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Different effects of ppGpp on *Escherichia coli* DNA replication *in vivo* and *in vitro*

Monika Maciąg-Dorszyńska, Agnieszka Szalewska-Pałasz, Grzegorz Węgrzyn*

Department of Molecular Biology, University of Gdańsk, Wita Stwosza 59, 80-308 Gdańsk, Poland

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ABSTRACT

Inhibition of *Escherichia coli* DNA replication by guanosine tetraphosphate (ppGpp) is demonstrated *in vitro*. This finding is compatible with impairment of the DnaG primase activity by this nucleotide. However, in agreement to previous reports, we were not able to detect a rapid inhibition of DNA synthesis in *E. coli* cells under the stringent control conditions, when intracellular ppGpp levels increase dramatically. We suggest that the process of ppGpp-mediated inhibition of DnaG activity may be masked in *E. coli* cells, which could provide a rationale for explanation of differences between ppGpp effects on DNA replication in *E. coli* and *Bacillus subtilis*.

Introduction

Guanosine tetraphosphate (ppGpp) is a specific nucleotide playing the role of a signal molecule involved in a global bacterial regulatory response to stress conditions, called the stringent response [1]. Although initially linked solely to amino acid starvation, the stringent response is now recognized as a process connected to various nutritional and environmental stresses [1,2]. For a long time, ppGpp had been considered as a signal molecule occurring exclusively in bacterial cells. However, recent analyses indicated occurrence of homologues of genes coding for enzymes of ppGpp metabolism in various organisms, from bacteria, through protists and plants, to animals, including *Homo sapiens* [3], implicating possible regulatory roles of ppGpp (in bacteria) or putative related nucleotides (in eukaryotes) in organisms from various domains of life. In addition to the stress response, ppGpp was reported to be one of the main regulators of the growth rate control in a model Gram-negative bacterium *Escherichia coli* [4]. Furthermore, recent studies on a model Gram-positive bacterium *Bacillus subtilis* led to the proposal that ppGpp is required to maintain physiological GTP levels even in the absence of starvation [5]. These recently published reports strongly suggest a global regulatory role for ppGpp, which is not restricted to conditions of nutrient limitation (when levels of this nucleotide are highly elevated).

In bacteria, shortly after the onset of starvation conditions, ppGpp is produced in large amounts [1]. In *E.* coli, this nucleotide directly interacts with RNA polymerase and modulates significantly its transcriptional properties. Therefore, dramatic changes in transcription of many genes are observed during the stringent response, and they are considered the primary effects of this cellular response, despite the fact that considerable changes in regulation of various cellular processes occur in starved cells [1,6,7]. The RNA polymerase-associated protein, DksA, was shown to be indispensable for the stringent response, and its role was suggested to enhance *in vivo* and in *vitro* effects of ppGpp, thus, DksA was proposed to be a co-factor of this regulation [8,9].

One of crucial processes which are severely affected under conditions of the stringent response is DNA replication. Specific inhibition of DNA synthesis was first described for chromosomes of *B. subtilis* and *E. coli* [10], but subsequent studies indicated that such a phenomenon occurs also in various other replicons (for reviews see Refs. [11,12]). Interestingly, for *E. coli* chromosome, the (p)ppGpp-mediated inhibition of replication was postulated to occur only at the initiation stage (Ref. [11] and references therein), whereas in *B. subtilis*, an arrest of the chromosomal replication forks was reported [10,13], strongly suggesting that ppGpp may impair DNA replication elongation. Subsequent studies demonstrated also a ppGpp-dependent cell cycle arrest at the stage of *E. coli* chromosome segregation [14], but no considerable inhibition of replication elongation could be detected in this bacterium.

A new light on the mechanism of ppGpp-mediated inhibition of DNA replication was shed by finding that *B. subtilis* primase activity is impaired by direct binding of this nulecotide [15]. These results suggested the molecular mechanism of negative regulation of replication elongation based on ineffective synthesis of primers. One could speculate that this might be potentially a reason for differences between effects of ppGpp on DNA replication in *B. subtilis* and *E. coli*. However, results of subsequent experiments, obtained by our group [16] and corroborated recently by others [17], led to the conclusion that

Abbreviations: ppGpp, guanosine tetraphosphate; pppGpp, guanosine pentaphosphate.

* Corresponding author. Tel.: +48 58 523 6024; fax: +48 58 523 5501.
E-mail address: wegrzyn@biotech.univ.gda.pl (G. Węgrzyn).

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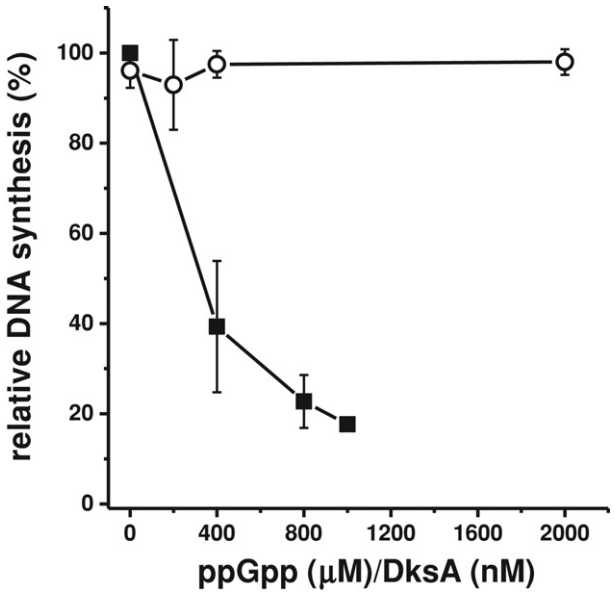

Fig. 1. Effects of ppGpp and DksA on *in vitro* DNA replication. The reactions were performed in the presence of the Fraction II from wild type bacteria and increasing ppGpp concentrations (closed squares), and the Fraction II isolated from the *dksA* mutant and increasing concentrations of DksA (open circles). The value obtained in experiments with [^{3}H]thymidine incorporation without additional factors was set as 100%. This value corresponds to 68 pmol of synthesized DNA. Mean values from three experiments with error bars representing SD are shown.

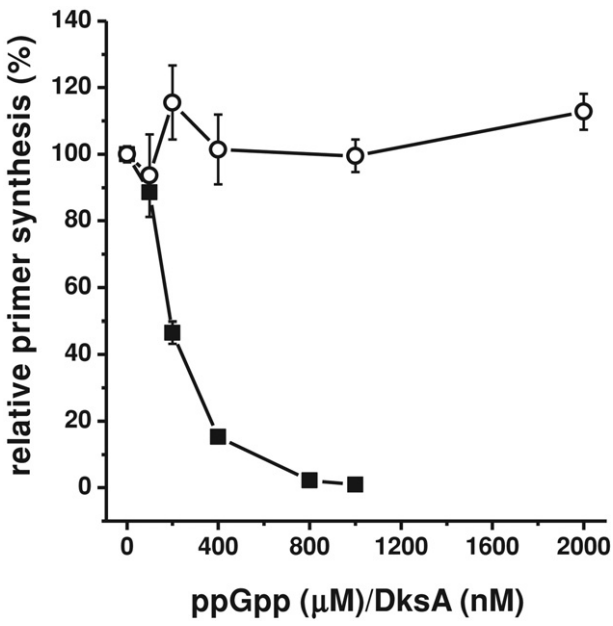

Fig. 2. Effects of ppGpp and DksA on the DnaG primase activity. Primer synthesis was performed by DnaG in the presence of either ppGpp (closed squares) or DksA (open circles). The synthesis with no additional factors was set as 100%. Mean values from three independent experiments with error bars representing SD are shown.

E. coli DnaG primase is also directly inhibited by ppGpp; this inhibition occurs most probably due to direct obstruction of the primase active site by ppGpp [17]. Therefore, the question appeared whether ppGpp-mediated negative regulation of DNA replication elongation may also occur in *E. coli*. To address this question, we have studied effects of ppGpp on *E. coli* DNA replication *in vitro* in comparison to effects of the stringent response on DNA synthesis *in vivo*.

Results

Until now, effects of ppGpp on *E. coli* DNA replication were tested *in vivo*, using stringent (wild-type) and relaxed (not able to produce ppGpp in amino acid-starved cells) strains [10,18,19]. It was speculated that ppGpp may influence *oriC*-initiated replication initiation indirectly, through changes in efficiency of transcription from promoters whose functions are important in either expression of genes coding for replication proteins or in transcriptional activation of the origin [18,20], similarly to the mechanism actually described for plasmids derived from bacteriophage λ [21]. Nevertheless, since results of those experiments strongly suggested that *E. coli* chromosome replication is inhibited during the stringent response at the stage of initiation rather than elongation, we have tested effects of ppGpp on *E. coli* DNA replication *in vitro*.

We have employed a semi-purified *in vitro* replication system, in which a cellular fraction containing all proteins necessary for the replication process (called Fraction II) is used [22]. We found a marked inhibition of DNA synthesis *in vitro* in the presence of ppGpp (Fig. 1). These results are compatible with the ppGpp-mediated inhibition of DnaG primase activity, reported previously [16] and confirmed in this work (Fig. 2).

Since DksA is considered as a co-factor of the stringent response, we asked whether it can affect DNA replication. The addition of purified DksA protein to the *in vitro* replication assay showed no effect of this protein (Fig. 1). Contrary to ppGpp, this protein also did not inhibit *E. coli* DnaG primase activity *in vitro* (Fig. 2).

In the light of the results of *in vitro* experiments, we have investigated kinetics of DNA replication in *E. coli* cells under conditions of amino acid starvation. Agents resulting in inhibition of DNA replication elongation cause a quick impairment in incorporation of labeled precursors, which is exemplified by the effects of the presence of DNA-intercalating antibiotic, mitomycin C (Fig. 3A). On the other hand, if only replication initiation is affected, minor effects on DNA synthesis can be observed shortly after induction of the inhibiting conditions. In fact, such a phenomenon was observed in amino acid-starved stringent strain (Fig. 3A), which massively produced ppGpp under these conditions (Fig. 3C), and whose growth was rapidly inhibited upon the starvation onset (Fig. 3B). A lack of both ppGpp synthetases (RelA and SpoT proteins) in the ppGpp-null strain leads to inability of ppGpp production (Fig. 3B) but did not influence DNA synthesis in starved and unstarved *E. coli* cells (Fig. 4). These results corroborate previously reported data [18] suggesting that ppGpp may influence *E. coli* chromosome replication *in vivo* only at the initiation stage.

Discussion

While the stringent response is a global response of vast majority of bacteria to nutritional stresses, it appears that specific regulatory mechanisms, mediated by ppGpp, the alarmone of this response, may differ between various species [1]. One example is DNA replication, the crucial cellular process, which is inhibited under stringent response conditions in both *E. coli* and *B. subtilis*, while its different stages are affected in these bacteria. It is generally accepted that replication initiation is specifically inhibited in *E. coli*, while the elongation process is affected in *B. subtilis* [13,18,23].

The discovery that *B. subtilis* primase is inhibited by ppGpp [15] implied that this may be a major mechanism for replication elongation impairment, which could also distinguish the regulatory processes occurring in *B. subtilis* and *E. coli*. However, *E. coli* primase was subsequently shown to be also inhibited by ppGpp [16,17].

In this report, we have demonstrated for the first time that ppGpp