FUNCTIONING OF *spoT* GENE PRODUCT IN *BACILLUS SUBTILIS* CELLS

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1. Introduction

Guanosine 5′-diphosphate, 3′-diphosphate (ppGpp) is one of the most important low M_r regulators of bacterial metabolism [1]. Concentration of ppGpp in *Escherichia coli* cells is regulated both at the levels of its synthesis and degradation. A sharp increase in the ppGpp pool size is observed during aminoacyl-tRNA limitation [2]. This is caused by the enhanced production of the ppGpp precursor guanosine 5′-triphosphate, 3′-diphosphate (pppGpp), catalyzed by the product of the *relA* gene [3–6], and is accompanied by pppGpp accumulation. The concentration of ppGpp under these conditions is 2–4-fold higher than the level of pppGpp [2,5].

The degradation of ppGpp in *E. coli* cells is governed by the product of the *spoT* gene [3–6]. The activity of this enzyme in vivo and in vitro is stimulated by Mn^{2+} [7–10]. Therefore, inactivation of the *spoT* gene product can be achieved by treatment with some agents chelating divalent metal ions. Under these conditions both in *relA*$^+$ and *relA* cells the ppGpp pool expands in the absence of pppGpp accumulation [11].

While the presence of (p)ppGpp was found in the cells of different bacterial species [12], the metabolism of these nucleotides was investigated only in *E. coli*. During aminoacyl-tRNA limitation in *Bacillus subtilis* cells the pppGpp pool is higher than that of ppGpp, which is in contrast to *E. coli* cells [13]. In order to characterize further (p)ppGpp metabolism in *B. subtilis* we studied the process of ppGpp degradation. For this purpose we used an ion chelator 1,10-phenanthroline and found a sharp accumulation of ppGpp, caused by its slow decay. This effect of phenanthroline was suppressed by Mn^{2+}. Thus the stability of ppGpp in *B. subtilis* seems to be under a control mechanism analogous to the *spoT* gene function in *E. coli*.

2. Materials and methods

2.1. *Bacterial strains and growth conditions*

Bacillus subtilis 168 IS58 *trpC*2, *lys*, *relA*$^+$ and IS56 *trpC*2, *lys*, *relA* [14] are identical to the strains BR16 *rel*$^+$ and BR16 *rel* [15] and were kindly provided by Dr E. Dubnau (USA). The cells were grown in Tris–glucose minimal medium as in [13], but without citrate and at 0.4 mM KH_2PO_4.

2.2. *Determination of protein synthesis, RNA accumulation and nucleotide pools*

All methods used were according to [13], with slight modifications. Exponentially growing cells at 0.25 A_{600} units were labelled with 4 μCi/ml, 5 μg/ml [^{3}H]leucine and 2.2 μCi/ml, 5 μg/ml [^{14}C]uracil or with 100 μCi/ml $H_3{}^{32}PO_4$. For measuring the rates of protein synthesis and RNA accumulation 100 μl samples were mixed with 5% trichloroacetic acid, and the acid-insoluble fraction was collected on glass-fiber filters GF-C (Whatman, England). Nucleotide pools were determined according to [2], after thin-layer chromatography on PEI-cellulose plates (Macherey-Nagel, FRG).

3. Results

The chelating agent 1,10-phenanthroline at >0.1 mM inhibits protein synthesis and RNA accumulation in *relA*$^+$ strain of *B. subtilis* (fig.1a,b). At the same time the intracellular concentration of (p)ppGpp significantly increases, with the ppGpp pool being 2–4-fold larger than the pppGpp level (fig.2a). The observed (p)ppGpp accumulation is not the result of induced amino acid starvation, because addition of the chelator to the cells, grown in the presence of all 20 amino acids, also elicits a distinct expansion of ppGpp pool (fig.2b).

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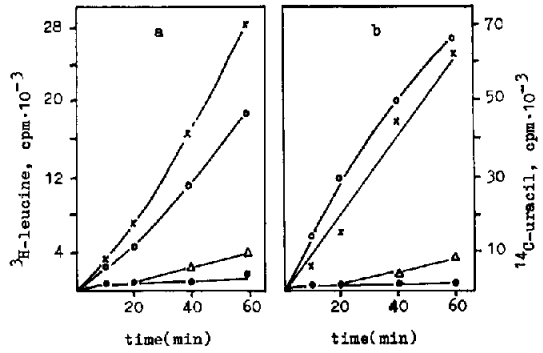

Fig.1. Protein synthesis and RNA accumulation in IS58 *relA*$^+$ cells treated with phenanthroline: (x———x) exponential growth; (●———●) + 0.5 mM phenanthroline (Reanal, Hungary) at zero time; (o———o) + 0.5 mM phenanthroline and 20 μM $MnCl_2 \cdot 4\,H_2O$ at zero time; (△———△) + 0.5 mM phenanthroline at zero time and 20 μM $MnCl_2 \cdot 4\,H_2O$ at +20 min.

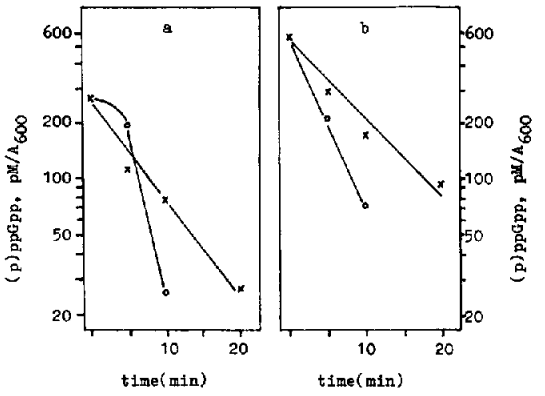

Fig.3. Decay of (p)ppGpp in IS58 *relA*$^+$ cells treated with phenanthroline. The cells were treated with 0.5 mM phenanthroline at −20 min; at zero time 100 μg chloramphenicol/ml (x———x) or 20 μM $MnCl_2 \cdot 4\,H_2O$ (o———o) was added; (left) pppGpp; (right) ppGpp.

During aminoacyl-tRNA limitation in *B. subtilis*, the protein synthesis inhibitor chloramphenicol caused a rapid breakdown of (p)ppGpp with half-lives of 20–40 s [13]. When chloramphenicol is added to phenanthroline-treated cells, a much slower degradation of ppGpp is observed with an estimated half-life of 5–7 min (fig.3b). The same rate of degradation of phenanthroline-induced ppGpp is observed after addition of another translation inhibitor tetracycline, at 10 μg/ml (not shown).

The inhibitory action of phenanthroline can be suppressed by the presence of Mn^{2+} at >1 μM. Simultaneous addition of Mn^{2+} and the chelator almost completely relieves the inhibition of protein synthesis and RNA accumulation (fig.1a,b). The addition of Mn^{2+} 20 min after phenanthroline leads to the rapid decline of (p)ppGpp pools (fig.3) and the concomitant restoration of protein synthesis and RNA accumulation (fig.1a,b). The effect of Mn^{2+} seems to be quite specific. Mg^{2+} are present in the medium at 1 mM and therefore not able to neutralize phenanthroline action. Ca^{2+}, Fe^{2+} or Zn^{2+} at 20 mM, 50 μM and 10 μM, respectively, do not alter the efficiency of inhibition caused by 0.5 mM phenanthroline (if [chelator] is lowered to 0.1 mM, Fe^{2+} are able to suppress the inhibition as well as Mn^{2+}; not shown).

In the cells of *B. subtilis relA* mutant treated with phenanthroline some increase in the ppGpp pool can be seen also (fig.4a). The decay of ppGpp under these

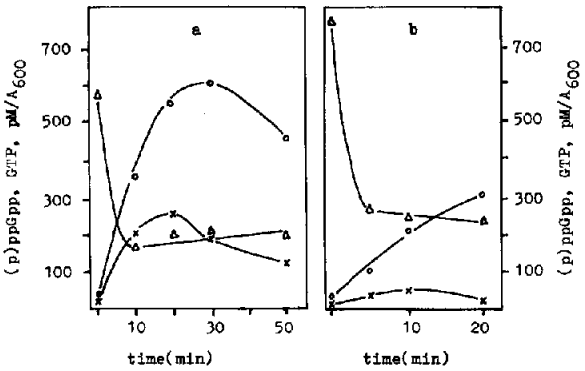

Fig.2. Guanine nucleotides pools in IS58 *relA*$^+$ cells treated with 0.5 mM phenanthroline: (x———x) pppGpp; (o———o) ppGpp; (△———△) GTP; (left) minimal medium with tryptophan and lysine; (right) minimal medium with 20 amino acids at 40 μg/ml each.

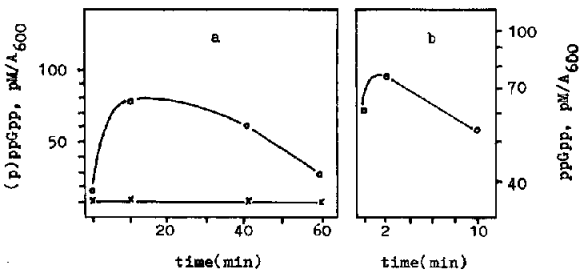

Fig.4. Pools and stability of pppGpp (x———x) and ppGpp (o———o) in IS56 *relA* cells treated with phenanthroline: (left) 0.5 mM phenanthroline was added at zero time; (right) 41 min later 100 μg chloramphenicol/ml was added to chelator-treated cells.

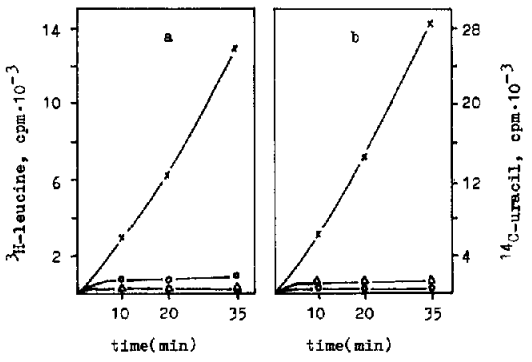

Fig.5. Protein synthesis and RNA accumulation in IS56 *relA* cells treated with phenanthroline: (x– – –x) exponential growth; (•– – –•) + 0.5 mM phenanthroline; (△– – –△) + 0.5 mM phenanthroline and 100 μg chloramphenicol/ml.

conditions, observed after chloramphenicol addition, is very slow (fig.4b), the same as in *relA*$^+$ cells.

Neither chloramphenicol nor tetracycline are able to prevent the inhibition of RNA accumulation in the presence of phenanthroline in *relA*$^+$ or *relA* cells (fig.5b). Furthermore the degree of protein synthesis and RNA accumulation inhibition in these cells is almost the same in spite of different levels of ppGpp (fig.1,5). Therefore the lack of RNA accumulation under these conditions is most probably not caused by the elevated pool of ppGpp.

4. Discussion

Here we show that 1,10-phenanthroline enhanced the stability of ppGpp in *B. subtilis* and evoked its accumulation in these cells. The degradation of ppGpp in *B. subtilis* is due to the activity of an enzyme, ppGpp-phosphohydrolase, which, by analogy with *E. coli*, may be called the product of *spoT* gene. Our results show that in *B. subtilis* as well as in *E. coli* cells the inactivation of *spoT* gene product takes place after treatment with a chelating agent and can be abolished by addition of Mn^{2+}. This suggests the involvement of similar mechanisms in ppGpp degradation in these bacteria. The existence of an Mn^{2+}-dependent enzyme capable of ppGpp degradation has been shown in *B. subtilis* cell extracts [16].

We conclude that ppGpp accumulation in *B. subtilis* can occur not only during aminoacyl-tRNA limitation, activating the *relA* gene product [13–15,17], but during inhibition of the *spoT* gene product functions as well. The stabilization of ppGpp is probably the only cause of its accumulation in *relA* cells, unable to accelerate ppGpp production [13,15,17], and the main reason for ppGpp pool expansion in the chelator-treated *relA*$^+$ cells. In the latter case some increase in the rate of ppGpp synthesis seems plausible, which is responsible for the difference in ppGpp levels in *relA*$^+$ and *relA* cells (fig.2a,4a) and for the unequal accumulation of (p)ppGpp in *relA*$^+$ cells grown in different media (fig.2).

A peculiar feature of *B. subtilis relA*$^+$ cells is the elevation of the pppGpp pool after phenanthroline addition (fig.2a). At least partially this is explained by the enhanced stability of this nucleotide (fig.3a). The possible role of the *spoT* gene product in pppGpp degradation in *B. subtilis* will be described separately.

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