ISOLATION OF A PYROPHOSPHORYLASE FROM *BACILLUS SUBTILIS* AND *BACILLUS STEAROTHERMOPHILUS* THAT SPECIFICALLY DEGRADES GUANOSINE 3'-DIPHOSPHATE 5'-DIPHOSPHATE

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1. Introduction

In the past few years evidence has been presented for the existence of guanosine 5'-diphosphate 3'-diphosphate (ppGpp) in microbial species other than *Escherichia coli.* Although the precise role of ppGpp in strains such as *Bacillus brevis, Bacillus subtilis, Bacillus stearothermophilus* and *Streptomyces* has not yet been established it is assumed that, as in *E. coli,* ppGpp functions as a pleitropic effector controlling numerous biosynthetic and catabolic pathways.

For understanding its role as regulating effector knowledge of the metabolism of ppGpp are essential. The in vitro synthesis of ppGpp has been shown to require an enzyme, named stringent factor [1]. In *E. coli* this factor is ribosome-bound and activated when uncharged tRNA is codon-specifically bound to ribosomes [2–4]. In taxonomically distant bacteria such as *Bacillus brevis* [5] or *Bacillus stearothermophilus* [6] ppGpp is synthesized in a ribosome-independent manner. Apparently the mechanism for triggering production of ppGpp varies with strains. In this study, we have investigated the in vitro degradation of ppGpp in *Bacillus stearothermophilus* and *Bacillus subtilis,* in particular we were interested whether ppGpp is degraded by a similar mechanism as has been reported for *E. coli* [7,8]. In the latter strain a manganese-dependent enzyme has been isolated [9] that released pyrophosphate from the 3'-position of ppGpp yielding ppG [10].

2. Materials and methods

Labeled substrates p$\overset{*}{p}$Gpp, ppG$\overset{*}{p}$p, ppGp$\overset{*}{p}$ and pp[^{3}H]Gpp were kindly provided by E.-A. Heinemeyer and according to [9]; ^{32}P-labeled ppGpp substrates are designated with an asterisk over the 'p'; protein was estimated as in [9].

2.1. *Growth conditions and preparation of the pyrophosphorylase*

B. subtilis cells [11] were kindly provided by Dr P. Fortnagel, Hamburg; *B. stearothermophilus* strain 799 cells by F. Godt. The latter strain was grown at 64°C in medium containing per liter: 10 g tryptone; 5 g yeast extract; 5 g NaCl; 10 μg $MnCl_2$; 5 g glucose. Cells were harvested at a density of 1.2 A_{650} units/ml, suspended in 3 vol. buffer (10 mM Tris–HCl (pH 7.7), 10 mM Mg-acetate, 6 mM 2-mercaptoethanol, 1 mM $MnCl_2$, and 2 μg/ml DNase) and homogenized in a laboratory pressure cell model 15 M (Gaulin Co., Everett, MA) at 16 000 p.s.i. After centrifugation at 8000 × *g* for 15 min, the supernatant fraction (9 ml) was centrifuged through a 3 ml sucrose cushion consisting of 2 M sucrose, 5 mM Mg-acetate, 2 mM $MnCl_2$, 2 mM dithiothreitol at 38 000 rev./min for 17 h using a Beckman rotor SW 41.

The gradient was fractionated from the top according to [7]. The upper 7–8 ml contained little ppGpp degrading activity and were not further processed; the following 3.5 ml were of yellow-

brownish color and consisted of membranes and ribosomes. This fraction, highly active in ppGpp degradation, was diluted 3-fold with 20 mM Tris–HCl buffer (pH 7.7), 1 mM $MnCl_2$, 5 mM Mg-acetate, 2 mM dithiothreitol, centrifuged at 60 000 × *g* for 30 min and the supernatant fraction at 200 000 × *g* for 12 h. The pellet of the high speed centrifugation step was dissolved in buffer (20 mM Tris–HCl (pH 7.7), 1 mM EDTA, 2 mM $MnCl_2$, 2 mM dithiothreitol, 10% sucrose, w/v); although this fraction consisted mainly of ribosomes the presence of membrane vesicles could not be excluded. The fraction was used as source for ppGpp degrading activity and contained 30–40 mg/ml of protein; it was stored in liquid nitrogen.

2.2. *Assay for ppGpp degradation*

The assay mixture (25 or 50 μl) consisted of 50 mM Tris–HCl (pH 7.8), 2 mM dithiothreitol, 3 mM $MnCl_2$, 0.2 mM ^{32}P- and/or ^{3}H-labeled ppGpp (spec. act. 0.5–2 Ci/mol) and the enzyme fraction as indicated. After incubation at 37°C for 15 min, the reaction was stopped by addition of 1–2 μl 44% formic acid. Aliquots were spotted on polyethyleneimine sheets together with cold ppGpp (Sanruku-Ocean Co., Tokyo) and/or guanosine nucleotides as markers and autoradiographed overnight. The sheets were developed one- (1.5 M KH_2PO_4, pH 3.5) or two-dimensionally (system 1, 1st dimension 0.5 M LiCl/4 M Na-formate, pH 3.4; 2nd dimension 1.5 M KH_2PO_4, pH 3.5; system 2, 1st dimension 1.5 M LiCl/2 M Na-formate, pH 3.4; 2nd dimension 1.5 M KH_2PO_4, pH 3.5).

3. Results and discussion

When cell-free extracts were prepared from *B. subtilis* or *B. stearothermophilus* and centrifuged as indicated in section 2 the main ppGpp degrading activity was found in fractions containing membranes and ribosomes [7]. Decay of ppGpp was optimal at 200–300 μg protein (*B. subtilis*) incubated at 37°C for 20 min (fig.1). The lower degrading activity observed with the protein fraction from *B. stearothermophilus* could be due to inactivation of the enzyme during preparation. Only little activity was found in the 105 000 × *g* supernatant fraction (not shown).

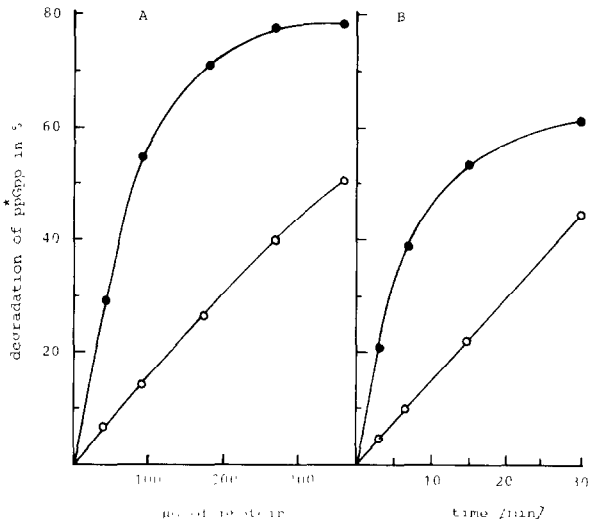

Fig.1. Concentration and time curve of the ppGpp degrading reaction. The protein fractions containing ppGpp degrading activity were isolated from *B. subtilis* (●——●) or *B. stearothermophilus* (○——○) as in section 2. After incubation at 37°C for 15 min aliquots were analyzed by one-dimensional chromatography (A). In (B) 90 μg protein from *B. subtilis* or 150 μg protein from *B. stearothermophilus* were assayed; at the time indicated 5 μl were withdrawn, stopped with 2 μl 44% formic acid and chromatographed.

In an attempt to identify the degradation products ^{32}P-labeled ppGpp and protein fractions from *B. subtilis or B. stearothermophilus* were incubated. Figure 2 shows an autoradiogram of a two-dimensional thin-layer chromatography of the breakdown products. It is apparent that ppG is the main product suggesting a similar decay mechanism as has been reported for *E. coli* [10]. This is confirmed by double-labeling experiments with $pp^{*}[^{3}H]Gpp$, $pp[^{3}H]G^{*}pp$ or $pp[^{3}H]Gpp^{*}$ as substrate. Table 1 shows that the molar ratio of ^{32}P- and ^{3}H-labeled ppG is 1:1 with $pp^{*}[^{3}H]Gpp$ but not with $pp[^{3}H]G^{*}pp$ or $pp[^{3}H]Gpp^{*}$. On the other hand with the latter two nucleotides as substrate ^{3}H-labeled ppG and ^{32}P-labeled pyrophosphate were the products. It is evident, that, similar to the ppGpp degrading enzyme from *E. coli*, the corresponding enzyme from the two *Bacillus* strains releases pyrophosphate from the 3′-position of ppGpp; it is referred to as pyrophosphorylase. It has been reported that the ppGpp degrading enzyme

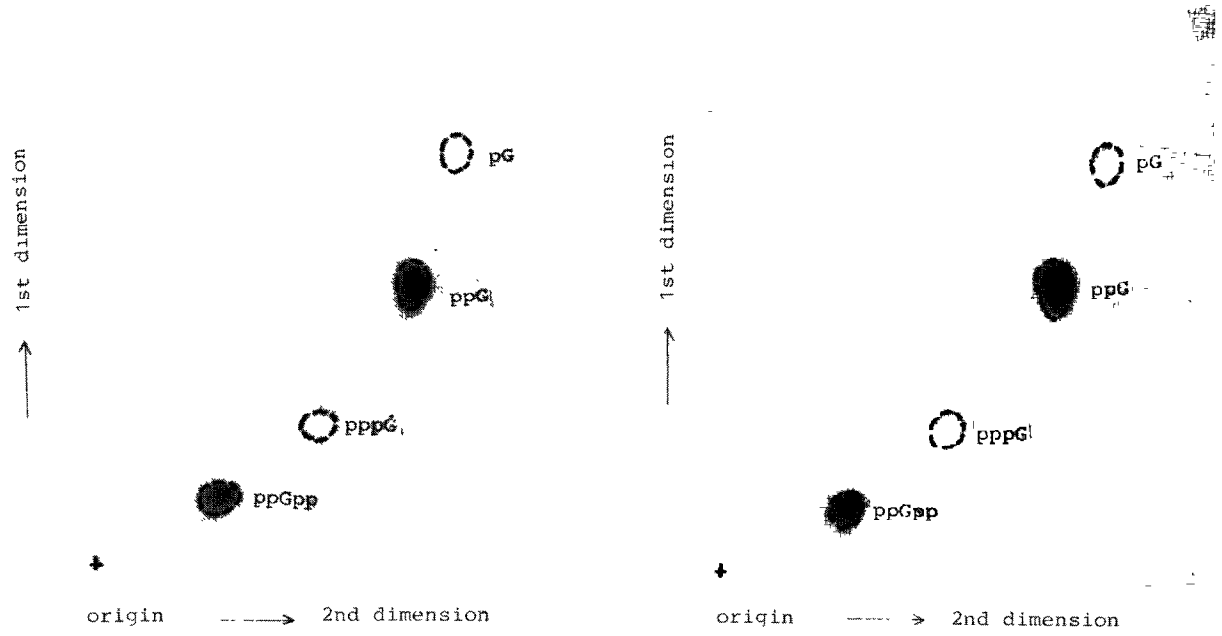

Fig.2. Two-dimensional thin-layer chromatography of the pp*Gpp degradation products. For assay conditions see section 2. Chromatography was carried out in system 1; the sheets were autoradiographed over-night. Unlabeled nucleotides were co-chromatographed and identified under ultraviolet light (dotted lines). The left panel shows degradation of ppGpp by the *B. stearothermophilus*, the right panel by the *B. subtilis* enzyme.

Table 1
Decay of ppGpp to ppG by a pyrophosphorylase isolated from *B. subtilis* or *B. stearothermophilus*

Double-labeled substrates assayed	Formation of ppG and $^{32}PP_i$ in nmol					
	B. subtilis			*B. stearothermophilus*		
	$^{32}PP_i$	ppG		ppG		$^{32}PP_i$
		^{3}H	^{32}P	^{3}H	^{32}P	
pp*[^{3}H]Gpp	0	6.3	6.5	6.1	5.8	0
pp[^{3}H]Gpp*	5.1	5.9	0.8			
pp[^{3}H]Gpp*				3.7	0.4	3.2

The ppGpp degrading reaction was carried out in the presence of the substrates indicated; the latter were double-labeled with ^{3}H in the base and ^{32}P in the 5′α, 3′α, or 3′β position. The assay mixture contained 100–150 μg ribosomal protein. Degradation products were analyzed by chromatography in system 1 and 2. ^{32}P-labeled pyrophosphate, identified by treatment with yeast inorganic pyrophosphatase (Sigma), was calculated from the percentage of pp[^{3}H]Gpp* (or pp[^{3}H]Gpp*) converted to ppG and ^{32}PPi

from *E. coli* depends on divalent cations, in particular on manganese ions [9]. As shown in table 2 the pyrophosphorylase from the two *Bacillus* strains shows a similar requirement for manganese ions. In contrast to the in vitro enzyme system from *E. coli* no monovalent cations were required.

For understanding its physiological role the subcellular distribution of the ppGpp degrading enzyme is of particular interest. Although so far this enzyme has been isolated from fractions containing mainly ribosomes an association with membrane vesicles cannot completely be excluded. In order to elucidate whether the pyrophosphorylase could be separated from ribosomes and/or membrane vesicles the protein fraction isolated as in section 2 was dialyzed against buffer of low ionic strength and subjected to sucrose density gradient centrifugation. A typical elution and activity profile of this protein fraction (*B. subtilis*) is depicted in fig.3A. Although ppGpp degrading activity could be separated from ribosomal subunits, a second peak

Table 2
Effect of divalent cations on the p*pGpp degrading reaction

Concentrations of divalent cations (mM)	Degradation of p*pGpp to p*pG in nmol					
	B. subtilis			*B. stearothermophilus*		
	Mn^{2+}	Mg^{2+}	Ca^{2+}	Mn^{2+}	Mg^{2+}	Ca^{2+}
0.8	2.18	1.86	1.92	1.04	0.22	0.52
1.6	4.89	1.45	1.95	1.15	0.42	0.58
3.2	5.16	2.28	1.65	0.98	0.97	0.38
6.0	4.26	0.88	1.03	1.07	0.88	0.18
12.0	2.83	0.68	0.68	0.91	0.62	0.09

All assays contained 1 mM EDTA. In the control assay where divalent cations were omitted 1.7 nmol ppGpp were degraded with the *B. subtilis* fraction and 0.61 nmol with the one from *B. stearothermophilus*. This residual activity was due to traces of manganese ions tightly bound to the ppGpp degrading enzyme which could not completely be chelated by 1 mM EDTA. For concentrations of enzyme see fig.1

emerged, that comigrated with or very close to 30 S ribosomal subunits. When the pyrophosphorylase, released into the top fractions of the gradient, was recentrifuged through a sucrose density gradient the ppGpp degrading activity coincided with the bovine serum albumin peak (fig.3B). Although an exact estimate of the molecular weight of the pyrophosphorylase isolated from the two *Bacillus* strains will only come from SDS–gel electrophoresis of the purified enzyme the data shown here suggest mol. wt

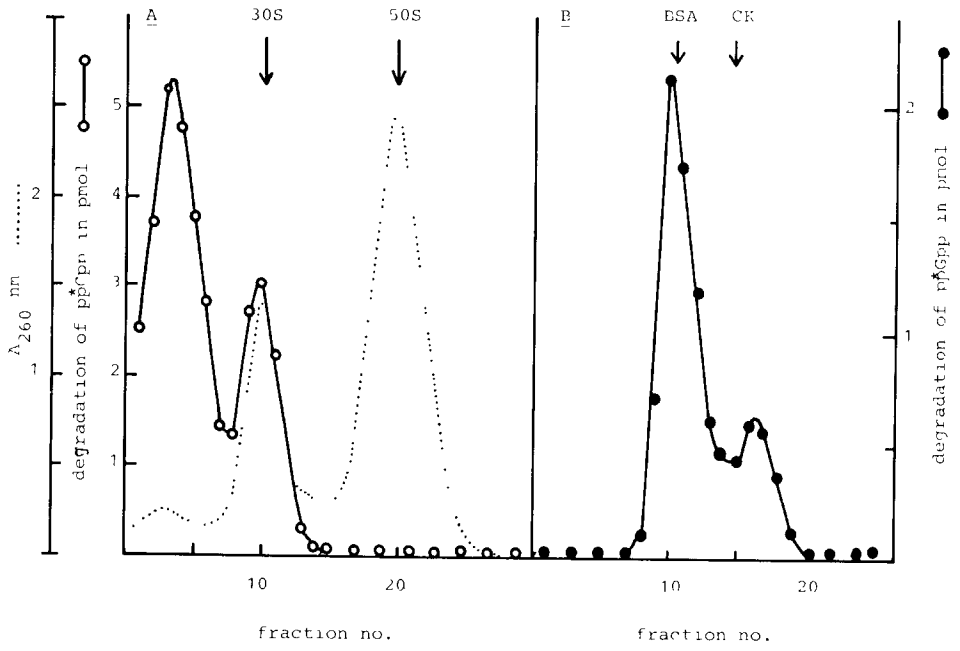

Fig.3

65 000–70 000. The association of the pyrophosphorylase with the ribosomal fraction and its apparent affinity to the 30 S ribosomal subunit could suggest that it is identical with one of the high-molecular weight ribosomal proteins. From the proteins of the small ribosomal subunit only S1 [12] and A-protein [12,13] have a comparable molecular weight. As shown in [14], however, *B. stearothermophilus* does not contain protein S1. Also, neither S1 nor A-protein from *E. coli* (kindly provided by Dr A. Subramanian, Berlin) degraded ppGpp.

The results presented here suggest that the mechanism for degradation of ppGpp in the two *Bacillus* strains and in *E. coli* [10] are identical. Similar to the *E. coli* enzyme the manganese-dependent *Bacillus* pyrophosphorylase releases coordinately two phosphates from the 3′-position of the nucleotide. Although the enzyme seems to have a high affinity to the ribosome, in particular to 30 S ribosomal subunits, ribosomes are not essential in the ppGpp degradation reaction.

Acknowledgement

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Fig.3. Sucrose density centrifugation of the pyrophosphorylase from *B. subtilis*. As pointed out in section 2 the fraction with the highest ppGpp degrading activity consisted of ribosomes and membranes. Although a particulate fraction had been removed by centrifugation (60 000 × *g*, 30 min) the pyrophosphorylase containing fraction may still have some membrane vesicles. To release pyrophosphorylase from any structural components 50 A_{260} units/ml of the fraction containing ribosomes and ppGpp degrading activity (for definition of the units see [15]) were dialyzed against 2 l buffer 1 (40 mM Tris–HCl buffer (pH 7.7), 25 mM NH_4Cl, 2 mM dithiothreitol, 0.1 mM Mg-acetate, 0.1 mM $MnCl_2$). This dialyzed fraction (1 ml) was applied to a linear sucrose gradient (10–30% sucrose, w/v, in buffer 1) and centrifuged at 20 000 rev./min for 17 h in a SW27 Beckman rotor. Fractions (1.2 ml) were collected from the top and A_{260} (dotted lines) was recorded. Aliquots (25 μl) of the fractions were assayed using 100 pmol $p\overset{*}{p}Gpp$ (0.3 Ci/mmol)/30 μl assay mixture (A). The top fractions obtained from 6 gradients were pooled, concentrated by $(NH_4)_2SO_4$ precipitation (45 g/100 ml), dissolved in 300 μl of and dialyzed against buffer 1. (B). The dialyzed pyrophosphorylase was layered on top of a 5.4 ml linear sucrose gradient (10–30%, w/v, in buffer 1) and centrifuged in an SW65 Beckman rotor at 59 000 rev./min for 23 h; bovine serum albumin (BSA) and creatine kinase (CK) were used as marker proteins. Fractions (210 μl) were collected from the top; 25 μl aliquots were assayed as described for (A). The arrows indicate the positions of the marker proteins.