A RIBOSOME-INDEPENDENT STRINGENT FACTOR FROM *BACILLUS STEAROTHERMOPHILUS* AND A LOW MOLECULAR WEIGHT SUBSTANCE INHIBITORY TO ITS ACTIVITY

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1. Introduction

In many bacterial strains the level of guanosine 5′-diphosphate, 3′-diphosphate (ppGpp) and the rate of rRNA and tRNA synthesis are apparently correlated suggesting that the stringent control system is involved in the process of regulation of ribosomal gene expression (reviewed [1,2]). In *relA*$^+$ strains of *Escherichia coli* (p)ppGpp is accumulated during starvation for the required amino acids which in turn leads to restriction of synthesis of rRNA, tRNA and certain species of mRNA. While *relA*$^-$ mutant strains no longer responded to amino acid starvation, they do restrict rRNA synthesis in response to nutritional changes during which they accumulate (p)ppGpp [3–5]. Apparently the trigger signal for (p)ppGpp production varies with strains. It has been shown [6] that in *relA*$^+$ strains synthesis of (p)ppGpp is catalyzed by the stringent factor which transfers pyrophosphate from pppA to (p)ppG, this ribosome-associated factor is activated when uncharged tRNA is codon-specifically bound to the ribosomes [7–9]. A similar mechanism has not yet been found in *relA*$^-$ strains [6,10] which may indicate that the factor from *relA*$^-$ strain is either highly labile and inactivated during preparation or that (p)ppGpp is synthesized via a different process. Such a different process has been found in *Bacillus brevis* [11] and *Streptomyces strains* [12] where (p)ppGpp is synthesized in a ribosome-independent manner. In contrast to the *E coli* stringent factor these factors are not associated with ribosomes, and soluble at low ionic conditions and not dependent on ribosomes for activity. We reported in vitro synthesis of (p)ppGpp by a ribosomal fraction isolated from *Bacillus stearothermophilus* [14]. As compared to the *E. coli* system the activity was rather low and only slightly stimulated by uncharged tRNA and mRNA. Detailed studies have revealed that only a small percentage of (p)ppGpp synthesizing activity is associated with the ribosomal fraction, > 80% of the stringent factor is present in the 105 000 × *g* supernatant fraction. The partial purification of this stringent factor from *B stearothermophilus*, which functions independently of ribosomes, mRNA and tRNA, is the subject of this paper.

2. Material and methods

[α-^{32}P]GTP or [γ-^{32}P]ATP were obtained from New England Nuclear. (p)ppGpp production was followed by two-dimensional thin-layer chromatography using polyethyleneimine coated sheets (Macherey, Nagel + Co., Duren), for details of the chromatographic systems see [13]. Cold ppGpp and pppGpp were purchased from Sanruku-Ocean Co., Tokyo and adenosine 5′(β,γ-imino) triphosphate and guanosine 5′(β,γ-imino) triphosphate from Boehringer Mannheim

2.1 *Assay for (p)ppGpp synthesis*

The standard assay system (50 μl) consisted of 20 mM Tris–HCl (pH 7.8), 10 mM Mg-acetate, 40 mM

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NH_4Cl, 2 mM dithiothreitol, 4 mM ATP, 0.4 mM [α-^{32}P]GTP or [γ-^{32}P]ATP (spec. act. 25 Ci/mol) and 20–50 μg stringent factor preparation obtained from the first peak of the DEAE-cellulose column (see fig.1). The mixture was incubated at 37°C for 1 h, then the reaction was stopped with formic acid and analyzed as in [13].

3. Results and discussion

3.1. *Partial purification of the stringent factor*

When crude cell extracts from *B. stearothermophilus* were fractionated by high-speed centrifugation (p)ppGpp synthesizing activity was found in the ribosomal but not in the S100 fraction (table 1). Fractionation of the S100 by ammonium sulfate precipitation, however, yielded a preparation highly active in guanosine polyphosphate production; table 1 indicates that most of the stringent factor activity is not associated with ribosomes. Thus, lack of activity in the S100 fraction may be due either to the presence of alkaline phosphatase or to inhibitor(s) which interfer(s) with (p)ppGpp synthesis.

The stringent factor was further purified by DEAE-cellulose chromatography using a linear KCl gradient. Two peaks with so far identical properties in the (p)ppGpp synthesizing reaction were obtained (fig.1). Whether the two forms represent chromatographic artefacts of one and the same factor or whether there exists heterogeneity of the stringent factor remains to be elucidated. In contrast to the *E coli* factor the one from *B stearothermophilus* is soluble in the absence

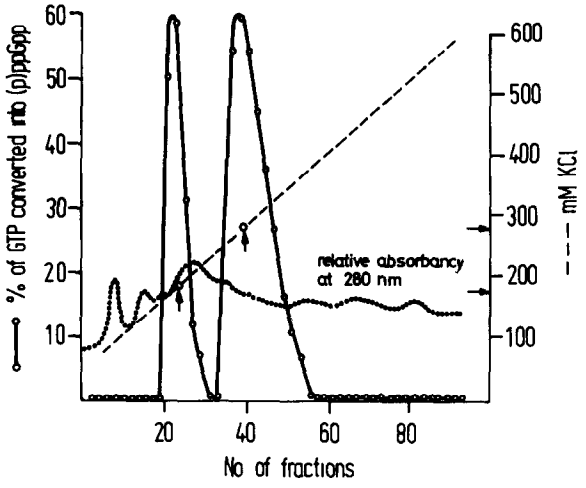

Fig 1. Purification of the stringent factor by DEAE-cellulose chromatography S100 fraction was adjusted with NaOH to pH 7 8, precipitated with 45 g ammonium sulfate/100 ml solution and centrifuged at 18 000 rev /min for 30 min in a Sorvall SS34 rotor. The protein pellet was re-extracted 3 times with 20%, then 3 times with 16% and finally with 12% ammonium sulfate solutions (w/w); all solutions were adjusted to pH 7 8 and contained 1 mM dithiothreitol The supernatant fractions of each step were combined and reprecipitated with 20 g ammonium sulfate/100 ml solution. The 16% and 12% fraction contained the stringent factor activity and were dialyzed against buffer A (20 mM Tris–HCl (pH 7 8), 2 mM dithiothreitol) containing 50 mM KCl. The 12% and 16% fractions were combined and chromatographed on a DEAE-cellulose (Whatman) column (9 × 20 cm) which had been equilibrated with buffer A containing 50 mM KCl The stringent factor was eluted from the column using a linear gradient of 50–600 mM KCl in buffer A (100 × 100 ml) The flow rate was 10 ml/h and the fraction vol , 0 4 ml, 10 μl aliquots were assayed for stringent factor activity in the assay system in section 2

Table 1
Purification of the stringent factor from *Bacillus stearothermophilus*

Purification steps	mg protein/ g cells	Spec act. (nmol.min^{-1} mg^{-1})	Units/ g cells
Ribosomal fraction	5 41	1.03	5 6
S100	25	0	0
$(NH_4)_2SO_4$ fraction	17 4	2.6	45 2

B. stearothermophilus strain 799 was grown at 64°C in medium containing per liter. 10 g tryptone, 5 g yeast extract, 5 g NaCl, 10 μg $MnCl_2$ and 5 g glucose. Cells were harvested at a density of 1.2 A_{650} units/ml, resuspended in 2.5 vol. buffer (10 mM Tris–HCl (pH 7 8), 10 mM Mg-acetate, 1 mM dithiothreitol, 2 μg DNase/ml) and disrupted at 16 000 p s.i using a French pressure cell. The cell extract was centrifuged at 18 000 rev /min for 30 min in a Sorvall rotor SS34 to remove cell debris and at 40 000 rev./min for 3.5 h in a Beckman Ti60 rotor to pellet ribosomes The top 2/3rds of the supernatant fraction was carefully collected and is referred to as S100, the lowest 1/3rd which is just above the ribosomal pellet was removed and not further analyzed. The ribosomal pellet was suspended in a small volume of water and stored in liquid nitrogen. The S100 fraction was precipitated with 45 g ammonium sulfate/100 ml solution. Specific activity is defined as nmol (p)ppGpp synthesized/min and mg protein and is calculated from the initial linear slope of the time course experiment for a given protein concentration

of NH_4^+ or K^+. The factor was stable for more than 3 months when stored at −80°C. The stringent factor of the first peak of the DEAE-cellulose column was used in subsequent experiments.

3.2. *Properties of the (p)ppGpp synthesizing reaction*

As stated above the stringent factor from *B. stearothermophilus* functions in the absence of the ribosome mRNA–tRNA complex. It requires as substrate ATP and GTP (or GDP) and, like its *E. coli* counterpart transfers the β,γ-moiety of ATP to the 3′-position of GDP or GTP yielding ppGpp and pppGpp, respectively. The two products were identified by one-(1.5 M KH_2PO_4, pH 3.5) or two-dimensional thin layer chromatography [System 1 1st dimension 1.5 M LiCl/2 M formic acid (pH 3.5); 2nd dimension 1.5 M KH_2PO_4 (pH 3.5); System 2: 1st dimension 0.5 M LiCl/4 M formic acid (pH 3.5); 2nd dimension 1.5 M KH_2PO_4 (pH 3.5)] and co-migrated with cold ppGpp and pppGpp visualized by ultraviolet absorption. Little or no activity was observed with adenosine 5′(β,γ-imino) triphosphate as phosphate donor or with 5′-GMP, guanosine 5′(β,γ-imino) triphosphate, guanosine 5′-tetraphosphate, 2′ dGTP, 3′GMP, 2′GMP, cyclic 3′, 5′GMP as phosphate acceptor (data not shown).

Like the counterparts from other bacteria [6,11] the *B. stearothermophilus* factor is quite specific in the phosphate transfer reaction. When other nucleotides were assayed in a competition test 0.2 mM 5′CDP

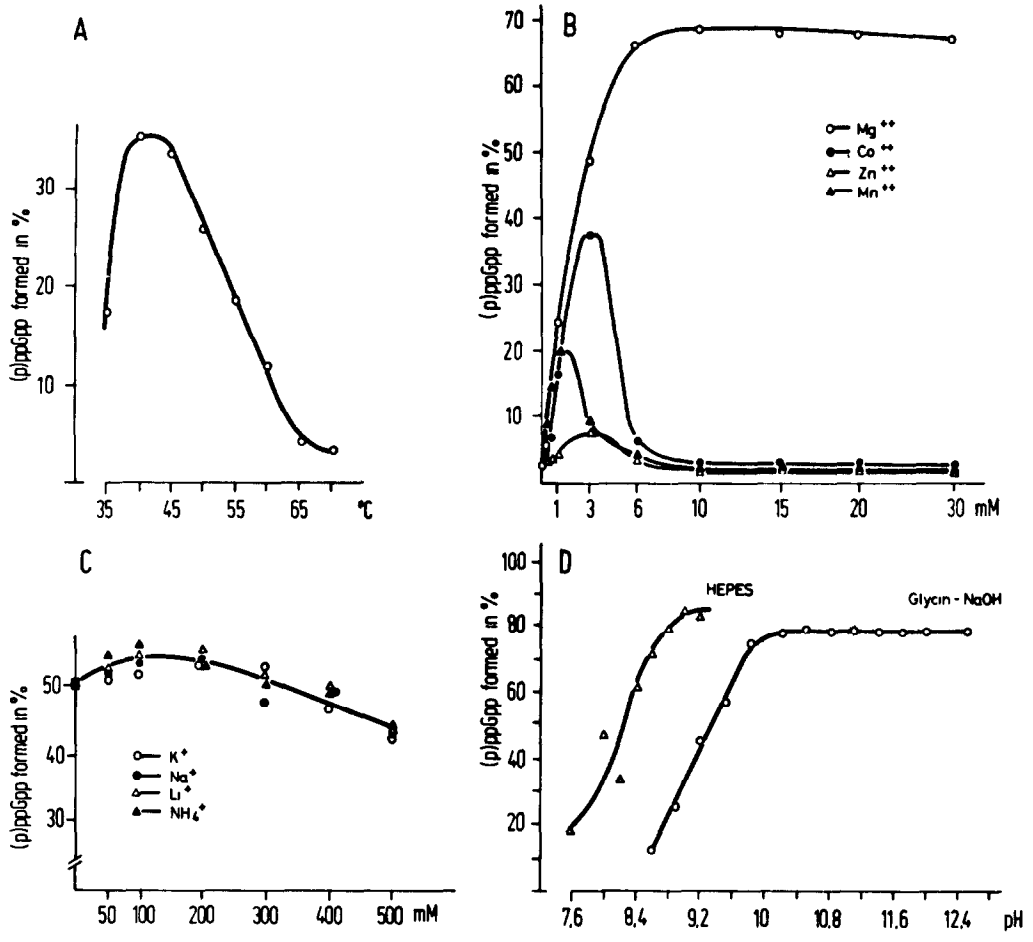

Fig.2. Dependence of synthesis of (p)ppGpp on temperature (A), di- (B) and monovalent (C) cations and pH (D). The assay conditions were as in section 2

inhibited the ppGpp synthesizing reaction to > 80%, 0.2 mM 5'CMP or 5'CTP to ~50%. At similar concentrations neither inosine nor uridine nucleotides restricted the synthesis of (p)ppGpp (data not shown). Figure 2 shows the dependence of the (p)ppGpp synthesizing reaction on various parameters. Apparently the reaction has a broad Mg^{2+} optimum between 3 mM and 30 mM which is in contrast to the *B. brevis* factor [11] but consistent with the one found in *Streptomyces* strains [12]. Co^{2+} and Mn^{2+} slightly stimulated the reaction. Optimal incubation temperature was between 40°C and 45°C and the pH optimum of the reaction was around 9 in Hepes buffer and > 9.6 in glycine–NaOH buffer. The monovalent cations tested were not essential for the reaction.

3.3. *Inhibition of the (p)ppGpp synthesizing reaction by ribosomes and a low molecular weight compound present in the S100 fraction*

During the isolation and purification of *B stearothermophilus* stringent factor we noticed that synthesis of (p)ppGpp was severely hampered when the crude factor was assayed in the presence of ribosomes. However, no such inhibition was observed when purified stringent factor was combined with ribosomes suggesting that an inhibitory component had been removed during purification. As shown in table 2 the inhibitor is present in the S100 fraction and functions when combined with *E. coli* or *B stearothermophilus*

Table 2
Effect of ribosomes and/or S100 on (p)ppGpp synthesis

Stringent factor assayed	(p)ppGpp synthesis (nmol)
Alone	14.2
+ Ribosomes	13.4
+ S100	13 6
+ Ribosomes + S100	1 0

The standard assay conditions were the same as in section 2 except that when ribosomes were present Mg^{2+} was increased to 20 mM and 7 9 pmol 50 S, 8 9 pmol 30 S, 6.25 μg poly(U) and 1 μg $tRNA^{Phe}_{yeast}$ were added to a 50 μl assay system, the latter two were routinely added although they were not essential for inhibition. Ribosomal subunits were prepared either from *E coli* or *B stearothermophilus*, ribosomal subunits from both strains gave similar results. Where indicated 40–70 μg S100 were added to a 50 μl assay mixture. Incubation was at 37°C for 30 min

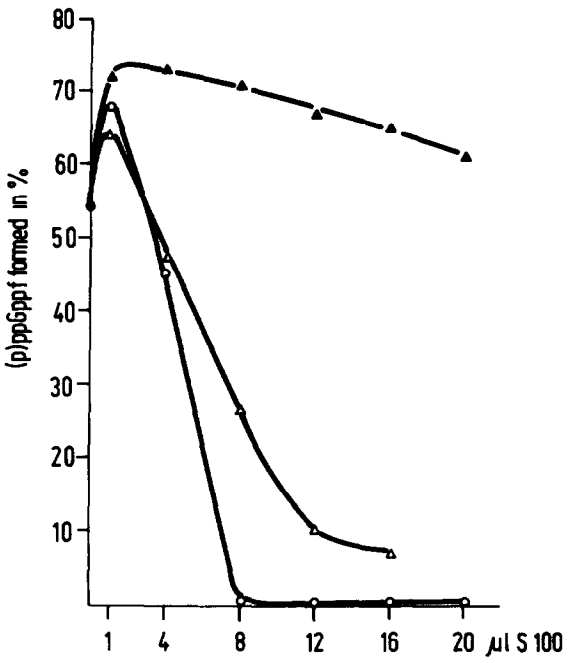

Fig.3 Inhibition of synthesis of (p)ppGpp by a low molecular weight component The assay conditions were as described in table 2 and included ribosomes, poly(U) and $tRNA^{Phe}_{yeast}$ Where indicated the S100 fraction (18 mg/ml) was boiled for 5 min, then cooled to 4°C and centrifuged to remove denaturated protein. S100 (o——o), heated S100 (Δ——Δ), S100 dialyzed against buffer A (see fig.1) (▲——▲)

ribosomes. It can be excluded that reduced synthesis of (p)ppGpp is due to rapid unspecific degradation, because (p)ppGpp produced by pre-incubation of the stringent factor system was not degraded when S100 was added. It should be noted that at higher concentrations of S100 the (p)ppGpp synthesizing reaction is blocked even in the absence of added ribosomes. Figure 3 shows that the inhibitor is heat-stable when boiled at 95°C for 5 min and can be removed by dialysis. Sephadex gel filtration experiments indicate that it has mol. wt 500–600. The compound revealed an A_{260} max

In summary an enzyme has been isolated from *B stearothermophilus* that catalyzes the pyrophosphate transfer from ATP to GDP or GTP yielding ppGpp and pppGpp, respectively. Ribosomes are not essential in this reaction. Synthesis of (p)ppGpp is inhibited by a heat-stable low molecular weight com-

pound which may be involved in the regulation of the intracellular level of (p)ppGpp through the interaction with ribosomes.

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References

[1] Nomura, M., Morgan, E. A. and Jaskunas, S R (1977) Ann. Rev. Gen 11, 297–347
[2] Richter, D. and Isono, K. (1977) Curr. Top Microbiol. Immunol. 76, 81–123.
[3] Lazzarini, R A , Cashel, M. and Gallant, J. (1971) J. Biol. Chem 246, 4381–4385
[4] Winslow, R. M. (1971) J. Biol Chem 246, 4872–4877
[5] Hansen, M T., Pato, M L , Molin, S., Fiil, N P and von Meyenburg (1975) J. Bacteriol. 122, 585–591
[6] Haseltine, W A., Block, R , Gilbert, W and Weber, K (1972) Nature 238, 381–384.
[7] Pedersen, F. S., Lund, E and Kjeldgaard, N. O. (1973) Nature New Biol. 243, 13–15
[8] Haseltine, W. A. and Block, R. (1973) Proc. Natl Acad Sci. USA 70, 1564–1568.
[9] Richter, D. (1976) Proc Natl Acad Sci USA 73, 707–711.
[10] Cochran, J W. and Byrne, R W (1974) J. Biol. Chem. 249, 353–360.
[11] Sy, J. and Akers, H (1976) Biochemistry 15, 4399–4403.
[12] Oki, T , Yoshimoto, A , Sato, S. and Takamatsu, A (1975) Biochim. Biophys Acta 410, 262–272.
[13] Schmale, H., Farnung, K , Fehr, S and Richter, D. (1977) Hoppe-Seyler's Z. Physiol. Chem. 358, 1613–1622
[14] Richter, D. and Isono, K (1974) FEBS Lett. 44, 270–273