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# Guanosine Pentaphosphate Synthetase from *Streptomyces antibioticus* Is Also a Polynucleotide Phosphorylase

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**The gene for the enzyme guanosine pentaphosphate synthetase I (GPSI) from *Streptomyces antibioticus* has been cloned and sequenced. The cloned gene functioned as a template in the streptomycete coupled transcription-translation system and directed the synthesis of a protein with the properties expected for GPSI. Sequencing of the cloned gene identified an open reading frame of 740 amino acids whose amino terminal sequence corresponded to the N terminus of purified GPSI. The GPSI protein sequence was found to possess significant homology to polynucleotide phosphorylase from *Escherichia coli*. Indeed, like *E. coli* polynucleotide phosphorylase, purified GPSI was shown to catalyze the polymerization of ADP and the phosphorolysis of poly(A). However, the *E. coli* enzyme was unable to catalyze the synthesis of guanosine pentaphosphate under conditions in which GPSI was highly active in that reaction. Overexpression of the cloned *gpsI* gene in *E. coli* led to an increase in both polynucleotide phosphorylase and guanosine pentaphosphate synthetase activities in the cloning host. The polynucleotide phosphorylase activities of GPSI and of the *E. coli* enzyme were strongly inhibited by dCDP, but the pppGpp synthetase activity of GPSI was not inhibited and indeed was slightly stimulated by dCDP. These results strongly support the identity of GPSI as a bifunctional enzyme capable of both pppGpp synthesis and polynucleotide phosphorylase activities.**

One of the most important experimental challenges related to the mechanism and regulation of antibiotic production is the identification of the signals that trigger the production of those antibiotics. One candidate for such a trigger molecule in antibiotic-producing members of the genus *Streptomyces* is the highly phosphorylated guanine nucleotide, guanosine tetraphosphate (ppGpp). There is strong evidence for the involvement of ppGpp as an effector of the stringent response in *Escherichia coli* and other bacteria (5). There are at least two pathways for the production of ppGpp in *E. coli*. The *relA* and *spoT* loci are responsible for the synthesis of ppGpp in the stringent responses to amino acid starvation and energy starvation, respectively (5, 15–17, 40).

Recently, evidence has been presented for a role of ppGpp in the regulation of antibiotic production in members of the genus *Streptomyces*. Bibb and coworkers have examined the role of ppGpp in *Streptomyces coelicolor* A3(2) during normal growth on antibiotic production medium and under conditions of amino acid starvation (35, 37). Their results are consistent with a role for ppGpp in signaling the initiation of antibiotic production in *S. coelicolor*, although recent results indicate that inactivation of a putative *relA* homolog in *S. coelicolor* does not abolish actinorhodin production (7). There is also a significant body of evidence suggesting a relationship between ppGpp and the onset of actinomycin production in *Streptomyces antibioticus*. Ochi isolated a relaxed (*relC*) mutant of *S. antibioticus* IMRU 3720 some years ago and demonstrated that this mutant contained reduced levels of ppGpp compared with the parental strain and was unable to synthesize actinomycin (28, 29). In addition, the relaxed mutant contained decreased levels of two enzymes involved in the biosynthesis of actinomycin, phenoxazinone synthase and actinomycin synthetase I (22, 28). In the parental strain of *S. antibioticus*, there was a significant increase in ppGpp levels just prior to the onset of actinomycin production (22). Thus, studies of wild-type *S. antibioticus* as well as relaxed mutants support a relationship between ppGpp production and actinomycin biosynthesis.

To further characterize the relationship between ppGpp and actinomycin biosynthesis in *S. antibioticus*, an enzyme capable of synthesizing pppGpp, the biosynthetic precursor of ppGpp, has been isolated. This enzyme, named guanosine pentaphosphate synthetase I (GPSI), showed some functional similarity to the RelA protein of *E. coli* but differed from RelA in several important respects. Thus, the activity of GPSI, unlike that of RelA, was not activated by ribosomes. Moreover, GPSI functioned effectively with GTP as a substrate, but again unlike RelA, the enzyme did not utilize GDP. Finally, the activity of GPSI could be stimulated by mild proteolysis with trypsin. Under similar reaction conditions, no stimulatory effect of trypsin on the activity of the RelA protein could be demonstrated (19, 20).

To further examine the role of ppGpp in regulating actinomycin biosynthesis, the gene for GPSI has been cloned and characterized. We describe below the properties of the cloned gene and the interesting finding that the amino acid sequence of GPSI bears significant similarity to that of the enzyme polynucleotide phosphorylase (PNPase) from *E. coli*. We demonstrate definitively that in addition to its activity as a pppGpp synthetase, GPSI from *S. antibioticus* also possesses PNPase activity.

## MATERIALS AND METHODS

**Growth of organisms.** *S. antibioticus* IMRU 3720 was grown on NZ-amine and galactose-glutamic acid media as described previously (8). *E. coli* strains DH5α (14) and XL1-Blue (Stratagene Cloning Systems) were cultured in Luria broth supplemented with antibiotics as necessary.

**DNA manipulations.** Plasmid and chromosomal DNAs were prepared and manipulated as described by Hopwood et al. (18) and earlier publications from our laboratories (21, 27).

**Cloning of the GPSI gene.** DNA fragments containing the coding sequence for GPSI were identified by Southern blotting of *S. antibioticus* total DNA by using as a probe a degenerate oligonucleotide, (A,G)TG-GAG-AAC-GAG-AC(G,C)-

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CAC-TAC-GC(G,C)-GAG-GC(G,C)-GT, prepared according to the amino terminal sequence of purified GPSI (MENETHYAEAV, see Fig. 3). Hybridization was carried out at 60°C in 3× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), and filters were washed twice in 2× SSC at 60°C. This analysis identified a ca. 7-kb *Bcl*I-*Bgl*II fragment, which was cloned in *E. coli* XL1-Blue by using the plasmid vector pBluescript SK+ (Stratagene Cloning Systems). Clones of interest were identified by colony hybridization, performed under the conditions described for Southern blotting. The resulting recombinant plasmid, pJSE301 (see Fig. 1), was analyzed in the streptomycete coupled transcription-translation system (see below). This analysis showed that the 7-kb insert of pJSE301 contained only the 5′ half of the *gpsI* gene (see Fig. 1). pJSE301 hybridized to a ca. 4-kb *Bam*HI fragment from the *S. antibioticus* genome which included ca. 2 kb of sequence downstream of the *Bcl*I-*Bgl*II fragment. A minilibrary was prepared in *E. coli* DH5α by cloning 3- to 5-kb *Bam*HI fragments of the *S. antibioticus* genome, obtained by excising the relevant region from an agarose gel of *Bam*HI-digested *S. antibioticus* DNA. The cloning vector used in these experiments was pIJ4642, a positive selection vector derived from pIJ699 (23) but which lacks the Tn*5* neomycin resistance gene. The function of pIJ4642 as a positive selection vector results from the fact that the plasmid produced by recircularization of the larger (core) fragment, obtained by *Bam*HI digestion and removal of the smaller fragment, is not viable. The core fragment of pIJ4642 was obtained by *Bam*HI digestion and fractionation of the digest by agarose gel electrophoresis. Following ligation of the genomic fragments to the pIJ4642 core and transformation, colonies of interest, selected on chloramphenicol, were identified by colony hybridization by using as a probe a *Bam*HI-*Xba*I fragment obtained from pJSE301. This analysis identified a recombinant plasmid containing the ca. 4-kb *Bam*HI insert, and this plasmid was designated pJSE302. The *Bam*HI insert was subsequently cloned into the *Bam*HI site of pBluescript SK+ in both orientations, generating pJSE303, with the open reading frame (ORF) oriented left to right as shown in Fig. 1, and pJSE304.

**DNA sequence determination and analysis.** DNA sequencing of both strands of the *gpsI* gene was done with the TaqTrack system from Promega by using single-stranded DNA as templates. The sequencing strategy involved the use of restriction sites in pBluescript SK+ and the 4-kb insert to generate smaller fragments and the use of custom sequencing primers. The *gpsI* sequence was analyzed using the FRAME (1) and BESTFIT (10) programs.

**Enzyme assays.** GPSI was purified from *S. antibioticus*, and methanol- and trypsin-dependent assays for GPSI were performed as described previously (19, 20) except as noted below. *E. coli* PNPase (nominal specific activity, 1.47 μmol of [$^{14}$C]ADP incorporated into poly(A) per min per mg of protein at 37°C) was obtained from Sigma Chemical Co., and polymerization and phosphorolysis assays were carried out under conditions similar to those described by Littauer and Kornberg (24) and Godefroy et al. (11). In the polymerization assays, 100-μl reaction mixtures contained 50 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, 5 mM unlabeled ADP, and 1 μCi of [2,8-$^3$H]ADP (25 Ci/mmol; New England Nuclear) (experiments of Fig. 6) or 0.04 μCi of [8-$^{14}$C]ADP (55 mCi/mmol; New England Nuclear) (experiments of Fig. 7). Reaction mixtures were incubated at 37°C for 15 to 30 min. Reactions were quenched and poly(A) was precipitated by the addition of 10% trichloroacetic acid; precipitates were collected on glass fiber filters and analyzed by liquid scintillation counting.

To measure phosphorolysis of poly(A), the $^3$H-polymer formed in a 300-μl reaction, performed as described above in a 30-min incubation, was isolated by phenol extraction of the reaction mixtures and ethanol precipitation in the presence of 5 M ammonium acetate. Phosphorolysis reaction mixtures (100 μl) contained 50 mM Tris-HCl (pH 8.0) 5 mM $MgCl_2$, 10 mM potassium phosphate buffer (pH 7), [$^3$H]poly(A) (45,000 cpm), and enzyme. Reaction mixtures were incubated for 30 min at 37°C and quenched by the addition of 10% trichloroacetic acid. Phosphorolysis was measured as the conversion of the [$^3$H]poly(A) to an acid soluble form.

In some experiments it was necessary to perform GPS and PNPase reactions under identical conditions. The basic reaction mixtures for these experiments were prepared as 2× stocks containing 160 mM Tris-HCl (pH 8.0), 8.8 mM ATP, 2.6 mM GTP, 30 mM ADP, and 20 mM $MgCl_2$. Final reaction mixtures were prepared with 0.5 volume of 2× stock with either a 40-μCi/ml final concentration of [α-$^{32}$P]GTP (GPSI assays), or a 20-μCi/ml final concentration of [8-$^{14}$C]ADP (PNPase polymerization assays), enzyme, and dCDP, as indicated in the legend to Fig. 8. Reaction mixtures were incubated at 30°C for GPSI assays and 37°C for PNPase assays, and reaction products were separated by thin layer chromatography on polyethyleneimine cellulose plates. For the experiments shown in Fig. 8, spots corresponding to [$^{14}$C]poly(A) or [$^{32}$P]pppGpp were cut from the thin layer plates and analyzed by liquid scintillation counting.

**Overexpression of the *gpsI* gene.** To clone the *gpsI* ORF for overexpression experiments, a PCR primer corresponding to the region near the amino terminus of the protein sequence was prepared. This primer, GGG-ACG-AAG-AAC-GGA-TCC-AAA-ACG-CTA-GTG-GAG, was designed to introduce a *Bam*HI site (underlined) beginning five codons upstream of the GTG initiation codon for *gpsI* (see Fig. 3). This primer was used in PCRs with pJSE303 (see below) as template, an extended pBluescript SK+ M13 reverse primer (AAC-AAT-TTC-ACA-CAG-GAA-ACA-GCT-ATG-ACC-ATG) as the second PCR primer, and Vent DNA polymerase (New England BioLabs). The product of this reaction was treated with Klenow enzyme and a mixture of all four deoxynucleoside triphosphates, and the resulting DNA fragment was cloned into pBluescript SK+ which had been digested with *Eco*RI and also filled in with Klenow polymerase and deoxynucleoside triphosphates. The resulting recombinant plasmid (pJSE370) was obtained by transformation of *E. coli* XL1-Blue. The *Bam*HI fragment containing the entire *gpsI* ORF was cloned into the *Bam*HI site of the *E. coli* expression vector pET11a (Novagen). The resulting plasmids representing both orientations of the *gpsI* ORF (pJSE371 and -372) were subsequently used to transform *E. coli* BL21(DE3) and BL21(DE3)pLysS for overexpression of *gpsI*. The orientation of the *gpsI* ORF which permitted expression from the T7 promoter in pET11a was that obtained in pJSE371. Expression of *gpsI* in this construct would be expected to produce a protein with an N-terminal extension of 17 amino acids, 14 of which are derived from the T7-Tag of pET11a and 3 of which are derived from the PCR primer used to prepare the *gpsI* ORF for cloning. Expression of the cloned *gpsI* ORF was induced by exposing 50-ml cultures of the relevant strains, grown in Luria broth, to 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Samples of 20 ml were harvested from the cultures before addition of IPTG and 2 h following addition. The cells were collected by centrifugation, washed with 10 ml of 50 mM Tris-HCl (pH 8.0)–2 mM EDTA, resuspended in 1 ml of 50 mM Tris-HCl (pH 8.0)–5 mM $MgCl_2$–5% glycerol, and disrupted by sonication for 10 s. Sonicates were centrifuged for 5 min in an Eppendorf centrifuge, and portions of the resulting supernatants were assayed for GPSI and PNPase activities as described above.

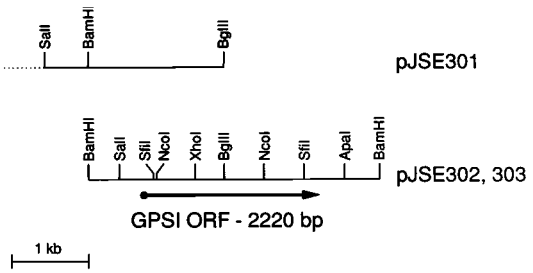


FIG. 1. Partial restriction maps of pJSE301 and pJSE302. The extent and orientation of the *gpsI* ORF are shown.

**Miscellaneous methods.** Coupled transcription-translation assays were performed and the products analyzed as described previously (21, 38). Protein was determined as described by Bradford (3) by using the BioRad reagent.

**Nucleotide sequence accession number.** The GPSI sequence has been assigned GenBank accession number U19858.

## RESULTS

**Cloning and identification of the *gpsI* gene.** As described in Materials and Methods, a 7-kb *Bcl*I-*Bgl*II fragment, containing a portion of the region encoding GPSI, was identified by Southern blotting by using as a probe a degenerate oligonucleotide corresponding to the amino terminus of purified GPSI. A partial restriction map of this fragment is shown in Fig. 1. A portion of the insert of pJSE301, extending from the rightmost *Bam*HI site (Fig. 1) to the *Xba*I site in the pBluescript SK+ polylinker, was used as a probe to identify a 4-kb *Bam*HI fragment containing ca. 2 kb of sequence downstream of the insert of pJSE301. A partial restriction map of this fragment, the insert of pJSE302, pJSE303, and pJSE304, is presented in Fig. 1.

The presence of the coding region for GPSI on the 4-kb *Bam*HI fragment was demonstrated initially by using the streptomycete coupled transcription-translation system. The results of this analysis are depicted in Fig. 2. The electrophoretic analysis of the coupled reactions shows that pJSE301, as indicated above, and pJSE302 functioned as templates in the coupled system (Fig. 2, lanes 3 and 5). Treatment of reaction mixtures with antibody to GPSI precipitated products related to GPSI (Fig. 2, lanes 9 and 11). pJSE301 directed the synthesis of a truncated derivative of GPSI with an $M_r$ of about 40,000 (Fig. 2, lanes 3 and 9). pJSE302 directed the synthesis of a polypeptide with the mobility of purified GPSI and which reacted with GPSI antibody (Fig. 2, lanes 5 and 11). The lower molecular weight polypeptides observed in lanes 9 and 11 of Fig. 2 also reacted with GPSI antibody and were presumably

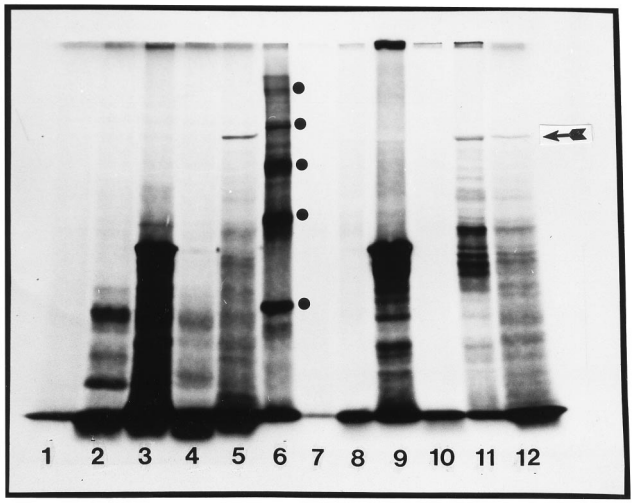


FIG. 2. Fluorogram of the products of coupled transcription-translation. Reactions were prepared and incubated as described (21) and products either were analyzed directly on SDS-PAGE gels (lanes 1 to 5 and lane 12) or were treated with antibody to GPSI and the immunoprecipitates were subjected to SDS-PAGE (lanes 7 to 11). Templates used were as follows: lanes 1 and 7, no template added; lanes 2 and 8, pBluescript SK+; lanes 3 and 9, pJSE301; lanes 4 and 10, pIJ4642; lanes 5, 11, and 12, pJSE302. Lane 6 contains radioactive size markers with apparent $M_r$s (from top to bottom) of 200,000, 92,500, 69,000, 46,000, and 30,000, indicated by dots. The arrow indicates the migration position of purified GPSI.

produced by nonphysiological initiation or termination of transcription or translation in the coupled system. Such products have been observed before in that system (21).

**DNA sequence analysis.** Nucleotide sequencing of the insert of pJSE303 verified the correspondence of the DNA sequence of the cloned gene and the amino terminal sequence of the purified protein. The nucleotide sequence of the cloned *gpsI* gene, extending from just upstream of the translation start to just beyond the translation stop codon, is shown in Fig. 3. The *gpsI* ORF is 2,220 bp in length and encodes a polypeptide of 740 amino acids with a calculated $M_r$ of 79,129. As indicated above, the amino terminal sequence of purified GPSI corresponds exactly to the protein sequence predicted by translation of the DNA sequence of the gene. FRAME analysis (1) indicated that the *gpsI* ORF has the characteristics expected for a streptomycete gene (data not shown). The ORF has a GC content of 69.1%. The translation start codon for the ORF is GTG, and a potential ribosome binding site (AGGAG) begins 14 bp upstream of the translation start. The *gpsI* sequence shows that in each of the degenerate third codon positions in the synthetic oligonucleotide used as a probe to identify the *gpsI* gene (see Materials and Methods), the actual base is C (Fig. 3).

The transcription start and end points for the *gpsI* gene have not yet been determined but preliminary primer extension studies suggest that the transcription start point lies upstream of the *Sal*I site of the insert of pJSE302 (Fig. 1). Thus, it is possible that the *gpsI* gene is a part of an operon, although we have no direct evidence for that possibility at this point. A hairpin structure which might function in transcription termination can be formed between nucleotides 2265 and 2296. Using the data of Tinoco et al. (39) and Borer et al. (2), the free energy of the hairpin is estimated to be −147 kJ/mol.

**Comparison of the GPSI sequence to known protein sequences.** Because of the function of GPSI in the synthesis of pppGpp, and because it has been demonstrated that *S. antibioticus* extracts can convert pppGpp to ppGpp (19), it seemed highly likely that the GPSI sequence would correspond to either the RelA or SpoT protein from *E. coli* (and other bacteria). When the protein databases were searched, however, no significant homology to any RelA or SpoT homolog was discovered. Rather, the *gpsI* gene was found to be strikingly similar to the gene that encodes an enzyme, polynucleotide phosphorylase (PNPase), from *E. coli*. The amino acid sequences of PNPase (32) and GPSI are aligned in Fig. 4. The overall identity between the two proteins is 44%, a striking degree of sequence conservation for proteins from two such widely separated bacterial genera. Figure 4 further shows that while there are regions in which the level of identity is as high as 70% (e.g., amino acids 61 to 120 of GPSI and 47 to 106 of PNPase), there is significant similarity throughout the sequences of the two proteins.

**Enzymatic activities of GPSI and *E. coli* PNPase.** Because GPSI is clearly a homolog of *E. coli* PNPase, it was of considerable interest to determine whether the two proteins possessed similar catalytic activities. To this end, the activity of *E. coli* PNPase was first tested in the methanol-dependent assay for pppGpp formation (19). The results of such an experiment are shown in Fig. 5, along with the results of standard assays using GPSI. It can be seen that at concentrations of PNPase which were equivalent to GPSI levels that produced significant amounts of pppGpp, no pppGpp was formed by the *E. coli* enzyme. PNPase concentrations which were nine times higher than the highest GPSI concentration tested still failed to show pppGpp synthetase activity (data not shown). Similar results were obtained in the trypsin-dependent assay. Under conditions where trypsin-stimulated pppGpp formation by GPSI could be easily detected, PNPase was inactive (data not shown). It should also be noted that PNPase did not function as a pppGpp synthetase in the absence of activators.

We next asked the question, does GPSI function as a polynucleotide phosphorylase? *E. coli* PNPase has been known for many years to catalyze the reversible polymerization of nucleoside diphosphates (11, 24). We therefore prepared reaction mixtures which permitted the measurement of both the polymerization and phosphorolysis activities of *E. coli* PNPase and examined whether GPSI would function in either of those assays. Results of the polymerization and phosphorolysis assays are shown in Fig. 6. In the polymerization assays, the ability of purified GPSI to convert [$^3$H]ADP into an acid insoluble form was demonstrated. In the phosphorolysis assays, the product of polymerization was used as a substrate for GPSI under conditions which favored the conversion of poly(A) to ADP. Figure 6 shows that both activities were observable with GPSI as the enzyme source. It should also be noted that *E. coli* PNPase converted the product obtained in the polymerization reactions with GPSI into an acid insoluble form (data not shown).

**Overexpression of *gpsI* increases both PNPase and GPSI activity in the cloning host.** To verify the dual enzymatic activities of GPSI, the cloned gene was overexpressed in *E. coli*. In these experiments, a PCR fragment was prepared with a primer derived from the sequence of pBluescript SK+ and a second primer corresponding to the region upstream of the *gpsI* translation start for GPSI but constructed to introduce a *Bam*HI site into the PCR product. This PCR fragment was first cloned into pBluescript SK+ and was then excised as a *Bam*HI fragment and cloned into pET11a for overexpression. Both orientations of the cloned *gpsI* ORF were obtained in these experiments. In pJSE372, the *gpsI* ORF is oriented in the direction opposite to that required for transcription from the T7 promoter, while in pJSE371, T7 promoter-directed tran-

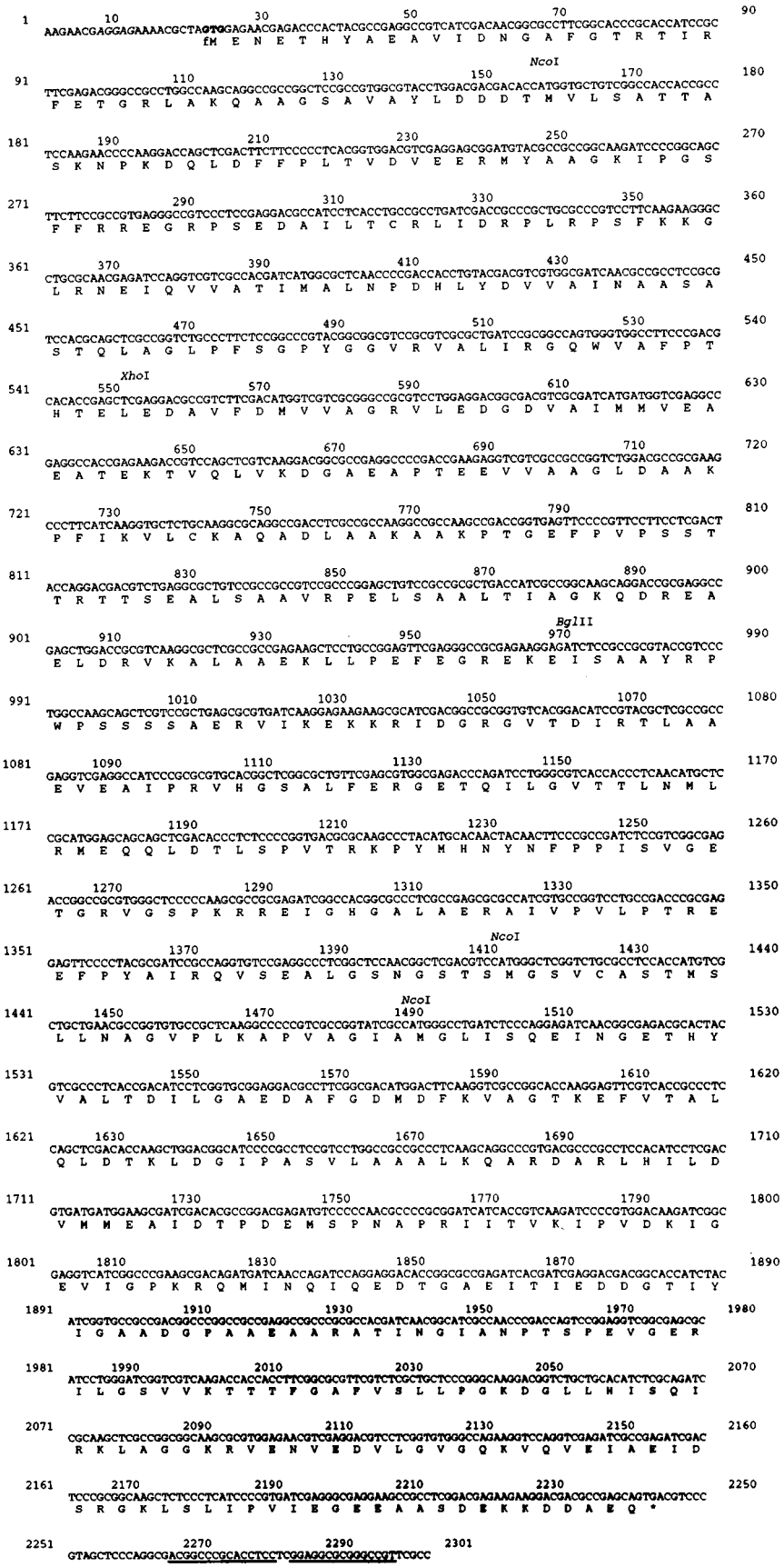


FIG. 3. Nucleotide sequence of the *gpsI* gene from *S. antibioticus*. Several relevant restriction sites are indicated in the sequence and the translation initiation and termination codons are indicated in bold type. A putative ribosome binding site is indicated in italics upstream of the translation start, and a potential hairpin structure downstream of the termination codon (asterisk) is underlined.

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  7 YAEAVIDNGAFGTRTIRFETGRLAKQAAGSAVAYLDDDTMVLSATTASKN 56
  1 LLNPIVRKFQYGQHTVTLETGMMARQATAAVMVSM.DDTAVFVTVVGQKK 49

 57 PKDQLDFFPLTVDVEERMYAAGKIPGSFFRREGRPSEDAILTCRLIDRPL 106
 50 AKPGQDFFPLTVNYQERTYAAGRIPGSFFRREGRPSEGETLIARLIDRPI 99

107 RPSFKKGLRNEIQVVATIMALNPDHLYDVVAINAASASTQLAGLPFSGPY 156
100 RPLFPEGFVNEVQVIATVVSVNPQVNPDIVAMIGASAALSLSGIPFNGPI 149

157 GGVRVALIRGQWVAFPTHTELEDAVFDMVVAGRVLEDGDVAIMMVEAEAT 206
150 GAARVGYINDQYVLNPTQDELKESKLDLVVAG.................T 182

207 EKTVQLVKDGAEAPTEEVVAAGLDAAKPFIKVLCKAQADLAAKAAKPTGE 256
183 EAAVLMVESEAQLLSEDQMLGAVVFGHEQQQVVIQNINELVKEAGKPRWD 232

257 FPVPSSTTRTTSEALSAAVRPELSAALTIAGKQDREAELDRVKALAAEKL 306
233 WQ.PEPVNEALNARVAALAEARLSDAYRITDKQERYAQVDVIKSETIATL 281

307 LPEFEG.REKEISAAYRPWPSSSSAERVIKEKKRIDGRGVTDIRTLAAEV 355
282 LAEDETLDENELGEILHAIEKNVVRSRVLAGEPRIDGREKDMIRGLDVRT 331

356 EAIPRVHGSALFERGETQILGVTTLNMLRMEQQLDTLSPVTRKPYMHNYN 405
332 GVLPRTHGSALFTRGETQALVTATLRTARDAQVLDELMGERTDTFLFHYN 381

406 FPPISVGETGRVGSPKRREIGHGALAERAIVPVLPTREEFPYAIRQVSEA 455
382 FPPYSVGETGMVGSPKRREIGHGRLAKRGVLAVMPDMDKFPYTVRVVSEI 431

456 LGSNGSTSMGSVCASTMSLLNAGVPLKAPVAGIAMGLISQEINGETHYVA 505
432 TESNGSSSMASVCGASLASMDAGVPIKAAVAGIAMGLVKEGDN....YVV 477

506 LTDILGAEDAFGDMDFKVAGTKEFVTALQLDTKLDGIPASVLAAALKQAR 555
478 LSDILGDEDHLGDMDFKVAGSRDGISALQMDIKIEGITKEIMQVALNQAK 527

556 DARLHILDVMMEAIDTP.DEMSPNAPRIITVKIPVDKIGEVIGPKRQMIN 604
528 GARLHILGVMEQAINAPRGDISEFAPRIHTIKINPDKIKDVIGKGGSVIR 577

605 QIQEDTGAEITIEDDGTIYIGAADGPAAEAARATINGIANPTSPEVGERI 654
578 ALTEETGTTIEIEDDGTVKIAATDGEKAKHAIRRIEEITAEI..EVGRVY 625

655 LGSVVKTTTPGAFVSLLPGKDGLLHISQIRKLAGGKRVENVEDVLGVGQK 704
626 TGKVTRIVDPGAFVAIGGGKEGLVHISQI....ADKRVEKVTDYLQMGQE 671

705 VQVEIAEIDSRG..KLSLIPVIEGEEAASDEKKDDAEQ 740
672 VPVKVLEVDRQGRIRLSIKEATEQSQPAAAPEAPAAEQ 709
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FIG. 4. BESTFIT comparison of the amino acid sequences of *S. antibioticus* GPSI (top sequence) and *E. coli* PNPase (bottom sequence). The sequences show 44% identity. Vertical lines indicate sequence identity and colons and dots indicate sequence similarity.

scription of the ORF can occur. The effects of induction of T7 RNA polymerase in the relevant strains are shown in Fig. 7. As can be seen, BL21(DE3)pLysS, which contains no plasmid, shows only low levels of PNPase and GPSI activities prior to treatment with IPTG. The same observations were obtained for BL21(DE3)pLysS containing pJSE371 or pJSE372. Treatment of the cultures with IPTG to induce expression of the cloned ORF led to only a slight increase in the specific activities of the enzymes in BL21(DE3)pLysS and in the corresponding strain containing pJSE372 (Fig. 7). However, in the strain containing pJSE371, a nearly 10-fold increase in enzyme

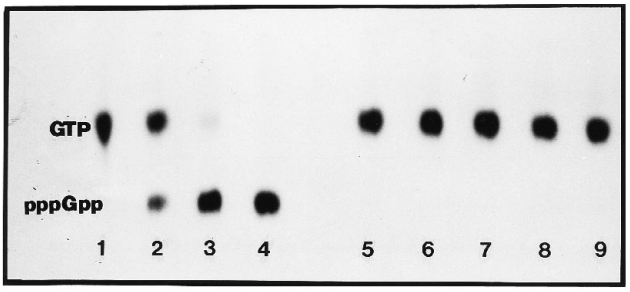


FIG. 5. GPS assays of GPSI and *E. coli* PNPase. GPS reactions were performed and analyzed as described previously (19) and in Materials and Methods. Lane 1, no enzyme added; lanes 2 to 4, reactions containing 0.95, 2.85, and 4.75 μg of purified GPSI, respectively; lanes 5 to 9, reactions containing 0.34, 0.68, 2.04, 3.4, and 6.8 μg of *E. coli* PNPase, respectively. The migration positions of GTP and pppGpp are indicated.

specific activity was observed for both GPSI and PNPase, compared with the strains containing no plasmid or pJSE372, 2 h after addition of IPTG. Thus, induction of expression of the cloned insert of pJSE371 led to a simultaneous increase in both GPSI and PNPase activities in the cloning host.

**Does GPSI use distinct catalytic domains for its pppGpp synthetase and PNPase activities?** The results presented in the preceding section support the intriguing possibility that GPSI possesses at least two catalytic domains, one for pppGpp synthesis and one for its PNPase activity. To test this possibility we took advantage of the observation that the activity of *E. coli* PNPase is inhibited by deoxynucleoside diphosphates (26, 33). Reaction mixtures were devised which contained ATP, GTP, and ADP and which were shown in preliminary assays to support both pppGpp formation and PNPase activity. The activities of GPSI and *E. coli* PNPase in the two reactions were measured in the presence of various concentrations of dCDP. Figure 8 shows that the dCDP strongly inhibited the polymerization activity of *E. coli* PNPase and of GPSI. In contrast, the GPS activity of GPSI was stimulated slightly by dCDP and no inhibition was observed even at the highest concentration tested.

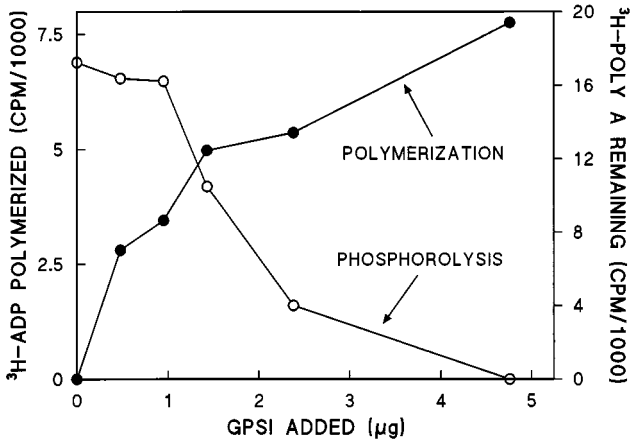


FIG. 6. Polymerization and phosphorolysis activities of GPSI. Reaction mixtures were prepared as described in Materials and Methods with [$^3$H]ADP (polymerization) and [$^3$H]poly(A) (phosphorolysis) as substrates and contained the indicated amounts of GPSI. Reaction mixtures were incubated for 30 min.

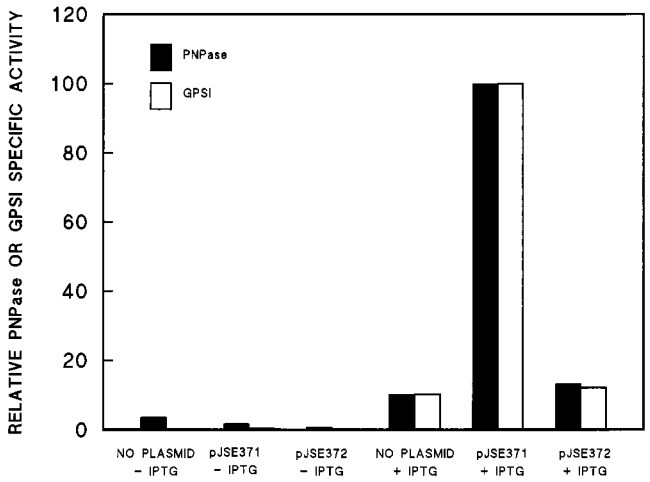


FIG. 7. GPSI and PNPase activities in *E. coli* BL21(DE3)pLysS containing or lacking pJSE371 or pJSE372. GPSI assays were performed with the polyethyleneimine cellulose thin layer assay, and PNPase assays were performed with [$^{14}$C]ADP (in a 15-min incubation) (see Materials and Methods for description). IPTG-induced cultures were harvested 2 h after IPTG treatment and cell extracts were assayed for the two enzyme activities. Results are expressed relative to the PNPase and GPSI values in the induced cultures containing pJSE372 (specific activities of 149,571 cpm/mg of protein for PNPase and 73.9 $\times$ 10$^6$ cpm/mg of protein for GPSI), set arbitrarily at a value of 100.

## DISCUSSION

The foregoing data argue that GPSI, purified from *S. antibioticus* as a pppGpp synthetase, is also a polynucleotide phosphorylase. GPSI is similar in size to *E. coli* PNPase, as measured by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) ($M_r$s of 88,000 and 86,000 for GPSI and *E. coli* PNPase, respectively) and as calculated by translation of the respective nucleotide sequences ($M_r$s of 79,000 and 77,000, respectively) (12, 32, and see above).

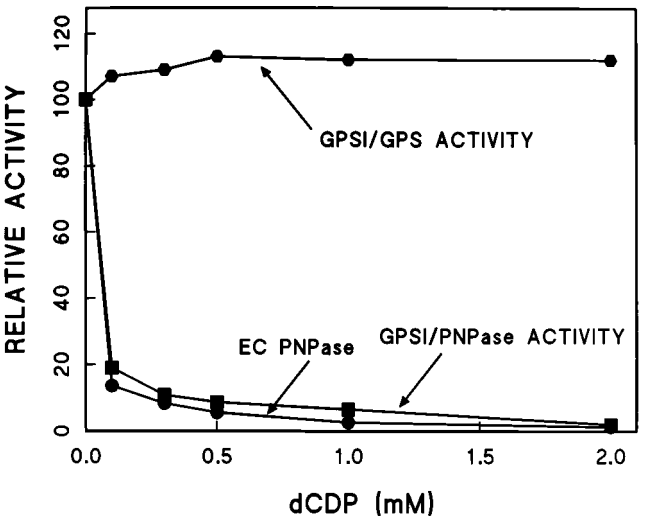


FIG. 8. Effects of dCDP on the activities of GPSI and PNPase. GPS and PNPase polymerization assays were performed as described in Materials and Methods in the presence of the indicated concentrations of dCDP with incubation for 30 min at 30 and 37°C, respectively. In the *E. coli* PNPase assay, 100% activity represents the incorporation of 3,335 cpm into poly(A), and for GPSI in the polymerization assay, 100% activity represents the incorporation of 3,589 cpm. For the GPS assay, 100% activity represents the incorporation of 196,000 cpm into pppGpp.

There is also a high level of sequence similarity between the two proteins (Fig. 4). The evidence supporting the conclusion that the GPSI preparations used in this study contained a single protein is as follows. First, in the final step of the GPSI purification procedure, anion exchange chromatography on QAE-Sepharose, the elution profile for pppGpp synthetase activity exactly matched the protein elution profile (19). Second, the purified protein showed a single band on one-dimensional SDS-PAGE and a single spot on two-dimensional gels (19 and unpublished results). Third, the one- and two-dimensional gel products give the same (and only a single) sequence when subjected to N-terminal amino acid sequencing. Fourth, the specific activity of GPSI in the methanol-dependent pppGpp synthetase assay is essentially identical to that of the RelA protein assayed under the same conditions (19, 30). If the GPSI preparation were contaminated, for example, with the RelA homolog at a level of 1%, the *S. antibioticus* RelA protein would have to be 100 times more active in the methanol-dependent assay than its *E. coli* counterpart. Finally, the fact that overexpression of the cloned *gpsI* gene in *E. coli* results in a simultaneous increase in both the GPSI and PNPase activities of the cloning hosts strongly supports the conclusion that the *gpsI* gene product possesses both enzymatic activities. Thus, the weight of the evidence favors the conclusion that GPSI is a bifunctional enzyme with both pppGpp synthetase and PNPase activities. While it is not proven that the PNPase and GPS functions of GPSI involve different catalytic sites, the facts that *E. coli* PNPase cannot be shown to catalyze pppGpp formation and that dCDP inhibits the PNPase but not the GPS activity of GPSI argue for the presence of separate catalytic domains on the latter enzyme.

The PNPase activity of GPSI may explain the fact that the enzyme does not convert GDP into ppGpp (19), unlike the RelA protein. GDP would presumably be converted instead into poly(G) by the PNPase activity of GPSI. The results presented in this report also raise questions related to the mechanism of pppGpp synthesis by GPSI. By analogy with the RelA protein, it has been suggested that GPSI catalyzes a pyrophosphoryl transfer from ATP to GTP to form pppGpp (19). Given the fact that GPSI functions as a polynucleotide phosphorylase, it seems possible that the formation of pppGpp occurs not by pyrophosphoryl transfer but by successive transfer of phosphate groups to GTP. It will be possible to examine this possibility using the purified enzyme and appropriate substrates and phosphoryl donors.

One of the interesting features shared by GPSI and PNPase is their susceptibility to digestion by endogenous proteases and by trypsin. Mild proteolysis of either protein with trypsin produces discrete products with $M_r$s in the range of 60,000 to 80,000 (12, 13, 20, 25). In the case of GPSI, it is clear that mild proteolysis activates the pppGpp synthetase activity (20), and it is also apparent that proteolysis of *E. coli* PNPase can be accomplished with the retention of enzyme activity, although the properties of the proteolyzed PNPase differ somewhat from those of the native enzyme (12, 25). Proteolysis of GPSI also produces a product with an $M_r$ of ca. 47,000 (20). Studies with antibody to GPSI strongly suggest that this degradation product is identical to the protein designated GPSII, which can also be purified from *S. antibioticus* and which has weak GPS activity (19). A protein of $M_r$ 48,000 is sometimes found in association with *E. coli* PNPase and has been referred to as the β subunit of PNPase, although it appears to have no effect on the activity of the enzyme (12, 25). Given the results just cited for GPSI, it is interesting to speculate that the PNPase β subunit is in fact a degradation product of the α subunit of the

enzyme ($M_r$ 86,000 measured by SDS-PAGE). This hypothesis can be tested in the same way as was done for GPSI. It is also noteworthy that SDS-PAGE analysis of GPSI and *E. coli* PNPase yields $M_r$ values that are larger than those calculated from the amino acid sequences of the proteins (19, 32, and see above). It has been suggested that the acidic nature of PNPase (pI = 5.0) is responsible for its aberrant electrophoretic mobility (32), and a similar explanation is possible for GPSI (pI = 4.9).

Why should a single enzyme possess both pppGpp synthetase and PNPase activities in *S. antibioticus*? It is well established that in *E. coli*, and presumably in other bacteria, polynucleotide phosphorylase functions as an exoribonuclease and plays a key role in the degradation of mRNA (6, 9, 31). Little information is available on the effects of ppGpp and the stringent response on mRNA metabolism. Recently, Sorensen et al. (34) presented evidence for the inhibition of mRNA synthesis by high levels of ppGpp. These authors argued that the decrease in mRNA levels would reduce the frequency of mistranslation in cells that are starved for particular amino acids. In a study of the effects of ppGpp on protein synthesis in vivo, Svitil et al. (36) found that *E. coli* cells with elevated levels of ppGpp contain a 65% lower level of translatable mRNA than do control cells. Taken together, these results suggest that decreased mRNA levels may be an important element of the stringent response. If so, it is possible that ppGpp affects not only the rate of mRNA synthesis but the rate of mRNA degradation as well. In this regard it is noteworthy that preliminary experiments in one of our laboratories (G.H.J.) indicate that induction of the stringent response in *E. coli* leads to an increased rate of mRNA degradation (unpublished results).

It is premature to construct models to explain the bifunctional character of GPSI, but several experimentally testable questions are raised by consideration of the properties of the enzyme. Both GPSI and PNPase bind RNA (12, 20, 25). In the case of GPSI, the binding of synthetic mRNA stimulates the pppGpp synthetase activity (20). Does mRNA binding to GPSI (perhaps as a prelude to mRNA degradation at the PNPase site) stimulate the production of (p)ppGpp in vivo? Conversely, does (p)ppGpp binding to GPSI stimulate mRNA degradation? Is there a single RNA binding site on GPSI, functioning in both the pppGpp synthetase and PNPase reactions, or are there two RNA binding sites on the enzyme? If the two active sites of GPSI do not function independently of each other, is there positive or negative cooperation between them? Ongoing studies with the cloned *gpsI* gene should provide answers to these questions and explanations for the dual functions of the protein.

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