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 ColEl-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.

ColEl-related plasmids are amplified during limitation of amino acids in the relaxed (relA~) *E. 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 ColEl-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 ColEl-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).

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), Na2HPO4·12H2O (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<sup>1</sup> |
| K12 CP79           | (arg, leu, thr, his, thi, relA) | This lab. | Arginine<sup>1</sup> |
| K12 CP79 F'        | (leu, thr, his, thi, [F' argA<sup>+</sup>, lysA<sup>+</sup>, thyA, relA<sup>+</sup>]) | This lab. | Leucine<sup>2</sup> |
| K12 NF822          | (arg, leu, thr, his, thi, relC) | C. Kari | Arginine<sup>1</sup> |
| K12 NF161          | (met, arg, spoT, relA) | B. Bachmann | Arginine<sup>4</sup> |
| K12 NF162          | (met, relA) | B. Bachmann | Arginine<sup>4</sup> |
| K12 BW113          | (thr, leu, thi) | R. Langhammer | Methionine<sup>7</sup> |
| K12 C600           | (thr, leu, thi) | This lab. | Leucine<sup>8</sup> |
| 15TAU CP107        | (arg, thy, ura, thi) | U. Mortenson | Arginine<sup>9</sup> |
| 15TAU CP143        | (arg, thy, ura, thi, relA) | U. Mortenson | Arginine<sup>9</sup> |
| B B'r              | (trp, thy) | B. Adler | Tryptophane<sup>10</sup> |
| K12 NF166          | (met, arg, spoT [F' argA<sup>+</sup>, lysA<sup>+</sup>, thyA, relA<sup>+</sup>]) | C. Kari | — |

Culture medium was supplemented with:

<sup>1</sup> Amino acids (arginine, leucine, histidine, threonine), each 50 μg/ml;  
<sup>2</sup> Arginine, histidine, threonine, each 50 μg/ml;  
<sup>3</sup> Arginine, leucine, threonine, each 50 μg/ml, histidine 5 μg/ml;  
<sup>4</sup> Histidine, threonine, each 50 μg/ml, leucine 5 μg/ml;  
<sup>5</sup> Leucine, threonine, each 50 μg/ml, histidine 5 μg/ml;  
<sup>6</sup> Methionine, arginine, each 25 μg/ml;  
<sup>7</sup> Methionine 5 μg/ml;  
<sup>8</sup> Threonine, leucine, each 25 μg/ml;  
<sup>9</sup> Arginine 25 μg/ml;  
<sup>10</sup> 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 | Log-phase | After limitation of amino acids | Amplification factor | ng pBR322/cell mass<sup>*</sup> | Amplification factor | ng pBR322/cell mass after treatment with chloramphenicol | Amplification factor |
|---------|-----------------------|-----------|-------------------------------|---------------------|-------------------------------|---------------------|--------------------------------------------------------|---------------------|
| CP78    | Arginine<sup>11</sup> | 120       | 130                           | 1.2                 | 620                           | 5.2                 | 680                                                    | 5.2                 |
| relA<sup>11</sup> | Arginine | 130       | 730                           | 5.6                 |                               |                     |                                                        |                     |
| CP79    | Arginine<sup>11</sup> | 130       | 600                           | 4.6                 |                               |                     |                                                        |                     |
| relA<sup>11</sup> | Leucine | 600       | 660                           | 5.1                 |                               |                     |                                                        |                     |
| CP79 F<sup>11</sup> (relA<sup>11</sup>)<sup>12</sup> | Leucine | 100      | 100                           | 1                   | 500                           | 5                   |                                                        |                     |
| NF822   | Arginine<sup>11</sup> | 100       | 450                           | 4.5                 | n.d.                          | n.d.                |                                                        | n.d.                |
| relA<sup>11</sup> | Arginine | 150       | 110                           | <1                  | 730                           | 4.9                 |                                                        |                     |
| relC    | Arginine<sup>11</sup> | 140       | 620                           | 4.4                 | 730                           | 5.2                 |                                                        |                     |
| NF161   | Arginine<sup>11</sup> | 210       | 800                           | 3.8                 | 1300                          | 6.2                 |                                                        |                     |
| relA<sup>11</sup> | Methionine | 300 | 500                           | 1.7                 | n.d.                          | n.d.                |                                                        | n.d.                |
| BW113   | Methionine<sup>11</sup> | 300 | 500                           | 1.7                 | 1700                          | 10.6                |                                                        |                     |
| relA<sup>11</sup> | Leucine | 160      | 780                           | 4.9                 | 1700                          | 10.6                |                                                        |                     |
| CP107   | Arginine<sup>11</sup> | 130       | 1620                          | 12.5                | 1250                          | 9.6                 |                                                        |                     |
| relA<sup>11</sup> | Arginine | 130       | 1620                          | 12.5                | 1250                          | 9.6                 |                                                        |                     |
| CP143   | Arginine<sup>11</sup> | 150       | 250                           | 1.7                 | 1250                          | 8.3                 |                                                        |                     |
| B/r<sup>11</sup> | 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<sup>+</sup>, lysA<sup>+</sup>, thyA, relA<sup>+</sup>) from the donor NF166 into the recipient CP79
Plasmid amplification in *Escherichia coli* *relA*

Fig. 1. Concentration of ppGpp after arginine limitation. t\_0 = concentration of ppGpp in the log-phase. t\_u = 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 ColEl-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 ColEl-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
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 ColEl-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 of plasmid DNA.
Plasmid amplification in Escherichia coli relA

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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