunits were used where 1 m-u. = 0·001 i.u. Protein concentrations were estimated by the method of Waddell (1956).

3. RESULTS

R1 and R6K transfer into PolA$^+$ and PolA$^-$ strains

The mating frequencies of 58-161/Sp (R1) and (R6K) donors with Po/A$^+$ and PolA$^-$ recipient strains are shown in Table 2.

The frequency of R1 transfer was found to be unaffected by the DNA polymerase I status of the recipient. However, the frequency of R6K transfer was reduced a thousandfold when the recient strain was *polA1*, However, investigation of the R^+ colonies obtained from this mating revealed that no true *polA1* (R6K) colonies

Table 2. *Recipient abilities of* polA$^+$ *and* polA$^-$ *strains with R6K and R1 donors**

Recipient strain	*polA* genotype	Temp.	Mating frequency† R6K	Mating frequency† R1
MM300	*polA*$^+$	37	$3{\cdot}1 \times 10^{-3}$	$4{\cdot}0 \times 10^{-2}$
JG138	*polA1*	37	$3{\cdot}9 \times 10^{-6}$‡	$4{\cdot}0 \times 10^{-2}$
MM386	*polA12*	30	$3{\cdot}6 \times 10^{-3}$	$3{\cdot}6 \times 10^{-2}$
MM386	*polA12*	42	$2{\cdot}6 \times 10^{-6}$‡	$3{\cdot}0 \times 10^{-2}$

* The donor strains were the R6K and R1 derivatives of 58-161/sp.

† Mating frequencies are expressed as R^+ colonies per input donor after a 5 h mating followed by selection for R^+ transconjugants.

‡ These mating frequencies were corrected for Met$^+$ donor revertants as outlined in the text.

had been obtained. The putative *polA1* (R6K) clones turned out to be either Met$^+$ revertants of the donor strain, PolA$^+$ revertants, Su^+_{am} *polA1* R^+ colonies or partially PolA$^+$ revertants that exhibited a sensitivity to methyl methane sulphonate and ultraviolet light intermediate between that of PolA$^+$ and PolA1 *E. coli* strains (D. J. Tweats, Ph.D thesis, 1975).

Investigations of the growth pattern of polA12 (*R6K*)

As a true *polA1* (R6K) strain could not be constructed, the properties of the *polA12* (R6K) strain constructed at 30 °C were investigated at the permissive and restrictive temperatures.

The growth patterns of MM386 and its R6K derivative were monitored at 30 and 42 °C using 1 % inocula of stationary phase cultures grown at 30 °C.

MM386 exhibited normal growth at both temperatures (Fig. 1*a*, *b*). However, the R^+ strain, though growing normally at 30 °C (Fig. 1*a*), when cultured at 42 °C ceased to grow after 3 h when the viable count reached $1{\cdot}5 \times 10^8$ cells/ml (Fig. 1*b*).

When MM386 (R6K) was grown at 42 °C for 5 h and then diluted 10-fold in nutrient broth at 42 °C the viable count of this freshly diluted culture increased for 3 h before stopping at a viable count of $1{\cdot}7 \times 10^8$ cells/ml (Fig. 1*b*). These results show that at 42 °C growth of the R^+ strain ceases at about $2{\cdot}0 \times 10^8$ bacteria/ml, whereas the same strain without the R-factor continues growth to 10^9 cells/ml at this temperature. Thus the presence of the R-factor causes this strain to cease growth prematurely. These results suggest that cultures of MM386 (R6K) accumulate a substance during incubation at 42 °C that inhibits growth.

Microscopic observation of MM386 and its R6K derivative grown at 30 and 42 °C revealed that both strains formed filamenting cells at both temperatures. However, cultures of MM386 (R6K) showed a marked increase in filamentation during growth at 42 °C not shown by the other three cultures.

Total counts made using a counting chamber were carried out on a culture of MM386 (R6K) growing at 30 °C and after subculture at 30 or 42 °C. When grown at 30 °C, the total count results agreed well with the viable counts. However, at 42 °C the total counts exceeded the viable counts by as much as four-fold (Table 3). The R⁻ strain gave good agreement between total and viable counts at both temperatures. Thus it appears that at 42 °C many cells of MM386 (R6K) are

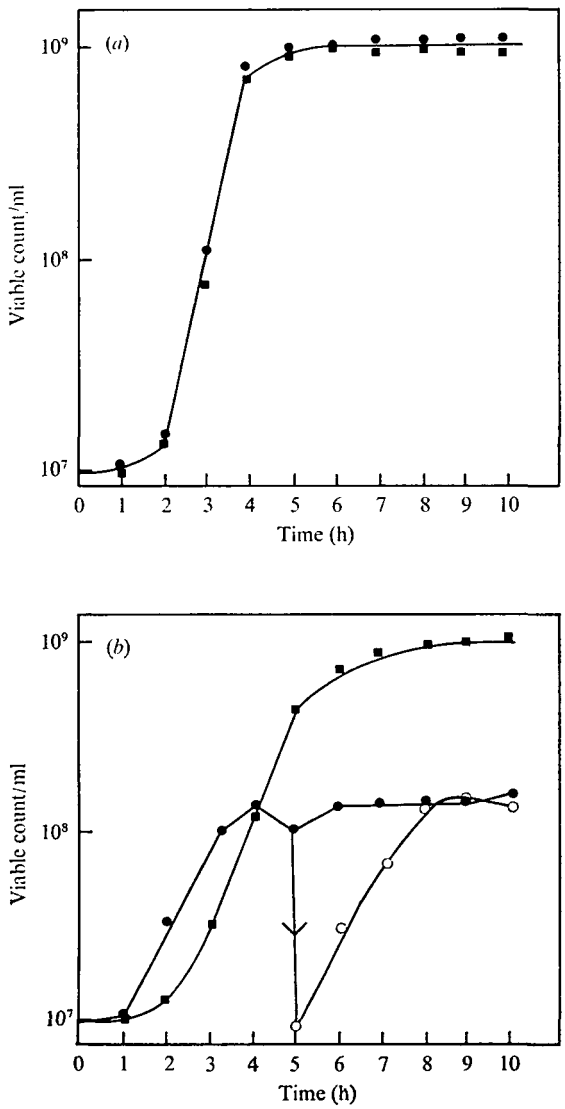

Fig. 1. Growth of MM386 (■) and MM386 (R6K) (●) (*a*) at 30 °C and (*b*) at 42 °C. Also shown in Figure 1(*b*) is the effect of a ten-fold dilution on a culture of MM386 (R6K) in premature stationary phase. A portion of the original culture was diluted ten-fold (arrow) after 5 h at 42 °C (○) and incubation continued at 42 °C for a further 5 h.

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unable to form colonies on solid media. It would seem this effect is due to the presence of R6K.

Table 3. *Total and viable counts of MM386 (R6K) grown at 30 and 42 °C*

	Viable count per ml		Total count per ml	
Time (hrs.)	30 °C	42 °C	30 °C	42 °C
0	$4{\cdot}4 \times 10^6$	$4{\cdot}4 \times 10^6$	$5{\cdot}7 \times 10^6$	$5{\cdot}7 \times 10^6$
4	$6{\cdot}3 \times 10^7$	$7{\cdot}3 \times 10^7$	$7{\cdot}6 \times 10^7$	$3{\cdot}8 \times 10^8$
8	$2{\cdot}6 \times 10^8$	$1{\cdot}5 \times 10^8$	$2{\cdot}8 \times 10^8$	$4{\cdot}6 \times 10^8$
27	$1{\cdot}0 \times 10^9$	$6{\cdot}5 \times 10^7$	$2{\cdot}0 \times 10^9$	$2{\cdot}2 \times 10^8$

MM386 (R6K) was incubated for 40 h at 30 °C, then a 1·0 % inoculum was diluted into fresh broth at 30 ° or 42 °C and incubation continued. At the times shown, the total and viable counts were estimated.

Analysis of β-lactamase levels of MM386 (R6K)

The level of β-lactamase in cultures of *E. coli* strains harbouring R6K correlates with the number of R-factor copies per cell (Kontomichalou *et al.* 1970; Macrina *et al.* 1974). Consequently MM386 (R6K) was grown at the permissive and restrictive temperatures and assayed for levels of β-lactamase activity to follow the fate of R6K replication during transition from DNA polymerase I sufficiency to deficiency. The β-lactamase activity from R6K was compared with that of R1 in J6-2 (*polA*$^+$) and in MM386 (*polA12*).

Normally R$^+$ *E. coli* strains produce only a cell-bound β-lactamase (Smith & Wyatt, 1974) but due to the large proportion of inviable filamenting bacteria encountered in cultures of MM386 (R6K), the β-lactamase levels of both the cell pellet and supernatant factions were investigated to detect any leakage of β-lactamase from the bacteria during growth.

The results obtained using 40 h cultures grown at 30 °C (Table 4), show that both MM386 and J6-2 strains harbouring R6K had similar β-lactamase levels that were much higher than those levels shown by the same strain harbouring R1. This difference has been ascribed to the presence of multiple copies of R6K per cell (Kontomichalou *et al.* 1970) compared to the 1-2 copies of R1 per cell (Hedges *et al.* 1974). The level of β-lactamase in culture supernatants from all strains tested at 30 °C was found to be less than 10 % of the respective intracellular levels in all cases.

The β-lactamase activities of cultures grown at 42 °C were also tested. Table 4 shows that the β-lactamase activities and percentage extracellular enzyme of R1-containing strains and that of J6-2 (R6K) were similar to the results obtained for these strains grown at 30 °C. However, when MM386 (R6K) was grown at 42 °C, half of the total β-lactamase activity was found in the supernatant. The intracellular level of β-lactamase was only one tenth of the level obtained at 30 °C with this strain and the level of total β-lactamase activity resembled that mediated by R1 in both hosts at both temperatures.

The β-lactamase levels of MM386 (R6K) cultures at 30 and 42 °C were investigated during various stages of the growth cycle to try to gain some knowledge

about the kinetics of R6K replication during the course of polymerase I deficiency. MM386 (R6K) was grown for 40 h at 30 °C in nutrient broth. One ml aliquots of this culture were subcultured into 100 ml amounts nutrient broth, which were then incubated at either 30 or 42 °C.

Table 4. *β-lactamase levels of J6-2 and MM386 R+ strains grown at 30 and 42 °C*

	Total β-lactamase activity (m-u./mg cell protein)		Extracellular enzyme activity (%)	
Strain	30 °C	42 °C	30 °C	42 °C
J6-2 (R1)	220	207	6·4	8·3
MM386 (R1)	263	253	9·4	9·1
J6-2 (R6K)	1188	889	5·4	7·4
MM386 (R6K)	1150	262	7·4	51·5

The R+ strains were grown for 40 h at 30 °C in nutrient broth, then a 1 % inoculum was diluted into fresh broth and incubation continued at 40 h at 30 or at 42 °C. The cultures were harvested by centrifugation and the levels of β-lactamase activity in the cell pellets and supernatant fluids estimated.

Table 5. *β-lactamase levels of MM386 (R6K) growing at 30 and 42 °C*

	Total β-lactamase activity (m-u./mg cell protein)		Extracellular enzyme activity (%)	
Time (h)	30 °C	42 °C	30 °C	42 °C
0	1811	1811	6·1	6·1
4	474	453	4·3	16·0
8	719	389	2·8	26·0
27	1620	278	7·1	61·8

MM386 (R6K) was grown for 40 h at 30 °C in nutrient broth, then a 1·0 % inoculum was diluted into fresh broth at 30 or 42 °C and incubation continued. At the times shown, the cultures were harvested by centrifugation and the levels of β-lactamase on the cell pellet and supernatant fluid estimated.

The results (Table 5) show that at 30 °C, the extracellular β-lactamase level did not exceed 7 % of the total β-lactamase level at any time. When MM386 (R6K) reached the early exponential phase (at 4 h) the level of β-lactamase activity had fallen about four-fold. This level progressively increased after 8 and 27 h incubation. At 27 h, the β-lactamase activity had returned to a level similar to that of the original starting culture. The variation we observed in the levels of β-lactamase activity correlated well with the changes in the R6K copy number that had been observed during the growth cycle by Kontomichalou *et al.* (1970) and Macrina *et al.* (1974).

In cultures grown at 42 °C (as for the 30 °C culture) there was a four-fold reduction in the level of β-lactamase activity as the bacteria entered the early exponential phase. Subsequently, however, unlike the 30 °C culture, the total β-lactamase activity continued to decline when incubated at 42 °C. The percentage extracellular enzyme progressively increased during growth, until after 27 h

incubation at 42 °C, the level of β-lactamase activity in the supernatant fluid exceeded that isolated from the cell pellet. At this time the cell bound β-lactamase activity was less than a tenth of that observed in the cells grown at 30 °C.

As the β-lactamase levels had fallen to a tenth of that seen in PolA$^+$ bacteria, experiments were carried out to test whether R6K was still present and transferrable after the inactivation of the polymerase I of the host cell by the restrictive temperature.

Maintenance of R6K in MM386 (R6K) at 42 °C

A 40 h culture of MM386 (R6K) grown at 30 °C was diluted to 1 % in prewarmed nutrient broth at 42 °C. Samples were plated at hourly intervals for 10 h and a further sample removed at 27 h. After incubation for 40 h at 30 °C, the plates were replica-plated onto media containing the antibiotics (ampicillin or streptomycin) to which R6K confers resistance. Transposition of the β-lactamase gene of R6K (TnA) into the chromosome would have been scored because plasmid elimination was monitored by measuring the loss of both antibiotic resistance genes carried by R6K. However, as every clone that had lost one of the two resistances was found to have simultaneously lost the other resistance, it would seem that insertion of TnA into the chromosome together with plasmid loss, occurred rarely if at all in this system.

In addition it was found that loss of the R-factor from MM386 at 42 °C at no time exceeded the R-factor loss in cultures grown at 30 °C (0·23 % R$^-$).

In a repeat experiment a culture of MM386 (R6K) grown at 30 °C was diluted to 1 % and incubated for 24 h at 42 °C then diluted again to 1 % followed by incubation at 42 °C for a further 24 h. As in the previous experiment no evidence for increased R-factor elimination was observed despite the fact that the bacteria had undergone twelve generations of growth at 42 °C. This compares with *polA12* (ColE1) cultured at 42 °C where after eight generations at the restrictive temperature, more than 60 % of the bacteria had lost the plasmid (Durkacz & Sherratt, 1973).

Transferability of R6K from MM386 (R6K) after exposure to 40 °C

As MM386 (R6K) does not carry auxotrophic markers (Table 1), a rifampicin resistant derivative of J6-2 was chosen as recipient. The matings were carried out as described in Materials and Methods.

In a control 5 h mating using an MM386 (R6K) donor culture grown overnight at 30 °C, R6K was transferred to J6-2 *rif* recipient cells at a frequency of $6{\cdot}2 \times 10^{-3}$ R$^+$ transconjugants per input donor, which compares well with the frequency of similar matings given in Table 2. A 5 h mating carried out using an MM386 (R6K) donor culture exposed to 42 °C for 27 h gave a transfer frequency of $5{\cdot}6 \times 10^{-4}$ R$^+$ transconjugants per input donor. Therefore, MM386 (R6K) is still capable of transferring its R-factor in conditions of polymerase I deficiency, although the mating frequency is reduced ten-fold compared with that from cells grown at 30°. This reduction may be related to the poor condition of the MM386 (R6K) cells after

exposure to 42°. However, these results shown that MM386 (R6K) retains not only antibiotic resistances but also its transfer properties after growth at 42°.

4. DISCUSSION

R1 was transferred to *E. coli polA1* without difficulty, whereas a *polA1* (R6K) strain could not be constructed. This finding was unusual as most R-factors that are self-transferring are normally maintained in *polA1* mutants, although the *Psuedomonas aeruginosa* R-factor RP1-1 and the *Proteus* plasmid *Plac* both have an absolute requirement for DNA polymerase I when present in *E. coli* cells (Moillo-Batt & Richmond, 1976 and Ambrosio, 1977, respectively).

The inability of *polA1* strains to accept or maintain R6K was investigated by observing the properties of a *polA12* (R6K) strain constructed at 30 °C, the permissive temperature. At this temperature, the strain appears to possess some properties of a *polA*$^+$ strains, although it is known to contain only 12% of the wild-type polymerizing activity of the polymerase I enzyme (Lehman & Chien, 1973).

The *polA12* (R6K) strain behaved normally at 30 °C, but at 42 °C cultures of this strain contained a large number of filamenting cells incapable of forming colonies. Therefore, it was not surprising that attempts to construct a *polA1* (R6K) strain failed, as any R6K$^+$ *polA1* cells that were formed would fail to develop into macroscopic colonies on solid media.

Terawaki *et al.* (1968) reported that when strains harbouring R*tsl*, an R-factor with temperature-sensitive replication defects, were exposed to the restrictive temperature, the division of the bacterial host became abnormal. Similarly the inhibition of ColE1 replication in host strains containing thermolabile DNA polymerase I growing at the restrictive temperature caused the formation of filamenting cells (Durkacz & Sherrat, 1973) and poor growth of the host strain (Kingsbury & Helinski, 1973). Therefore, the abnormal growth of *polA12* (R6K) cells at 42 °C could suggest that R6K replication may also be impaired under these conditions.

R6K was not eliminated from *polA12* (R6K) even after twelve generations of growth at 42 °C. By comparison ColE1 replication ceases shortly after the DNA polymerase I enzyme of its host is inactivated and the plasmid becomes diluted out of the divided cells in the culture until it is lost altogether (Kingsbury & Helinski, 1970; Durkacz & Sherratt, 1973). Furthermore, the response of R6K to polymerase I deficiency does not easily fit into the plasmid classes described by Grindley & Kelley (1976) based on the response of non-conjugative plasmids in hosts with *polA1* mutations.

R6K replication under conditions of DNA polymerase I deficiency was studied by following the level of R6K-determined β-lactamase in cultures of *polA12* (R6K) grown at the permissive and restrictive temperatures. At 30 °C the level of β-lactamase appeared to be unaffected by the *polA* genotype of the host. However, after 24 h at 42 °C, the level of β-lactamase from *polA12* (R6K) cultures (including extracellular enzyme) had fallen to a level similar to that shown by *polA12* (R1).

Both R6K and R1 are known to specify a β-lactamase enzyme with identical properties (Dale & Smith, 1971; Hedges *et al.* 1974). It is also known that this enzyme is extremely thermostable (J. T. Smith, unpublished) and hence the results at 42 °C cannot be explained by thermal inactivation of the β-lactamase. Indeed the results obtained with *polA12* (R1) provide direct evidence in support of this view.

R1 is normally present as 1-2 copies per *E. coli* cell (Helinski, 1973). Therefore, R6K may also be maintained as 1-2 R-factor copies per *E. coli* cell when DNA polymerase I is in short supply. This interpretation leads to the conclusion that R6K requires significant quantities of the polymerase in order to proceed from single to multiple R-factor copies per cell and that stringent R6K replication can continue under the control of other DNA polymerases in mutants deficient in DNA polymerase I.

An alternative explanation could be that the plasmid DNA is transcribed less efficiently during DNA polymerase I insufficiency. Such interference with transcription can be achieved by transferring R6K from *E. coli* into *Proteus mirabilis* (Smith, 1969).

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