A Ribosome-Independent, Soluble Stringent Factor-Like Enzyme Isolated from a *Bacillus brevis*†

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ABSTRACT: A ribosome-independent synthesis of guanosine 5′,3′-polyphosphates has been found in the soluble fraction of *Bacillus brevis* (ATCC 8185) extracts. The partially purified enzyme catalyzes the formation of both guanosine 5′-diphosphate 3′-diphosphate and guanosine 5′-triphosphate 3′-diphosphate, does not require 20% methanol to stimulate the rate of reaction, and is not stimulated by complexing with ribosomes of either *Escherichia coli* or *B. brevis*. The *B. brevis* enzyme system is not inhibited by RNase A or thiostrepton, and is only slightly inhibited by tetracycline. The pyrophosphoryl donor specificity of the *B. brevis* enzyme is similar to that of the *E. coli* ribosome-stringent factor system.

Stringent strains of *Escherichia coli*, when starved of a required amino acid, restrict the synthesis of its ribosomal RNA and, at the same time, accumulate a high concentration of ppGpp and pppGpp[1] (Cashel, 1969). The phenomenon of RNA synthesis dependent on the availability of amino acid is termed stringent response, and it is absent in mutant relaxed strains. In response to other growth-restricting conditions, such as limitation of carbon or nitrogen source, *E. coli* also show "stringent response" and accumulation of ppGpp (for a review see Cashel and Gallant, 1974). Guanosine 5′,3′-polyphosphate has been implicated as the regulatory nucleotide in the inhibition of ribosomal RNA synthesis under these limiting conditions (Cashel, 1969; Lazzarini et al., 1971). The nucleotide has also been found to inhibit, in vitro, the activity of several enzymes (Gallant et al., 1971; Merlie and Pizer, 1973; Polakis et al., 1973) as well as the transport of purine and pyrimidine (Hochstadt-Ozer and Cashel, 1972).

The in vivo synthesis of ppGpp and pppGpp has been associated with the protein synthesizing machinery since accumulation of guanosine 5′,3′-polyphosphate following amino acid starvation was found to be inhibited by antibiotics that interfered with protein synthesis (Lund and Kjeldgaard, 1972). In vitro, the biosynthesis of the guanosine 5′,3′-polyphosphates, ppGpp and pppGpp, has been characterized as being catalyzed by a protein present in the high salt wash of ribosomes of stringent strains of *Escherichia coli* in both a ribosome-dependent and ribosome-independent reaction (Haseltine et al., 1972; Sy et al., 1973). In both reactions, ATP donates its β,γ-pyrophosphate to be inserted into the 3′ position of GTP or GDP in forming the 5′,3′-polyphosphates (Sy and Lipmann, 1973). The ribosomal reaction requires the presence of uncharged tRNA in the acceptor site of a ribosome–mRNA complex for activity (Pedersen et al., 1973; Haseltine and Block, 1973), whereas in the nonribosomal reaction, a temperature lower than 30 °C and 20% methanol or some acidic proteins are required for stimulation of the low background activity (Sy et al., 1973).

We have reported previously (Sy et al., 1974) on the interchangeability of *Bacillus brevis* ribosomes and *E. coli* ribosomes in stimulating *E. coli* stringent factor. We noted that *B. brevis* sucrose-washed ribosomes, in the absence of added stringent factor, synthesized little ppGpp or pppGpp, in contrast to the sucrose-washed ribosomes of stringent *E. coli*. While attempting to clarify this anomaly, the author found that the major synthetic activity of the guanosine 5′,3′-polyphosphates in a *B. brevis* extract resided in the 100 000*g* supernatant fluid and that the enzymatic activity was not stimulated by the ribosome-uncharged tRNA-mRNA complex.

Materials and Methods

E. coli K-19 (stringent) ribosomes and stringent factor were obtained as described (Sy and Lipmann, 1973; Sy et al., 1973). *B. brevis* (ATCC 8185) was a gift from Dr. H. Akers. [α-^{32}P]GTP was purchased from ICN and New England Nuclear Corp.

Preparation of B. brevis Extracts. Late log phase *B. brevis* cells (15 g) were suspended in 16 ml of buffer A (10 mM Tris-OAc (pH 7.8), 14 mM $Mg(OAc)_2$, 60 mM KCl, and 1 mM dithiothreitol) containing 16 μg of electrophoretically pure DNase (Worthington). The cell suspension was passed through a French press at 18 000 psi, and the homogenate was centrifuged at 15 000*g* for 30 min. The supernate (S-30) was then centrifuged at 45 000 rpm for 2.5 h in a Spinco 50 rotor. The resulting supernate (S-100) was saved and the pellets, which consisted of a clear layer at the bottom of the centrifuge tube (R2) and a reddish fluffy layer (R1), were resuspended separately in buffer A.

Partial Purification of Stringent Factor from B. brevis. Ammonium sulfate was added to the 100 000*g* supernatant fraction (18 ml) to 45% saturation. The precipitate that formed was isolated by centrifugation and redissolved in 50 mM Tris-OAc (pH 8.1), 1 mM dithiothreitol, and 0.1 mM EDTA, and was then dialyzed for 5 h against 20 mM Tris-OAc (pH 8.1) and 1 mM dithiothreitol. Any precipitate that formed during dialysis was centrifuged off and discharged. The enzyme was further purified by DEAE-cellu-

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[1] Abbreviations used are: ppGpp, guanosine 5′-diphosphate 3′-diphosphate; pppGpp, guanosine 5′-triphosphate 3′-diphosphate; ATPγS, adenosine 5′-(3-thio)triphosphate; AMPPCP, adenosine 5′-(β,γ-methylene)triphosphate. An s superior to the p, e.g. ^{s}p, denotes a phosphothioate in that position.

Table I: Distribution of Guanosine 5',3'-Polyphosphate Synthetic Activity.[a]

Fractions	Vol (ml)	Act. (nmol of GTP Converted/ml of Enzyme Fraction per min) + Ribosomal Complex	Act. (nmol of GTP Converted/ml of Enzyme Fraction per min) − Ribosomal Complex
Crude extract	210	0.63 (133)	1.08 (228)
S-100 fraction	125	0.93 (117)	1.79 (224)
Ribosome (R1)	68	0.50 (34)	0.28 (19)
Ribosome (R2)	16	0.68 (11)	0.31 (5)

[a] Guanosine polyphosphate synthesis was assayed as described under Materials and Methods. The reaction mixture, in 25 μl, contained: 50 mM Tris-OAc, pH 8.1; 5 mM dithiothreitol; 10 mM $Mg(OAc)_2$; 0.2 mM [α-^{32}P]GTP (23 Ci/mol); 2 mM ATP; and 2 μl of various fractions. For assays in the presence of the ribosomal complex the reaction mixture contained, in addition, 38 μg of ammonium chloride washed *E. coli* ribosomes and 1.25 μg each of tRNA and poly(A,U,G), and the final Mg^{2+} concentration was 15 mM. Incubation was at 30 °C for 50 min and was terminated by the addition of HCOOH. The numbers in parentheses represent the total synthetic activity of the fraction.

Table II: Effect of Protein Synthesis Inhibitors on Guanosine Polyphosphate Synthesis.[a]

Additions	% GTP Converted to ppGpp + pppGpp *B. brevis* System	% GTP Converted to ppGpp + pppGpp *E. coli* Ribosomal System
None	45.1	52.9
+ RNase A (0.8 mg/ml)	45.0	3.3
+ Thiostrepton (4×10^{-6} M)	44.0	7.2
+ Tetracycline (88 μg/ml)	42.6	6.8

[a] Guanosine polyphosphate synthesis was assayed as described under Materials and Methods. The reaction mixture contained, in 25 μl: 33 mM Tris-OAc, pH 8.1; 3 mM dithiothreitol; 20 mM $Mg(OAc)_2$; 0.2 mM [α-^{32}P]GTP (20 Ci/mol); 2 mM ATP; for the *B. brevis* system, 30 μg of ammonium sulfate fraction; and for the *E. coli* ribosomal system, 15 μg of ammonium chloride ribosomes, 1 μg each of tRNA and poly(A,U,G), and 0.7 μg of fraction II stringent factor. RNase A and the antibiotics were added at the indicated concentrations. Incubation was at 30 °C for 60 min and was terminated by the addition of HCOOH.

lose column chromatography. The 0–45% ammonium sulfate fraction (228 mg) was fractionated on a 15-ml column of Whatman DE-52 cellulose that was equilibrated with 0.1 M KCl, 20 mM Tris-OAc (pH 8.1), and 1 mM dithiothreitol. The column was then washed with 20 ml of equilibration buffer, and the proteins were eluted stepwise with 15 ml each of 0.15, 0.2, 0.25, 0.3, and 0.5 M KCl in 20 mM Tris-OAc (pH 8.1) and 1 mM dithiothreitol. The major part of enzymatic activity was eluted with the 0.3 M KCl fraction. The proteins in the 0.3 M KCl fraction were precipitated by the addition of ammonium sulfate to 50% saturation. The precipitate was dissolved in 50 mM Tris-OAc (pH 8.1), 1 mM dithiothreitol, and 0.1 mM EDTA, and then dialyzed against 20 mM Tris-OAc (pH 8.1) and 1 mM dithiothreitol. The enzyme fraction was stored in small aliquots in liquid nitrogen.

Assays for the Synthesis of Guanosine Polyphosphates. The assays were carried out at 30 °C for 60 min in 25 μl of reaction mixture containing 50 mM Tris-OAc (pH 8.1), 4 mM dithiothreitol, 10 mM $Mg(OAc)_2$, 0.20 mM [α-^{32}P]GTP (10–40 Ci/mmol), 2 mM ATP, and the appropriate enzyme fractions. The reactions were stopped by the addition of HCOOH, and the guanosine polyphosphates formed were analyzed by thin-layer chromatography on polyethyleneimine cellulose as previously described (Sy et al., 1973; Cashel and Kalbacher, 1970).

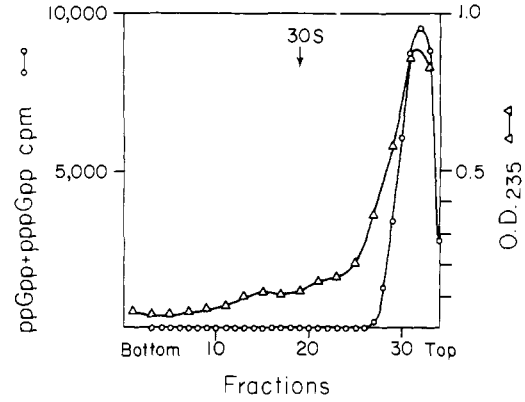

FIGURE 1: Sucrose gradient centrifugation of *B. brevis* stringent factor. The 0–40% ammonium sulfate fraction (4.7 mg) of the *B. brevis* S-100 fraction was centrifuged in a SW56 rotor at 50 000 rpm for 2 h on a 10–30% sucrose gradient containing 20 mM Tris-OAc (pH 8.1) and 1 mM dithiothreitol. Six-drop fractions were collected from the bottom and assayed for guanosine polyphosphate synthesis (○). In a final volume of 50 μl that contained 33 mM Tris-OAc (pH 8.1), 3 mM dithiothreitol, 20 mM $Mg(OAc)_2$, 0.2 mM [α-^{32}P]GTP (23 Ci/mol), 2 mM ATP, and 20-μl aliquots of the gradient fractions were incubated at 30 °C for 1 h. Incubation was stopped by the addition of HCOOH, and the guanosine polyphosphates formed were assayed as described under Materials and Methods; (△) represents absorption at 235 nm.

Results

E. coli stringent factor has been reported to be present only in the ribosomal fraction (Haseltine et al., 1972; Cochran and Byrne, 1974), but recent experiments (Block and Haseltine, 1974) have indicated that it can also be found in the membrane and S-100 fractions. Its distribution between these fractions has been found to be dependent on the strain of *E. coli* used (Y. Ogawa, unpublished results). The *E. coli* strain (K-19) that we have used routinely contained as much as 40% of its stringent factor activity in the S-100 fraction. To account for the low synthetic activity of the guanosine polyphosphates in the *B. brevis* ribosome fraction, stringent factor activity in different fractions of *B. brevis* extracts was tested. Table I shows that when assayed in the ribosomal system, the major activity is found in the S-100 fraction. Surprisingly, the results indicate that addition of ribosomes inhibited the synthesis of guanosine polyphosphates in the different S-100 fractions. The ribosome-independent activity in the S-100 fraction accounts for nearly all the activity found in the crude extract.

To prove that this synthetic activity is indeed ribosome-independent, the 45% ammonium sulfate fraction of S-100 was subjected to sucrose density gradient centrifugation (Figure 1). All the synthetic activity was found in the top few fractions that represent the low molecular weight protein region. The ribosome-independent nature of the reaction is further shown by its being unaffected by the inhibitors studied in Table II. RNase A, thiostrepton, and tetracycline severely curtailed the synthesis of guanosine polyphosphates in the *E. coli* ribosomal system while they had practically no effect in the *B. brevis* system.

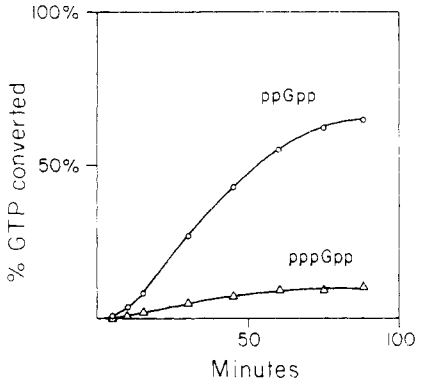

FIGURE 2: Time course of guanosine polyphosphate formation. DEAE-cellulose fractionated enzyme (11 μg) was incubated at 30 °C in 50 μl containing 50 mM Tris-OAc (pH 8.1), 4 mM dithiothreitol, 10 mM $Mg(OAc)_2$, 0.2 mM [α-^{32}P]GTP (37 Ci/mol), and 2 mM ATP. At the indicated time points 5-μl aliquots were removed and the reaction was stopped by the addition of HCOOH. The guanosine polyphosphates formed were then assayed as described under Materials and Methods.

Table III: Nucleotide Specificity of the Pyrophosphoryl Donor.[a]

Nucleotide	GTP Converted to ppGpp + pppGpp (%)
ATP	66.3
dATP	0.6
ATPγS	10.8
AMPPCP	0
CTP	0
UTP	0

[a] Guanosine polyphosphate synthesis was assayed as described under Materials and Methods. The reaction mixture contained, in 25 μl: 50 mM Tris-OAc, pH 8.1; 4 mM dithiothreitol; 10 mM $Mg(OAc)_2$; 0.2 mM [α-^{32}P]GTP (37 Ci/mol); 11 μg of DEAE-cellulose fractionated enzyme; and various indicated nucleotides at 4 mM each. Incubation was at 30 °C for 60 min and was terminated by the addition of HCOOH.

Table IV: Effect of Ribosomal Complexes on the Activity of *B. brevis* Stringent Factor.[a]

Additions	% GTP Converted to ppGpp + pppGpp at μg of Enzyme Added: 11	22
None	24.8	50.2
+ *E. coli* ribosomes	31.3	59.7
+ *E. coli* ribosomes + poly(A,U,G) + tRNA	27.5	63.1
+ *E. coli* ribosomes + poly(A,U,G) + tRNA in 20 mM Mg^{2+}	9.2	44.2
+ *B. brevis* ribosomes	16.3	55.6
+ *B. brevis* ribosomes + poly(A,U,G) + tRNA	24.3	46.5
+ *B. brevis* ribosomes + poly(A,U,G) + tRNA in 20 mM Mg^{2+}	4.8	24.8

[a] Guanosine polyphosphate synthesis was assayed as described under Materials and Methods. The reaction mixture contained, in 25 μl: 50 mM Tris-OAc, pH 8.1; 4 mM dithiothreitol; 10 mM $Mg(OAc)_2$; 0.2 mM [α-^{32}P]GTP (36 Ci/mol); 2 mM ATP; and 11 or 22 μg of the DEAE-cellulose fractionated enzyme as indicated. Where indicated, 15 μg of *E. coli* ammonium chloride washed ribosome had been added or 28 μg of *B. brevis* sucrose-washed ribosomes and 1.4 μg each of tRNA and poly(A,U,G). Reactions were performed at 30 °C for 60 min, and were terminated by the addition of HCOOH.

Table V: Effect of Methanol on *B. brevis* Pyrophosphotransferase.[a]

Methanol	% GTP Converted to ppGpp + pppGpp: 10 min	30 min
−	34.5	76.4
+	3.8	46.7

[a] Guanosine polyphosphate synthesis was assayed as described under Materials and Methods. The reaction mixture contained in 25 μl: 50 mM Tris-OAc, pH 8.1; 4 mM dithiothreitol; 2 mM ATP; 0.2 mM [α-^{32}P]GTP (4 Ci/mol); 2.2 mM $Mg(OAc)_2$; 100 mM NH_4Cl; 1 mg/ml, bovine serum albumin; 20 μg of DEAE-cellulose fractionated enzyme; and 15% methanol as indicated. Incubation was at 30 °C and was terminated at the indicated time with HCOOH.

The *B. brevis* system has a pH optimum between 8 and 8.5. In the presence of 2.2 mM triphosphates it requires 5 mM Mg^{2+} for optimal activity, but a Mg^{2+} concentration higher than 20 mM is inhibitory. The optimal temperature for the reaction is 30 °C, but the reaction will proceed at 50% activity at 37 °C. The products of the system were identified to be guanosine 5′,3′-polyphosphates by the following criteria: (1) ^{32}P-labeled products isolated by DEAE-cellulose chromatography (Sy and Lipmann, 1973) migrated indistinguishably from ultraviolet (uv)-spotted authentic ppGpp and pppGpp, isolated from the *E. coli* ribosomal system, in a two-dimensional thin-layer chromatography system (Cashel and Kalbacher, 1970); (2) the added pyrophosphate in the products had the high sensitivity to acid hydrolysis indicative of 3′ substitution, as found in the products of the *E. coli* system (Sy and Lipmann, 1973); and (3) similar products were obtained when [^{3}H]GDP or [γ-^{32}P]ATP were used as the radioactive-labeled substrates instead of [α-^{32}P]GTP.

Further purification of the enzyme was achieved by DEAE-cellulose fractionation, the enzyme eluting at 0.3 M KCl. The time course of ppGpp + pppGpp synthesis by this enzyme fraction is shown in Figure 2. After a lag of 5 min, the synthesis was nearly linear for an additional 50 min for both ppGpp and pppGpp; due to a remaining contamination with GTPase (see Discussion), the major product of the reaction was ppGpp.

The *B. brevis* system has a similar specificity for the pyrophosphoryl donor to that seen in the *E. coli* system (Cochran and Byrne, 1974; Sy, 1975) (Table III). dATP is a poor substitute for ATP, and adenosine 5′-(β,γ-methylene)triphosphate cannot replace ATP, but adenosine 5′-(3-thio)triphosphate is a fairly good pyrophosphoryl donor, yielding ppGp^{s}p and pppGp^{s}p. Neither CTP nor UTP is a pyrophosphoryl donor.

The effect of the partially purified *B. brevis* system on the ribosomal systems from *E. coli* and *B. brevis* was determined at two different Mg^{2+} concentrations and using different enzyme concentrations (Table IV). In the presence or absence of poly(A,U,G) + tRNA, neither *E. coli* ribosomes nor *B. brevis* ribosomes affected the synthetic activity of the DEAE-cellulose purified enzyme when assayed at 10 mM Mg^{2+}. Similar results were obtained at the two different concentrations of enzyme used. However, when Mg^{2+} concentration in the assay system was raised to 20 mM Mg^{2+},

the presence of *E. coli* or *B. brevis* ribosomes inhibited the enzymatic activity; this inhibition at high Mg^{2+} concentration should explain the lower activity obtained in Table I where crude extracts and S-100 fractions were assayed in the presence of added ribosomes and of 15 mM Mg^{2+}.

The effect of methanol on the synthetic activity of the DEAE-cellulose purified *B. brevis* enzyme was determined to ascertain if any stimulation of enzymatic activity could be found. Table V shows that addition of 15% methanol to the reaction mixture inhibited the activity of the *B. brevis* enzyme, a result in contrast to the near 20-fold activation of *E. coli* stringent factor by methanol (Sy et al., 1973; Block and Haseltine, 1975).

Discussion

We have previously shown the enzymatic nature of *E. coli* stringent factor (Sy et al., 1973). Its synthetic rate is stimulated 20-fold by 20% methanol and 400-fold by the ribosome–mRNA–uncharged tRNA complex (Block and Haseltine, 1975). The results reported here indicate that *B. brevis* cells contain an enzyme that is capable of synthesizing guanosine 5′,3′-polyphosphates but is not stimulated by either *E. coli* or *B. brevis* ribosomal complexes. *B. brevis* (ATCC 8185) ribosomes contain very little stringent factor activity; however, they will stimulate *E. coli* stringent factor as well as *E. coli* ribosomes (Sy et al., 1974). The *B. brevis* enzyme also differs from *E. coli* stringent factor in several ways: it is not stimulated by addition of methanol; it is a supernatant protein, in contrast to the *E. coli* enzyme which is found predominantly in the ribosomal wash; *E. coli* stringent factor is nearly insoluble in low ionic strength buffer, whereas the *B. brevis* enzyme is quite soluble.

The major interfering activity in the assay of guanosine 5′,3′-polyphosphate synthesis in the crude extract, S-100, and 45% ammonium sulfate fractions is a highly active phosphatase, which is completely removed during DEAE-cellulose fractionation since phosphatase does not bind to the DEAE-cellulose column at 0.1 M KCl. After purification through DEAE-cellulose chromatography, the *B. brevis* enzyme still contains GTPase, which causes the resulting product to be mostly ppGpp (Figure 2); however, after further purification through hydroxylapatite, the major product is pppGpp when GTP is used as the substrate (data not shown).

Stringent response has been identified in *Bacillus subtilis* (Gallant and Margason, 1972; Swanton and Edlin, 1972; Fortnagel and Bergmann, 1974). In vitro synthesis of ppGpp + pppGpp was also assayed with ribosomes from both *B. subtilis* and *Bacillus stearothermophilus* (Fortnagel and Bergmann, 1974; Richter and Isono, 1974). Activity was found to be low, however, in these in vitro systems as compared to the *E. coli* ribosomal system. Our finding that the major synthetic activity of *B. brevis* is located in the S-100 fraction may be an explanation for such low activity in the ribosomal fraction. The finding of a ribosome-independent system in *B. brevis* may also be of relevance to explain the presence of guanosine polyphosphate synthesis in relaxed strains of *E. coli* (Lazzarini et al., 1971; Harshman and Yamazaki, 1971). Attempts are under way to obtain amino acid deficient mutants of *B. brevis* that correspond to the stringent and relaxed types found with *E. coli*.

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