RelA mutation and pBR322 plasmid amplification in amino acid-starved cells of *Escherichia coli*

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Summary

Plasmid pBR322 is amplified following amino-acid limitation in *Escherichia coli relA* hosts. In *relA*$^+$ hosts there was no significant amplification or a much smaller one. Plasmid amplification is due to the *relA* mutation; when the *relA*$^+$ allele is transferred into the *relA* mutant CP79 this strain no longer amplifies plasmid DNA during amino acid starvation. It is concluded that ppGpp is a negative effector of plasmid replication. Amplification is temperature dependent, being maximal at 32 °C and negligible at 37 °C.

1. Introduction

The molecular mechanism of the replication of ColE1-related plasmids has been elucidated in its major details (Cesareni & Banner, 1985; Tomizawa, 1986), however, our knowledge of the physiology of plasmid replication is still rather limited.

ColE1-related plasmids are amplified during limitation of amino acids in the relaxed (*relA*$^-$) *Escherichia coli* K12 strain CP79 (Hecker *et al.*, 1983; 1988); but not in its stringently controlled isogenic counterpart CP78. This led us to suggest that ppGpp accumulation which follows amino acid starvation in *relA*$^+$ strains may have a negative effect on the replication of ColE1-related plasmids (Hecker *et al.* 1983).

Lin-Chao & Bremer (1986) did not confirm these results. They found an even higher plasmid amplification in *E. coli* B/K2 *relA*$^+$ than in the isogenic *relA* strains after deprivation of amino acids.

Because of these conflicting results we analysed several other isogenic *E. coli relA*$^+$/*relA* strain pairs in order to exclude that in the *E. coli* strain CP79 the plasmid amplification we observed is *relA*-independent. The results of this study give further evidence that *E. coli relA* strains are able to amplify ColE1-related plasmids. Recently these results were confirmed by Guzman *et al.* (1988) who found that deprivation of isoleucine in a Rel$^-$ strain gives rise to amplification of pBR322 with a better yield than that following treatment with chloramphenicol (Hecker *et al.*, 1985).

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2. Material and methods

The strains used in this investigation, their relevant characteristics and the source are listed in Table 1. All strains harboured the plasmid pBR322.

The *E. coli* cells were grown at 30 °C in a synthetic medium (Mitchell and Lucas-Lenard 1980) supplemented with thiamine (10 mg/l), glucose (6 g/l), $Na_2HPO_4 . 12H_2O$ (322 mg/l) and different concentrations of amino acids (see Table 1). The cells ceased growth as a result of amino acid exhaustion.

Alternatively amino acid starvation was brought about by: (i) transferring growing cells into amino acid free medium (see Lin-Chao and Bremer, 1986); (ii) the addition of valine (1 mg/ml) to logarithmically growing cells which triggers isoleucine limitation.

For chloramphenicol (Cm) amplification cultures were grown to an absorbance (OD 500) of 0·3 to 0·5 and Cm was added to a final concentration of 10 to 50 μg/ml (see Hecker *et al.*, 1985). Plasmid DNA content was measured as described by Frenkel and Bremer (1986). The concentration of ppGpp in the cells was measured according to Cashel *et al.* (1969).

3. Results

We measured plasmid DNA content in several otherwise isogenic *relA*$^+$ and *relA* strains after amino acid starvation (Table 2). In all strain pairs studied there was a much higher plasmid content in amino acid-starved cells of *E. coli relA* than in the isogenic *relA*$^+$ counterparts (Fig. 1, Table 2). In the *relA* strains CP79 and NF162 an about 5-fold increase of pBR322

Table 1. E. coli *strains*

Strains (genotype)		Supplements	Source	Growth inhibited by starvation for
K12	CP78	(arg, leu, thr, his, thi)	This lab.	Arginine[1]
K12	CP79	(arg, leu, thr, his, thi, *relA*)	This lab.	Arginine[1] Leucine[2] Histidine[3]
K12	CP79 F′	(leu, thr, his, thi, [F′ argA$^+$, lysA$^+$, thyA, *relA*$^+$])	This lab.	Leucine[4] Histidine[5]
K12	NF822	(arg, leu, thr, his, thi, *relC*)	C. Kari	Arginine[1]
K12	NF161	(met, arg, spoT)	B. Bachmann	Arginine[6]
K12	NF162	(met, arg, spoT, *relA*)	B. Bachmann	Arginine[6]
K12	BW113	(met, *relA*)	R. Langhammer	Methionine[7]
K12	C600	(thr, leu, thi)	This lab.	Leucine[8]
15TAU	CP107	(arg, thy, ura, thi)	U. Mortenson	Arginine[9]
15TAU	CP143	(arg, thy, ura, thi, *relA*)	U. Mortenson	Arginine[9]
B	B/r	(trp, thy)	B. Adler	Tryptophane[10]
K12	NF166	(met, arg, spoT [F′ argA$^+$, lysA$^+$, thyA, *relA*$^+$])	C. Kari	—

Culture medium was supplemented with:
[1] Amino acids (arginine, leucine, histidine, threonine), each 50 μg/ml; [2] Arginine, histidine, threonine, each 50 μg/ml; leucine 5 μg/ml; [3] Arginine, leucine, threonine, each 50 μg/ml, histidine 5 μg/ml; [4] Histidine, threonine, each 50 μg/ml, leucine 5 μg/ml; [5] Leucine, threonine, each 50 μg/ml, histidine 5 μg/ml; [6] Methionine, arginine, each 25 μg/ml; [7] Methionine 5 μg/ml; [8] Threonine, leucine, each 25 μg/ml; [9] Arginine 25 μg/ml; [10] Tryptophane 2·5 μg/ml.

Table 2. *Plasmid DNA content of several* E. coli *strains after limitation of amino acids and chloramphenicol treatment in comparison with log-phase cells at 30 °C*

Strains	Amino acid limitation	ng pBR322/cell mass*		Amplification factor	ng pBR322/cell mass after treatment with chloramphenicol	Amplification factor
		Log-phase	After limitation of amino acids			
CP78 *relA*$^+$	Arginine	120	130	1·2	620	5·2
CP79 *relA*	Arginine Leucine Histidine	130	730 600 660	5·6 4·6 5·1	680	5·2
CP79 F′ (*relA*$^+$)**	Leucine Histidine	100	100 105	1 1	500	5
NF822 *relA*$^+$ *relC*	Arginine	100	450	4·5	n.d.	n.d.
NF161 *relA*$^+$	Arginine	150	110	< 1	730	4·9
NF162 *relA*	Arginine	140	620	4·4	730	5·2
BW113 *relA*	Methionine	210	800	3·8	1300	6·2
C600 *relA*$^+$	Leucine	300	500	1·7	n.d.	n.d.
CP107 *relA*$^+$	Arginine	160	780	4·9	1700	10·6
CP143 *relA*	Arginine	130	1620	12·5	1 250	9·6
B/r *relA*$^+$	Tryptophane	150	250	1·7	1250	8·3

n.d. = no data.
* Cell mass: 1 ml of the bacterial culture (OD500 = 1)
** This strain was obtained after conjugational transfer of the F′ plasmid (argA$^+$, lysA$^+$, thyA, *relA*$^+$) from the donor NF166 into the recipient CP79

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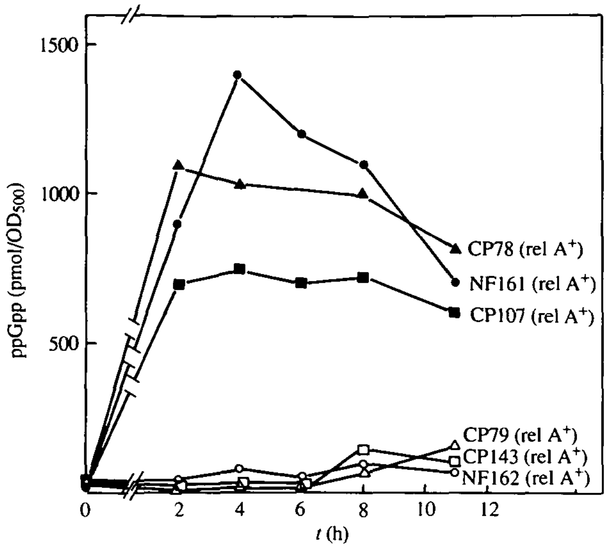

Fig. 1. Concentration of ppGpp after arginine limitation. t_0 = concentration of ppGpp in the log-phase. t_{1-11} = hours after cessation of growth due to arginine limitation.

plasmid DNA was measured, but in the amino acid-starved cells of the isogenic strains CP78 and NF161 plasmid level remained constant (Table 2).

The highest amplification factor was obtained with the *relA* mutant CP143. After deprivation of arginine this *E. coli* 15TAU strain amplified the plasmid 10 to 15-fold. Surprisingly the plasmid level increased in its stringently controlled counterpart CP107 as well. This strain also failed to show the typical stringent response concerning the control of stable RNA synthesis. The reason for this atypical behaviour remains to be elucidated.

The plasmid pBR322 was also amplified in the *E. coli relA$^+$relC* strain NF822, which does not increase its ppGpp pool in response to amino acid limitation (see Friesen *et al.* 1974; Parker *et al.* 1976).

In most cases amino acid starvation was achieved by the exhaustion of arginine; however amplification rate appeared to be independent of the limiting amino acid as similar plasmid yields were observed upon starvation for arginine, leucine, histidine or methionine (Table 2).

In a previous paper we reported that in the *E. coli relA* strain CP79 similar plasmid yields were obtained when cells were starved for amino acids or treated with Cm (Hecker et al., 1985). The same results were found using the *relA* mutants NF162, BW113 and CP143 (Table 2).

In kinetic experiments we observed that plasmid amplification in *E. coli* CP79 was initiated as growth stopped and continued for 8 to 10 hours (Fig. 2A). The same changes in plasmid concentration were detected when the cells were transferred from a growth medium containing all required amino acids into an amino acid-free medium (fig. 2B) or when valine was added to growing cells, but in this case the plasmid content increased 3 to 4-fold only (Fig. 2C).

Plasmid replication was also influenced by the growth temperature. In E. coli CP79 the maximum amplification factor (8-fold) was observed at 32 °C. At 37 °C the plasmid yield increased only 2-fold (Fig. 3).

In order to obtain further evidence that the differences in plasmid accumulation seen in Fig. 2 were due to *relA*-dependent differences in the ppGpp pool we introduced the *RelA$^+$* gene into *E. coli relA* CP79 on an F′ plasmid (*argA$^+$*, *lysA$^+$*, *thyA*, *relA$^+$*). After conjugational transfer this strain accumulated ppGpp and lost the ability to amplify the plasmid pBR322 (see Table 2).

4. Discussion

In this investigation the negative correlation between the ppGpp level and the replication of ColE1-related plasmids in amino acid-starved cells of *E. coli* proposed in a previous paper (Hecker et al., 1983) has been confirmed. When protein synthesis is inhibited with Cm both *relA* and *relA$^+$* strains amplify the plasmid. When protein synthesis is inhibited by amino-acid starvation only *relA* strains amplify. Since the only difference between *relA* and *relA$^+$* strains after amino-acid starvation is in the production of ppGpp (which is not produced after Cm addition), ppGpp might be responsible for the differences between the strains and thus might act as a negative regulator of plasmid replication.

All *relA* strains tested in this study amplified pBR322 plasmid DNA in response to amino acid starvation. Therefore we may conclude that the amplification of ColE1-related plasmids we observed is really caused by the *relA* mutation. Further evidence for our conclusion is given in this paper. When the *relA$^+$* allele was transferred into *E. coli* CP79 this *relA* strain acquired the capability to accumulate ppGpp and could no longer amplify the plasmid.

E. coli relC strains do not accumulate ppGpp during amino acid starvation in spite of an intact *relA* gene product because the ribosomal protein L11 engaged in ppGpp production is mutated (Friesen *et al.* 1974; Parker *et al.*, 1976). We found that the *relA$^+$relC* strain NF822 amplified the plasmid pBR322 under conditions of amino acid starvation. This result indicates that ppGpp itself and not an unknown function of the *relA* gene product prevents plasmid replication.

The results of Lin-Chao & Bremer (1986) are at variance with our conclusion. These authors described only a small increase in plasmid concentration in amino acid-starved cells of *E. coli relA* B or *relA* K12 strains at 37 °C.

We found that the growth temperature is a crucial factor for plasmid enrichment. In the *E. coli* K12 strain CP79 the highest plasmid yield was obtained at 32 °C. At 37 °C, in accordance with the results of Lin-Chao & Bremer (1986), the plasmid amplification was negligible. The different growth temperatures might

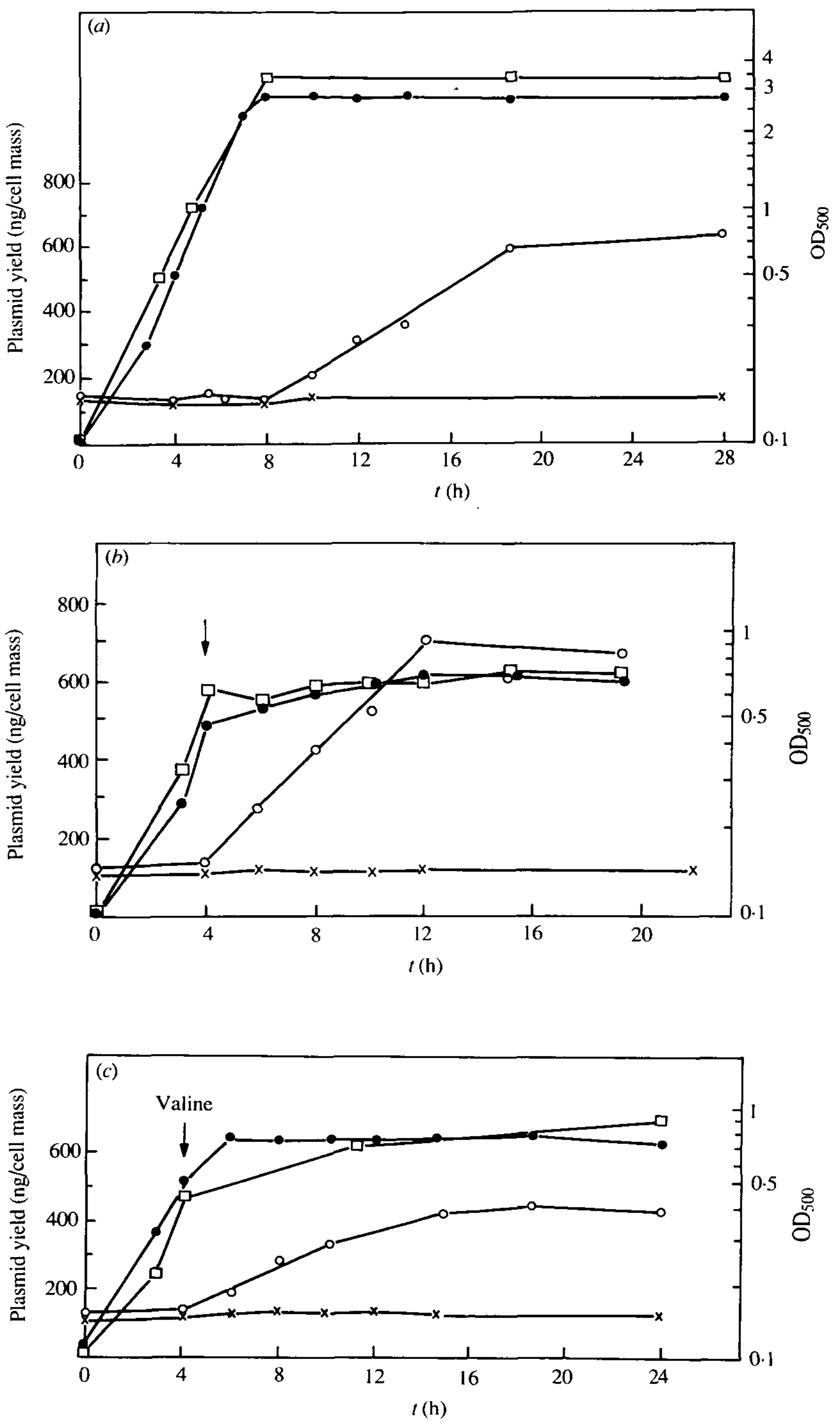

Fig. 2. Amplification of pBR322 DNA in the *relA*⁺ strain CP78 (–×–) and in the *relA* mutant CP79 (–○–) under conditions of amino acid limitation. Culture mass (OD500, CP78 –□–, CP79 –●–) (*a*) The *E. coli* cells ceased growth as a result of arginine exhaustion. Amino acid limitation was induced by: (*b*) transferring growing cells into amino acid free medium (arrow); (*c*) the addition of valine (1 mg/ml) to logarithmically growing cells.

explain the different results got by Lin-Chao and Bremer and in our studies. It has to be elucidated if there is any relation between growth temperature, ppGpp concentration and plasmid replication (see Ryals *et al.*, 1982).

The molecular mechanism of the ppGpp mediated negative control of plasmid replication is still unknown. At 37 °C Lin-Chao & Bremer (1986) did not detect any influence of ppGpp on the synthesis and accumulation of RNAI and RNAII which regulate the replication of ColE1-related plasmids (Davison, 1984; Cesareni & Banner, 1985; Tomizawa, 1986). Similar experiments will need to be done under conditions which results in plasmid amplification.

These results show that *relA* mutants of *E. coli* may be suitable hosts for the production of large amounts

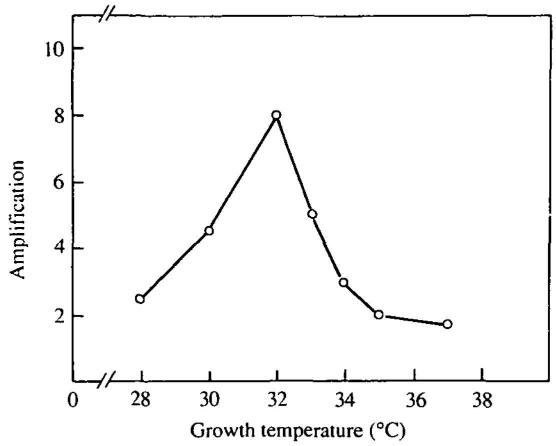

Fig. 3. Amplification of pBR322 DNA in the *relA* mutant CP79 after arginine exhaustion as a function of growth temperature.

of ColE1-related plasmids. The highest plasmid yield was reached with the *E. coli* strain CP143 which could amplify the plasmid pBR322 10 to 15-fold. Fermentation studies on plasmid production using this strain are being carried out (Hofmann *et al.*, in prep.).

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