Polyamine regulation of stringent control in a polyamine-auxotrophic strain of *Escherichia coli*

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1. *Escherichia coli* BGA8, a mutant unable to synthesize putrescine, behaves as stringent or relaxed according to the presence or absence of polyamine, respectively, in the culture medium. 2. The relaxed synthesis of RNA can be reverted back to stringent by addition of putrescine or spermidine. 3. The stringent response depends on the concentration of the polyamine in the culture medium. 4. The formation of guanosine 3'-diphosphate 5'-diphosphate elicited by amino acid starvation is stimulated at least 40-fold in putrescine-supplemented bacteria and only about 2-fold in putrescine-depleted cells.

The coupling of RNA and protein synthesis is a mechanism which helps to maintain the growth balance in a cell. When the supply of a required amino acid is interrupted, the accumulation of both protein and stable RNA is arrested in stringent strains. However, in relaxed mutants, RNA synthesis is not halted to the same extent as protein synthesis (Cashel, 1975; Nierlich, 1978; Gallant, 1979; Cozzzone, 1980).

It has been proposed (Raina *et al.*, 1967; Cohen *et al.*, 1967; Cohen, 1971) that the intracellular ratio of spermidine to putrescine in *Escherichia coli* controls the synthesis of stable RNA during amino acid starvation. On the other hand, Srinivasan *et al.* (1973), working with a pair of closely related *rel*⁺ and *rel*⁻ strains with a conditional requirement for putrescine, found that during amino acid starvation the *rel*⁺ and *rel*⁻ cells remained stringent or relaxed, respectively, either with or without polyamine supplementation. They postulated an indirect role of polyamines in RNA synthesis, and reached the conclusion that the ratio of spermidine to putrescine was not the factor responsible for the synthesis of stable RNA.

This paper reports results obtained with another polyamine-auxotrophic strain, *E. coli* BGA8. In this mutant, unable to synthesize putrescine, a phenotypic effect of polyamines on the expression

of the stringent control was observed. This strain showed a perfect stringent response when grown in the presence of polyamines, but behaved as relaxed if it had been previously starved for putrescine. The relaxed cells could be reverted back to stringency by addition of putrescine or spermidine to the culture. After starvation for an amino acid, accumulation of ppGpp was larger in polyamine-supplemented than in polyamine-depleted cells.

Experimental

Materials

E. coli BGA8 is a polyamine-auxotrophic strain deficient in ornithine decarboxylase and agmatinase (*speB speC*), which has been described previously (Goldemberg & Algranati, 1981). *E. coli* D18, a mutant deficient in *S*-adenosylmethionine decarboxylase (*speD*) (Tabor *et al.*, 1978), was kindly provided by Dr. C. Tabor.

Minimal growth medium (without glycine and ornithine) and procedures for polyamine depletion were as previously reported (Goldemberg & Algranati, 1977). Isoleucine starvation was achieved by addition of L-valine (300 µg/ml) to the culture.

[2-¹⁴C]Uracil (55 Ci/mol) and [5,6-³H]uridine (42 Ci/mmol) were obtained from New England Nuclear Corp.; carrier-free [³²P]P_i was provided by the Argentine Atomic Energy Commission.

Methods

RNA synthesis was estimated by incorporation of [¹⁴C]uracil into trichloroacetic acid-insoluble material.

Abbreviations used: ppGpp, guanosine 3'-diphosphate 5'-diphosphate; pppGpp, guanosine 3'-diphosphate 5'-triphosphate; Mops, 4-morpholinopropane-sulphonic acid; PEI-cellulose, polyethylenimine-cellulose.

To measure net RNA synthesis and the UTP internal pools, cells were grown in Mops medium (Neidhardt *et al.*, 1974) with the addition of glucose, the required amino acids, thiamin and 0.2mM-phosphate. [^{32}P]P_i (20 $\mu\text{Ci/ml}$ of culture) was added to label the culture for several generations; when A_{490} reached 0.15, putrescine was added to a portion of the culture. After incubation for 1 h, portions were treated with valine for 15 min, and samples were taken to measure RNA synthesis, as well as UTP pools. RNA synthesis was determined in 500 μl of the culture by incubation with [^3H]uridine (8 μM , 20 $\mu\text{Ci/ml}$) for 30 s; 200 μl samples (in duplicate) of this mixture were precipitated with 10% (w/v) trichloroacetic acid containing 1 mg of unlabelled uridine/ml. Net RNA synthesis was calculated as indicated by Boyle & Sells (1976). For determination of the UTP pools, 60 μl of the culture was incubated with [^3H]uridine (50 μM , 0.2 mCi/ml) for 30 s, and two 25 μl portions were each treated with 25 μl of cold 2M-formic acid. After 40 min at 4°C and centrifugation (Eppendorf Microfuge), 20 μl portions of the supernatants, as well as standards, were spotted on PEI-cellulose plates (Schleicher and Schüll, Dassel, W. Germany). Development of the t.l.c. was performed with 0.85M-KH₂PO₄ (pH 3.4) (Cashel *et al.*, 1969), after a previous run with methanol. The nucleotides were detected under u.v. light, scraped off the plate, extracted with 0.1 ml of 5M-NH₃ for 30 min and counted for radioactivity with Bray's (1960) solution. The specific radioactivities of the UTP pools were calculated by determining the ratio of ^3H to ^{32}P radioactivity in the UTP spots (Morris, 1973).

For determination of ppGpp, cultures grown in Mops medium without polyamine were labelled for more than one generation with carrier-free [^{32}P]P_i (100 $\mu\text{Ci/ml}$), putrescine was added to half the cell suspension, and after incubation for 1 h valine was added to portions of polyamine-depleted and supplemented cultures. Samples (50 μl) were taken after 15–30 min of amino acid starvation, and the

reaction was terminated by the addition of 10 μl of 25% (v/v) formic acid (Igarashi *et al.*, 1983). After 30 min in the cold and centrifugation in an Eppendorf Microfuge, samples of the supernatant fluids, as well as standards, were spotted on PEI-cellulose plates (Polygram Cel 300 PEI; Macherey Nagel and Co., Düren, West Germany). Two-dimensional t.l.c. was performed with 4M-formic acid/1M-LiCl in the first dimension, and 1.5M-KH₂PO₄ (pH 3.5) in the second (Ruppen & Switzer, 1983). After autoradiography, ppGpp and pppGpp were located, and counted for radioactivity as described by Ruppen & Switzer (1983).

Results

Isoleucine starvation is easily accomplished in *E. coli* K12 derivatives by the addition of L-valine, which causes feedback inhibition of the first enzyme in the isoleucine pathway (Ramakrishnan & Adelberg, 1965).

Fig. 1 shows the effect of isoleucine deprivation on growth rate and RNA synthesis in *E. coli* BGA8, a polyamine-auxotrophic mutant cultivated in the absence and presence of putrescine. The polyamine-supplemented cells showed a dramatic decrease not only in growth rate (Fig. 1b) but also in RNA accumulation (Fig. 1d). In contrast, in the polyamine-depleted cells growth was halted, but [^{14}C]uracil incorporation into RNA was only partially inhibited (Figs. 1a and 1c).

Similar results were obtained when the culture was starved for a required amino acid by rapid filtration and resuspension in medium without the amino acid.

It could be argued that the higher incorporation of [^{14}C]uracil into trichloroacetic acid-insoluble material observed in the polyamine-depleted culture starved for isoleucine might be due to a decrease in the UTP endogenous pool. To determine if the change in growth conditions could lead to a difference in the specific radioactivity of the nucleotide pools, polyamine-depleted cells labelled

Table 1. Effect of valine addition on ppGpp and pppGpp accumulation in polyamine-depleted and supplemented cells. *E. coli* BGA8 cells were labelled with [^{32}P]P_i, treated with putrescine (1 h) and valine (10 min) and then analysed for ppGpp and pppGpp as described in the Experimental section. The nucleotide concentrations were calculated from the ^{32}P radioactivity of a sample of the labelled culture spotted on a disc of PEI-cellulose and counted. Values are averages of duplicate experiments.

Putrescine in the culture	ppGpp (pmol/100 μl of formic acid extract)		pppGpp (pmol/100 μl of formic acid extract)	
	No valine	+ valine	No valine	+ valine
–	0.63	2.03	<0.1	0.27
+	<0.1	3.63	<0.1	0.63

Fig. 1. Effect of isoleucine starvation on growth rate and RNA synthesis in polyamine-depleted or supplemented bacteria. Polyamine-depleted *E. coli* BGA8 cells were cultivated without polyamine (a) or supplemented with putrescine when A_{490} reached 0.15 (b) (arrow P); 1 h later, valine was added to a portion of each culture (arrow V), and after 15 min (arrow S) 3 ml samples of putrescine-unsupplemented and supplemented bacteria were withdrawn (c and d respectively) for determination of RNA synthesis. These cells were further incubated with [14 C]uracil (0.018 μ mol, 0.1 μ Ci/ml of culture), and 0.5 ml samples were taken at different intervals to measure RNA synthesis as indicated in the Experimental section. Cell growth (a and b) was monitored by the A_{490} . Bacteria were grown in the absence (○) or in the presence (●) of valine.

with [32 P] P_i were cultivated in the absence or presence of putrescine for 1 h, incubated with or without valine for 15 min and pulsed with [3 H]-uridine for 30 s. The values obtained in the absence of valine indicated a 23% expansion of the UTP pool after putrescine supplementation, in agreement with the results reported by Morris (1973) for *E. coli* DK6. When the cells were starved for isoleucine, the specific radioactivity of the UTP pool remained unchanged in the polyamine-depleted culture, whereas in the presence of putrescine it was decreased by 28%. Taking these specific radioactivity values into account, net RNA synthesis was found to decrease by more than 90% in the

presence of putrescine, and by only 28% in its absence, when bacteria were starved for isoleucine. Therefore it may be concluded that the sustained [14 C]uracil incorporation into RNA observed in amino acid-starved cells grown in the absence of polyamine was not due to an increase in specific radioactivity of [14 C]UTP elicited by shrinkage of the nucleotide pool.

The relaxed response evoked in polyamine-starved bacteria could be reverted back to stringent by addition of putrescine. Fig. 2 illustrates an experiment in which, after addition of putrescine, samples were taken at different intervals to measure the capacity of the cells to shut off RNA

Fig. 2. Capacity of polyamine-depleted cells to recover stringent control after putrescine addition

Polyamine-depleted cells growing in the absence of polyamine (○) were supplemented with putrescine (●) at zero time. At the indicated intervals, samples were taken to measure [^{14}C]uracil incorporation into RNA after 30 min incubation, in both the presence and the absence of valine; relaxation percentage was calculated as the ratio of incorporation (+valine)/(-valine).

Fig. 3. Recovery of stringent control in polyamine-starved bacteria after addition of putrescine after valine treatment

Polyamine-depleted bacteria were incubated for 15 min in the presence or in the absence of valine. At zero time, putrescine was added to a portion of isoleucine-starved and unstarved cells. At the indicated intervals, samples were taken to measure [^{14}C]uracil incorporation into RNA. Relaxation percentages were calculated for polyamine-depleted (○) and supplemented (●) cells as in Fig. 2.

synthesis when deprived of isoleucine. A return to the stringent response began immediately after putrescine addition, whereas the polyamine-starved culture kept the original relaxed rate. If the culture was pretreated with valine before putrescine was added, there was a certain delay in the restoration of stringency evoked by the polyamine (Fig. 3).

The stringent response depended on the concentration of polyamine in the culture medium. Fig. 4 presents the decreasing percentage of relaxation obtained when the cells were supplemented with increasing concentrations of putrescine. When spermidine replaced putrescine, the restoration of stringency became even more efficient.

E. coli D18 is a *speD* mutant unable to synthesize spermidine (Tabor *et al.*, 1978). Depletion of this polyamine in *E. coli* D18 did not alter the stringent response, nor was there any significant change when spermidine was added back to the culture.

In stringent bacteria the absence of a required amino acid elicits the accumulation of ppGpp and pppGpp; these nucleotides are not found in relaxed strains under similar conditions (Cashel, 1975; Nierlich, 1978; Gallant, 1979). In *E. coli*

Fig. 4. Effect of different concentrations of putrescine and spermidine on the restoration of stringency in a polyamine-depleted culture

Polyamine-depleted bacteria were supplemented with different concentrations of putrescine (○) or spermidine (●), and after 1 h valine was added to a part of the culture for 15 min. RNA synthesis was measured in the presence and in the absence of valine, and relaxation percentages were calculated as in Fig. 2.

BGA8 starved for isoleucine there was a slight formation of ppGpp in the absence of putrescine. Polyamine addition to the culture stimulated the nucleotide accumulation about 2-fold; pppGpp was also evident. In polyamine-depleted cells not starved for isoleucine, very small amounts of ppGpp were present, but the nucleotide disappeared after addition of putrescine (Table 1).

Discussion

Previous studies with some *E. coli* strains which require polyamines for optimal growth have indicated an abnormal ribosomal distribution pattern when cells are deprived of putrescine, as well as a decrease in growth rate and macromolecular synthesis (Algranati *et al.*, 1975; Algranati & Goldemberg, 1977). The defect was located in the 30S subunit, probably at the level of the RNA (Algranati *et al.*, 1976; Igarashi *et al.*, 1979), though a decrease in protein S1 has also been reported by Igarashi *et al.* (1979). Furthermore, the inhibitory effect of streptomycin and other aminoglycoside antibiotics on growth and protein synthesis was also dependent on the presence of polyamines in the culture medium (Goldemberg & Algranati, 1981).

The results reported in the present paper show another example of polyamine requirement, namely to maintain the balance between RNA and protein synthesis during amino acid starvation. The non-co-ordinate regulation of RNA synthesis elicited by the lack of an amino acid in relaxed strains was observed in *E. coli* BGA8 cultures in the absence of polyamines. The same cells showed a stringent response if putrescine was added to the culture medium. The restoration of stringent control was concentration dependent.

The sustained RNA synthesis observed in putrescine-depleted cells derived of isoleucine could be reverted by addition of polyamine to the culture (Fig. 2). However, if the bacteria were treated with valine before putrescine was supplied to restore stringency, the recovery was slower, with a certain lag before it started (Fig. 3). This might indicate that the cell requires some time to adjust to the correct functioning of its controls after addition of polyamine.

The results reported in this paper are not in agreement with those obtained by Srinivasan *et al.* (1973), working with a pair of isogenic *rel*⁺ and *rel*⁻ polyamine-requiring mutants; they found no difference in stringent control elicited by polyamines.

It has been proposed that the intracellular accumulation of spermidine in *E. coli* parallels that of RNA in a variety of growth conditions (Raina *et al.*, 1967; Cohen *et al.*, 1967; Cohen, 1971). This

idea is not supported by the results obtained with *E. coli* strains BGA8 and D18, maybe because both strains are mutants blocked in the polyamine-biosynthetic pathway. Moreover, the various effects obtained with the different strains may also indicate that polyamines affect RNA synthesis in more than one way.

The formation of ppGpp and pppGpp during the stringent response was stimulated about 2-fold in strain BGA8 growing in the presence of putrescine. In polyamine-depleted bacteria, there was also an accumulation of ppGpp in the absence of isoleucine. It should be pointed out that there was a small amount of the nucleotide in the polyamine-depleted cells even before addition of valine, but ppGpp was no longer detectable after putrescine supplementation (Table 1). This unusual observation, as well as the stimulation of ppGpp accumulation after isoleucine deprivation, even though the cells showed a relaxed behaviour, might be attributed to the defective ribosomes of *E. coli* BGA8 (Algranati & Goldemberg, 1977). The impaired assembly of the subparticles would then possibly affect the regulation of the stringent factor. Igarashi *et al.* (1983), working with a mutant unable to synthesize putrescine and spermidine, found that the formation of ppGpp was also stimulated about 2-fold by spermidine *in vivo* either before or after amino acid starvation.

There are several examples in the literature in which a bacterium carrying a mutation in a locus different from the *rel* gene shows the relaxed phenotype under non-permissive conditions (Atherly & Suchanek, 1971; Sokawa *et al.*, 1972; Atherly & Menninger, 1972). *E. coli* BGA8 is a streptomycin-sensitive mutant blocked in the polyamine-biosynthetic pathway. Under non-permissive conditions, namely in the absence of polyamines, the strain behaves abnormally, not only in response to the lack of an amino acid as described above, but also in the presence of several aminoglycoside antibiotics (Goldemberg & Algranati, 1981).

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References

- Algranati, I. D. & Goldemberg, S. H. (1977) *Trends Biochem. Sci.* **2**, 272-274
- Algranati, I. D., Echandi, G., Goldemberg, S. H., Cunningham-Rundles, S. & Maas, W. K. (1975) *J. Bacteriol.* **124**, 1122-1127

- Algranati, I. D., Echandi, G., García-Patrone, M., González, N. S. & Goldemberg, S. H. (1976) *Arch. Biol. Med. Exp.* **10**, 49–60
- Atherly, A. G. & Menninger, J. R. (1972) *Nature (London) New Biol.* **240**, 245–246
- Atherly, A. G. & Suchanek, M. C. (1971) *J. Bacteriol.* **108**, 627–638
- Boyle, S. M. & Sells, B. H. (1976) *Arch. Biochem. Biophys.* **172**, 215–223
- Bray, G. A. (1960) *Anal. Biochem.* **1**, 279–285
- Cashel, M. (1975) *Annu. Rev. Microbiol.* **24**, 301–318
- Cashel, M., Lazzarini, R. A. & Kalbacher, B. (1969) *J. Chromatogr.* **40**, 103–109
- Cohen, S. S. (1971) *Introduction to the Polyamines*, Prentice-Hall, Englewood Cliffs
- Cohen, S. S., Hoffner, N., Jansen, M., Moore, M. & Raina, A. (1967) *Proc. Natl. Acad. Sci. U.S.A.* **57**, 721–728
- Cozzzone, A. J. (1980) *Biochimie* **62**, 647–664
- Gallant, J. A. (1979) *Annu. Rev. Genet.* **13**, 393–415
- Goldemberg, S. H. & Algranati, I. D. (1977) *Mol. Cell. Biochem.* **16**, 71–77
- Goldemberg, S. H. & Algranati, I. D. (1981) *Eur. J. Biochem.* **117**, 251–255
- Igarashi, K., Kashiwagi, K., Kishida, K., Watanabe, Y., Kogo, A. & Hirose, S. (1979) *Eur. J. Biochem.* **93**, 345–353
- Igarashi, K., Mitsui, K., Kubota, M., Shirakuma, M., Ohnishi, R. & Hirose, S. (1983) *Biochim. Biophys. Acta* **755**, 326–331
- Morris, D. R. (1973) in *Polyamines in Normal and Neoplastic Growth* (Russell, D. H., ed.), pp. 111–122, Raven Press, New York
- Neidhardt, F. C., Bloch, P. L. & Smith, D. F. (1974) *J. Bacteriol.* **119**, 736–747
- Nierlich, D. P. (1978) *Annu. Rev. Microbiol.* **32**, 393–432
- Raina, A., Jansen, M. & Cohen, S. S. (1967) *J. Bacteriol.* **94**, 1684–1696
- Ramakrishnan, T. & Adelberg, E. A. (1965) *J. Bacteriol.* **89**, 654–660
- Ruppen, M. E. & Switzer, R. L. (1983) *J. Bacteriol.* **155**, 56–63
- Sokawa, Y., Sokawa, J. & Kaziro, Y. (1972) *Nature (London) New Biol.* **240**, 242–245
- Srinivasan, P. R., Young, D. V. & Mass, W. (1973) *J. Bacteriol.* **116**, 648–655
- Tabor, C. W., Tabor, H. & Hafner, E. W. (1978) *J. Biol. Chem.* **253**, 3671–3676