THE SYNTHESIS OF MS 1 AND MS 2 BY BACILLUS SUBTILIS

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Summary

B. subtilis accumulates guanosine tetra- (ppGpp) and guanosine pentaphosphate (pppGpp) upon amino acid starvation or carbon step down, but not during the transition from exponential growth to sporulation in nutrient sporulation medium. Synthesis of both nucleotides was achieved in vitro in a system containing 70 S ribosomes, ATP, and GDP or GTP respectively. The formation of ppGpp and pppGpp is sensitive to the antibiotic fusidic acid.

Introduction

MS 1 (ppGpp) and MS 2 (pppGpp) were shown to accumulate when stringent E. coli cells are starved for an amino acid [1-3]. Both nucleotides are synthesized in vitro on E. coli ribosomes from ATP and GDP or GTP respectively [4]. The stringent - relaxed specificity originates from ribosome associated stringent factor [5]. MS 1 can inhibit in vitro the formation of the initiation complex containing formylmethionyl-tRNA, 70 S ribosomes, ApUpG or R 17 RNA [6], or the poly U - directed synthesis of polyphenylalanine [7]. The in vitro function of ppGpp emphasizes a coupling between protein synthesis and transcription since it inhibits, in complex with TuTs (a protein synthesis elongation factor), synthesis of ribosomal and transfer RNA [8]. In addition, MS 1 affects transcription by a non-specific inhibition of RNA polymerase activity [9]. Furthermore, ppGpp influences purine metabolism; it decreases the p-ribosyl-pp

dependent transport of purine nucleotides by blocking the membrane bound phosphoribosyl transferase activity [10], and it blocks de novo biosynthesis of purine nucleotides via the inhibition of IMP-dehydrogenase and adenylosuccinate synthase [11].

Material and Methods

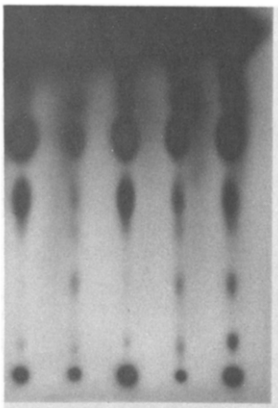
Detailed information is given in the legends to the figures.

Results

Synthesis of MS 1 and MS 2 by Bacillus subtilis in vivo.

Upon amino acid starvation or carbon step down, stringent [12] Bacillus subtilis forms two nucleotides with abnormal chromatographic mobility on PEI cellulose. Their R_f-values were similar to those reported for MS 1 (ppGpp) and MS 2 (pppGpp) produced by E. coli [1]. Accumulation of both nucleotides was triggered after transfering cells of the standard strain 60015 (try^-, met^-) from minimal medium containing glucose, glutamate and malate as carbon sources into the same medium lacking glucose (carbon step down)(Fig. 1). The omission of malate had no effect: it did not stimulate the production in the case of glucose step down, nor could the production be stimulated by only malate removal. If the standard strain was starved for the essential amino acids tryptophan and methionine both nucleotides accumulated (Fig. 1). Addition of 1×10^{-4} M fusidic acid completely inhibited the formation of the nucleotides.

The capacity for their synthesis was found throughout exponential growth and sporulation in nutrient sporulation medium (NSM)[12]. This was demonstrated after transfering equal numbers of cells, as judged from their optical density, into minimal medium without essen-



	nmol P_i/ml				
GDP ATP	985.9	343.9	1142.7	761.9	1164.7
GTP	241.7	117.6	290.4	147.9	218.5
MS 1	13.5	31.9	15.1	38.1	43.0
MS 2	19.3	21.6	16.3	30.3	47.6
Origin					
	1	2	3	4	5

Figure 1

Synthesis of MS upon carbon step down and amino acid starvation B. subtilis 60 015 (try$^-$, met$^-$) was grown in a synthetic sporulation medium containing 10 mM glucose, 10 mM malate, 25 mM glutamate, 3 mM citrate, 0.6 µM tryptophan, 0.3 µM methionine, 3 mM $MnCl_2$, 6 mM $MgCl_2$, 10 mM $CaCl_2$, 0.6 mM $(NH_4)_2SO_4$, 50 mM tris-Cl pH 6.5, and 2.3 mM K_2HPO_4 to mid log phase. ^{32}P was added (specific activity 40 Ci/mol). After one hour at 37° aliquots were transfered to medium with the following modifications: 1, none; 2, without glucose and malate (carbon step down); 3, without malate; 4, without glucose (carbon step down); 5, without tryptophan and methionine (amino acid step down); each in the presence of the same amount of ^{32}P. After 10 min at 37° 0.1 volume 4 M formic acid was added to the aerated cultures. They were cooled to 0° and stored for 30 min in ice. Cells were removed by centrifugation and 5 µl of the clear supernatant extracts were spotted onto Macherey and Nagel polyethyleneimine cellulose plates and chromatographed in 1.5 M KH_2PO_4 pH 3.4 as described by Cashel et al. [16]. Spots were cut from the plates, incubated for 1 hour in 0.5 ml of 0.5 M ammonia in scintillation vials and counted in Bray's solution.

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tial amino acids (Fig. 2). Whereas constant amounts of MS 2 were found, the capacity to form MS 1 decreased significantly with the end of exponential growth. The background level of both nucleotides during growth and the early stages of sporulation in NSM was determined by directly extracting cells grown in the presence of 25 µCi/ml ^{32}P without manipulation of the culture prior to extraction with 0.4 M formic acid. Until three hours after exponential growth ceased (T_3) neither

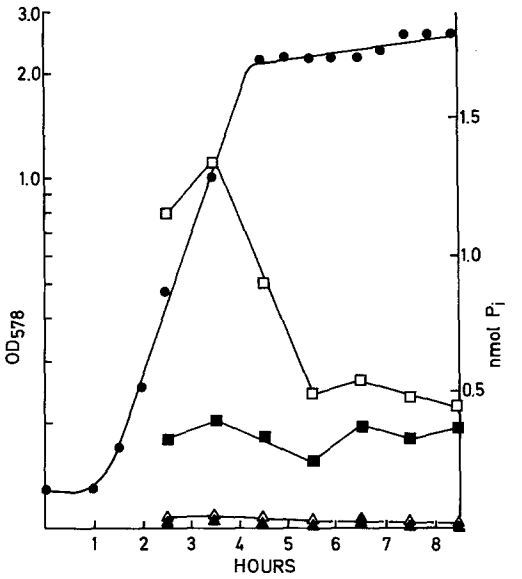

Figure 2

Synthesis of MS upon amino acid starvation
B. subtilis 60 015 was grown in NSM with 25 μCi/ml ^{32}P corresponding to 1.6 Ci/mol of phosphate. At 1 hour intervals 2.5 OD-units of the culture were withdrawn and suspended in 1 ml medium containing 10 mM tris-Cl pH 7.1, 10 mM glutamate, 10 mM glucose and 25 μCi/ml ^{32}P and were shaken at 37° for an additional 10 min. Formic acid extracts of cells were obtained and treated as described in Fig. 1. MS 1 and MS 2 backgrounds were determined by directly extracting aliquots of the culture containing 25 μCi/ml ^{32}P with 0.4 M formic acid.
● OD at 578 nm, □ MS 1, ■ MS 2, △ MS 1 background, ▲ MS 2 background

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MS 1 nor MS 2 increased above a constant low background level.

Synthesis of MS 1 and MS 2 in vitro.

It can be seen from Fig. 3 that both abnormal nucleotides were built in vitro when 5'-GTP and 5'-ATP were incubated in the presence of ribosomes. ADP instead of ATP or GTP alone were ineffective. Addition of 100 000 x g supernatant did not stimulate the production of the nucleotides. Apparently protein factors necessary for protein synthesis and for MS production were isolated complexed

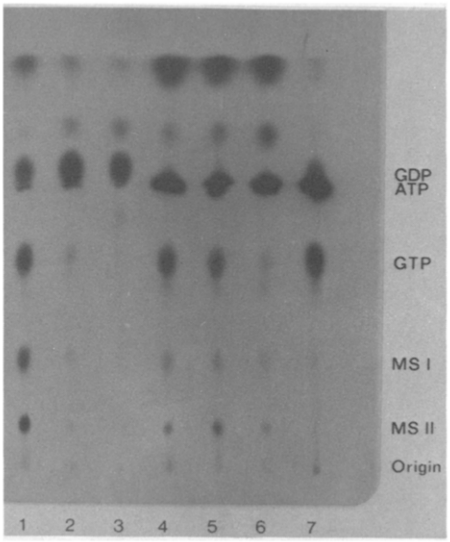

Figure 3

Requirements for MS synthesis in vitro

	nucleotides incubated							
Track	^{14}C-GTP	GTP	GDP	^{32}P-ATP	ATP	ADP	MS 1	MS 2
1	+	–	–	–	+	–	+	+
2	+	–	–	–	–	+	–	–
3	+	–	–	–	–	–	–	–
4	–	–	+	+	–	–	+	+
5	–	+	–	+	–	–	+	++
6	–	–	–	+	–	–	traces	traces
7	+	standard		+	standard			

100 µl reaction mixtures contained 30 mM tris-Cl pH 7.3, 27 mM NH_4Cl, 8 mM NaCl, 10 mM $MgCl_2$, 6 mM β-mercaptoethanol, 0.5 mM spermidine, 0,55 mM guanine nucleotide as indicated, 2.2 mM adenine nucleotide as indicated and 1.1 mg ribosomes prepared as described [13]. 8-^{14}C-GTP was obtained from Amersham Buchler and used at a specific activity of 5.7 Ci/mol. γ-^{32}P-ATP was prepared according to Glynn and Chappell [15]. It was used at 73 000 cpm per assay. Incubation was for 30 min at 37°. The reaction was terminated by chilling in ice and adding 2 µl of 98% formic acid. After centrifugation at 8 000 x g for 2 min 10 µl samples were spotted on PEI cellulose plates and chromatographed as described in Fig. 1.

with the ribosomes [13]. Both nucleotides in question contain guanine as nucleobase, since they were radioactively labelled if 8-^{14}C-GTP was added. Phosphorylation originated from 5'-ATP since ^{32}P

was introduced from γ-^{32}P labelled ATP. The Rf-values were identical with those reported for the guanosine tetraphosphate ppGpp and the pentaphosphate pppGpp [1]. We therefore assume that in the B. subtilis system, similar to E. coli, pyrophosphate is transfered onto the 3' position of 5'-GTP respectively 5'-GDP [17]. GDP gave rise to MS 1 and unsignificant amounts of MS 2. GTP in contrast gave rise not only to MS 2 but also to MS 1. This can be brought about after GTP is partially converted to GDP via ribosome associated G-factor [13, 14]. Subsequently it is transformed into MS 1 (Fig. 4). Addition of fusidic acid blocked the synthesis of both nucleotides. It prevented degradation of ATP in the in vitro system (Fig. 4).

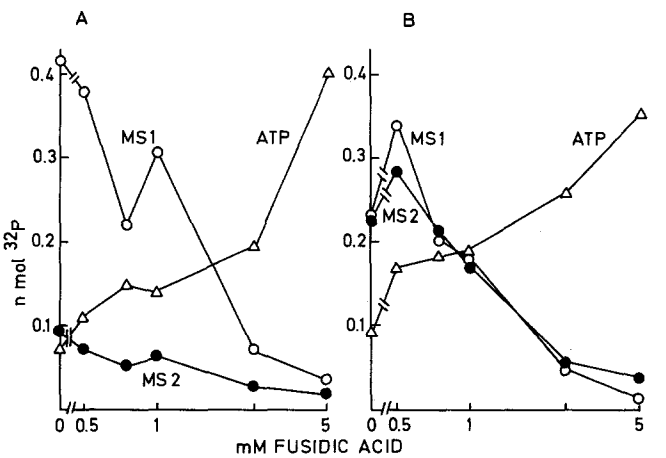

Figure 4

Fusidic acid influence on MS synthesis in vitro

50 µl reaction mixture contained 54 mM tris-Cl pH 7.8, 24 mM K-acetate, 10 mM Mg-acetate, 0.5 mM dithiothreitol, 0.5 mM spermidine, 0.6 mM γ-^{32}P-ATP with 10^6 cpm, 1 mM guanine nucleotide as indicated, 1.2 mg ribosomes (cf. Fig. 3) and varying amounts of fusidic acid as indicated. Incubation was for 1 hour; development of chromatograms and counting of spots was as described in Fig. 1.
A: Incubation with 1 mM GDP
B: Incubation with 1 mM GTP

Discussion

Bacillus subtilis can be stimulated to form guanosinetetra- or guanosinepentaphosphate, the magic spot compounds MS 1 and MS 2 of Cashel and Gallant [1]. Both nucleotides accumulate in vivo under carbon step down conditions or amino acid starvation. The amounts of the individual nucleotides formed depend on the pool size of GDP and GTP on one side and of ATP on the other. Carbon step down conditions for example reduce the GTP pool to a greater extend than does amino acid starvation. In the later case protein synthesis stops immedeately; consequently GTP consuming reactions cease.

In NSM both MS 1 and MS 2 are not made above a constant low background level when exponential growth ceases and the cells enter the sporulation period, although the capacity to synthesize both is present throughout growth and sporulation. Apparently the shortage which causes growth to cease is not sufficient to stop protein synthesis to such an extend that the formation of MS 1 and MS 2 is triggered. The process of spore formation does require the constant synthesis of new proteins. Although the rate of protein synthesis is reduced as compared to growth, obviously the rigid conditions for a stringent response are not reached.

A reduced level of MS 1 was found when sporulating cells were starved for amino acids compared to exponentially growing cells. This must be correlated to a reduction of the number of mRNA engaged ribosomes [18]. The synthesis of MS however strictly depends on mRNA bound ribosomes and in addition the presence of uncharged tRNA [5].

Both MS 1 and MS 2 are synthesized in vitro from ATP and

GTP in the presence of 70 S ribosomes. Experiments with this *in vitro* system demonstrated that guanine is a constituent of the nucleotides and that phosphorylation originates from ATP. Their production is sensitive to fusidic acid, an inhibitor of EF-G mediated translocation in protein synthesis. These results obtained in vitro are similar to those described for *E. coli*. We therefore conclude that the nucleotides found in *B. subtilis* in fact are MS 1 and MS 2.

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