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Cloning the *spoT* Gene of *Escherichia coli*: Identification of the *spoT* Gene Product

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We have isolated five specialized transducing lambda bacteriophages (λd*pyrE spoT*) carrying the *pyrE* and *spoT* genes of *Escherichia coli*. A fragment from one of these phages was used as the source of DNA to clone the *spoT* and *pyrE* genes on a multicopy plasmid, pBR322. Insertions and deletions in this plasmid were obtained. These plasmids were used to transform a minicell-producing strain, and the gene products synthesized were determined. Our experiments demonstrate that the *spoT* and *pyrE* genes are separated by about 4 megadaltons and suggest that the *spoT* gene product is a protein whose molecular weight is 80,000. The strain in which the *spoT*$^+$ allele is carried on a plasmid produced nine times more *spoT* gene activity than a normal *spoT*$^+$ strain when assayed in crude extracts. This strain was used to prepare partially purified gene product, guanosine 5′-diphosphate,3′-diphosphate pyrophosphatase. The enzyme has the following characteristics. (i) It hydrolyzes pyrophosphate from the 5′-pyrophosphate of guanosine 5′-diphosphate, 3′-diphosphate, yielding GDP and pyrophosphate. (ii) Its activity is strongly stimulated by Mn^{2+} and slightly stimulated by salt. (iii) Its activity is inhibited by uncharged tRNA. There are also two additional activities in the cell extract which degrade guanosine in 5′-diphosphate,3′-diphosphate in vitro but which are not specified by the *spoT* gene.

Guanosine 5′-diphosphate,3′-diphosphate (ppGpp) and guanosine 5′-triphosphate,3′-diphosphate (pppGpp) accumulate in *Escherichia coli* during amino acid starvation. They are generally regarded as pleiotropic regulatory molecules controlling several biosynthetic pathways (6). The synthesis of ppGpp is catalyzed by the enzyme ATP:GTP pyrophosphate transferase (stringent factor), the *relA* gene product (2, 5, 11, 14), which is activated by a ribosome-mRNA complex when uncharged tRNA occupies the aminoacyl-tRNA site (15). In addition to the *relA* gene, there are three genes known to affect the synthesis of these nucleotides. The first is *relB* which specifies a ribosome-associated protein (10, 21, 22, 28). The second is *relC*, which codes for ribosomal protein L11 (13, 32). The third is *relX*, which affects the basal level of ppGpp and the accumulation of this nucleotide upon carbon source starvation (31). At least two other genes are concerned with pppGpp and ppGpp metabolism. The first of these, *gpp*, governs the conversion of pppGpp to ppGpp (C. R. Somerville, personal communication). The second is *spoT*, which governs the conversion of ppGpp by hydrolysis of the 3′-pyrophosphoryl group and produces GDP plus pyrophosphate (12, 17, 20, 39; J. Justesen, Ph. D. thesis, University of Aarhus, Aarhus, Denmark, 1978).

In this paper, we concentrate on the *spoT* gene. We describe its cloning and the use of the cloned gene for partial purification and characterization of the gene product.

MATERIALS AND METHODS

Strains, plasmids, and culture conditions. Bacterial strains and plasmids used in this experiment are described in Table 1 and Table 2, respectively. Cells were grown in either L broth (23) or MOPS medium (29) supplemented with glucose (0.2%), required amino acids (50 μg/ml), uracil (20 μg/ml), and thiamine (10 μg/ml). All cell cultures were grown in a gyratory water bath (New Brunswick Scientific Corp.) at 33°C when working with lysogens, 42°C when pSC304, a temperature-sensitive, Tn*3*-carrying plasmid (similar to pSC204 [18]), was used, or 37°C otherwise. pBR322 (3) was given us by H. Boyer, and pSC304 was given by S. Cohen. Antibiotic concentrations were 50 μg of ampicillin (Ap) per ml and 20 μg of tetracycline (Tc) per ml.

Phage and plasmid DNA preparation. Bacteriophage growth and purification and phage DNA purification were carried out as described previously (27). Plasmids were amplified by growth of the culture in chloramphenicol (250 μg/ml [8]). Covalently closed circular (CCC) plasmid DNA was purified by CsCl-

TABLE 1. *Strains*[a]

Strain	Genotype	Reference/source
NF637	Δ(*gal-attλ-bio-uvrB*) *thi*	J. Schrenk (37)
NF929	*thr leu his argH pyrE thi*	Fiil et al. (12)
NF930	NF929 *spoT*	Fiil et al. (12)
AT2538	*thr leu his argE pro ara pyrE thi mtl xyl gal lacY str* λ *supE*	B. Bachmann
χ1488	F⁻ *minA purE supE pdxC minB his metC rpsL* T3ʳ *xyl ilv cycB cycA hsdR*	R. Curtiss III
JF650	AT2538 Nalʳ (spontaneous)	This study
JF447	JF650 (λcI857*S7*; λcI857*S7*d*pyrE spoT*447)	This study
JF450	JF650 (λcI857*S7*; λcI857*S7*d*pyrE spoT*450)	This study
JF451	JF650 (λcI857*S7*; λcI857*S7*d*pyrE spoT*451)	This study
JF452	JF650 (λcI857*S7*; λcI857*S7*d*pyrE spoT*452)	This study
JF454	JF650 (λcI857*S7*; λcI857*S7*d*pyrE spoT*454)	This study
JF1599	NF930 pGA1 (Tcʳ *pyrE spoT*)	This study
JF1600	NF930 pGA2 (Tcʳ *pyrE*)	This study
JF1601	NF930 pGA3 (Tcʳ *spoT*)	This study

[a] *E. coli* genes carried on plasmids or specialized transducing phages are the wild-type alleles.

TABLE 2. *Plasmids*[a]

Plasmid	Relevant genotype	Reference/source
pBR322	Tcʳ Apʳ	3
pSC304	Tn*3* temperature sensitive	Similar to pSC204 (18), except nonpermissive for replication at 42°C
pGA1	Tcʳ *pyrE spoT*	7.3-MDal *Pst*I fragment from λd*spoT pyrE*454 inserted in *Pst*I site of pBR322
pGA2	Tcʳ *pyrE*	Ligation of 5.2-MDal *Eco*RI fragment of pGA1
pGA3	Tcʳ *spoT*	Ligation of 4.7-MDal *Bam*HI fragments from pGA1 partially digested with *Bam*HI
pGA5	Apʳ	4.2-MDal *Bam*HI fragment from pGA1 inserted in *Bam*HI site of pBR322
pGA8	Tcʳ Apʳ *spoT*::Tn*3*	Transposition of Tn*3* from pSC304 to pGA3
pGA10	Apʳ Δ*spoT*	1.5-MDal *Eco*RI-*Sal*I fragment from pGA1 inserted in *Eco*RI-*Sal*I site of pBR322
pGA11	Tcʳ *pyrE* Δ*spoT*	Ligation of 8.05-MDal *Sal*I fragment from pGA1
pGA13	Tcʳ Apʳ *pyrE spoT*::Tn*3*	Transposition of Tn*3* from pSC304 to pGA1
pGA19	Tcʳ *pyrE* Δ*spoT*	Spontaneous deletion of pGA1
pGA20	Tcʳ *pyrE* Δ*spoT*	Spontaneous deletion of pGA1
pGA21	Tcʳ *pyrE* Δ*spoT*	Spontaneous deletion of pGA13 by excision of Tn*3*

[a] *E. coli* genes carried on plasmids are the wild-type alleles except where a deletion (Δ) or an insertion (In3) is indicated.

ethidium bromide density gradient ultracentrifugation of cleared lysates (9). For rapid determination and characterization of restriction enzyme digestion patterns, a small amount of DNA was purified from 5-ml cultures. DNA was extracted from the cleared lysate with phenol, washed with chloroform, and then precipitated by the addition of two volumes of ethanol. After precipitation at −20°C overnight, DNA was pelleted by centrifugation and suspended in 50 μl of water.

Restriction endonuclease digestion, electrophoresis, ligation, and DNA transformation. Conditions for restriction endonuclease digestion and DNA ligation by T4 ligase were those suggested by the manufacturer (New England Biolabs). Electrophoresis of DNA was carried out in 0.7% agarose gel on a vertical slab gel apparatus in electrophoresis buffer containing 0.04 M Tris·OAc (pH 8.3), 0.02 M Na·OAc, and 1 mM Na_2 EDTA at 40 V (38). Transformation of $CaCl_2$-treated *E. coli* cells was based on the method of Mandel and Higa (26).

Determination of nucleotide pools. Cells grown exponentially in low-phosphate (0.2 mM) MOPS medium with requirements and [^{32}P]orthophosphoric acid (100 μCi/ml) were starved of isoleucine by the addition of L-valine (500 μg/ml). Nucleotide pool measurements and determination of decay rates were carried out as described earlier (5, 7, 12).

Preparation and radioactive labeling of minicells. Minicells were prepared by a modification of the method of Roozen et al. (35). Minicell-producing bacteria (χ1488) were grown in 200 ml of L broth to an absorbance at 600 nm of approximately 1.0. The cells were collected by centrifugation at 10,000 × *g* for 5 min. The pellet was vigorously suspended in 40 ml of minicell buffer (50 mM Tris-hydrochloride, pH 7.5, 0.1 M NaCl, and 1 mM EDTA). This cell suspension was centrifuged at 3,000 × *g* for 1 min, and the supernatant

was centrifuged again as described above. These short centrifugations remove most of the normal-sized cells, leaving only about 1 cell per 1,000 minicells. The final supernatant from these centrifugations was centrifuged at 10,000 × *g* for 5 min, and the pellet was suspended in 1 ml of minicell buffer. The suspension was layered on top of a 26-ml, 5 to 30% sucrose gradient in minicell buffer and centrifuged at 4,000 × *g* for 10 min in a swinging-bucket rotor (IEC, PR-6000). The minicell band which formed one third of the distance from the top was collected, centrifuged at 10,000 × *g* for 5 min, and suspended in minicell buffer containing 25% glycerol. The minicells, stored at −70°C, were stable for at least 3 months without obvious loss of activity. They contained less than 1 cell per 10^6 minicells. For radioactive labeling, 10^9 minicells were incubated at 37°C for 20 min with either (i) 5 μCi of [^{35}S]methionine (760 Ci/mmol; Amersham/Searle) in 100 μl of MOPS containing 0.2% glucose, adenine and uracil, each at 20 μg/ml, thiamine at 10 μg/ml, pyridoxine at 0.8 μg/ml, and at 50 μg/ml, 19 amino acids lacking methionine; or (ii) 5 μCi of ^{14}C-amino acid mixture (50 mCi/mg-atom of carbon; Amersham/Searle) in 100 μl of the same medium without amino acids. The radioactive minicells were pelleted and lysed either (i) by boiling for 5 min in sodium dodecyl sulfate (SDS) sample buffer (125 mM Tris-hydrochloride, pH 6.8, 3% SDS, 10% glycerol, and 5% 2-mercaptoethanol) for SDS protein gel analysis, or (ii) freezing and thawing several times in water for two-dimensional protein gel analysis.

Assay condition for the ppGpp-degrading activity. Standard assays were carried out in 15 μl of reaction mixture containing 10 μl of protein sample and 5 μl of assay buffer (4 mM Tris·OAc, pH 7.8, 3 mM $MnCl_2$, 6.4 mM Mg·$(OAC)_2$, 24 mM K·OAc, 0.4 mM dithiothreitol, bovine serum albumin at 0.2 mg/ml, 0.2 mM ppGpp, and radioactive ppGp^{32}p). After incubation at 37°C for 1 h, 10-μl portions were applied to polyethyleneimine-cellulose thin-layer plates (Brinkmann) and developed with 1.5 M potassium phosphate, pH 3.4 (7). The spots of ppGpp and its degradation products, localized by overnight exposure on X-ray films, were cut out and counted in a liquid scintillation spectrometer.

Purification of ppGpp-ase. Cells were grown in L broth with pH maintained at 7.0 and were harvested at an absorbance at 600 nm of 4 to 5. A 5-g amount of cells was suspended in 10 ml of buffer A [10 mM Tris·OAc, pH 7.8, 14 mM Mg·$(OAc)_2$, 60 mM K·OAc, 0.5 mM EDTA, and 1 mM dithiothreitol] and disrupted by passing through a French press. The crude cell lysate was centrifuged at 30,000 × *g* for 30 min. This supernatant fraction is referred to as crude extract. To the crude extract 0.106 g of ammonium sulfate per ml (Schwarz-Mann) was added to make a 20% final concentration. After 1 h of precipitation with stirring, the mixture was centrifuged at 10,000 × *g* for 10 min. Ammonium sulfate (0.113 g/ml; final concentration, 40%) was added to the supernatant, followed by precipitation for 1 h and centrifugation as described above. The precipitate was suspended in 25 ml of buffer A and centrifuged at 100,000 × *g* for 2 h. We have previously observed that most of the activity precipitated between 20 and 40% saturation with ammonium sulfate (Justesen, Ph. D. thesis, 1978). The supernatant (S100) was applied to a Bio-Gel P-300 column (2.5 by 45 cm) equilibrated with a buffer (20 mM Tris-hydrochloride, pH 8.0, 300 mM NaCl, 5 mM $MgCl_2$, and 0.2 mM dithiothreitol). The column was washed at a rate of 5 ml/h with one volume of the same buffer.

ppGpp-ase was further purified by DEAE-cellulose column chromatography. The fractions of ppGpp-ase activity from the Bio-Gel P-300 column were pooled and applied to a 40-ml DEAE-cellulose (Whatman DE52) column equilibrated with 20 mM Tris-hydrochloride (pH 8.0) containing 20 mM NaCl and 2 mM dithiothreitol. The column was washed with 100 ml of equilibration buffer, and then eluted with a linear 400 ml, 0 to 400 mM NaCl gradient in the same buffer.

Other methods. Bacteriophage P1 transduction (27), SDS-polyacrylamide gel electrophoresis (19), two-dimensional gel electrophoresis (30), and preparation of ppGpp^{32}p (Justesen, Ph.D. thesis, 1978) performed as described. Protein concentration was measured by the method which involved the binding of Coomassie brilliant blue G-250 to protein as described by Bradford (4).

Plasmids were cured as described by Miller (27). A fresh overnight culture was diluted 5 × 10^6-fold in L broth (pH 7.6) containing acridine orange (75 μg/ml). After overnight incubation in the dark, suitable dilutions of the culture were spread on L broth agar. When the colonies appeared, they were replica plated on L broth agar supplemented with tetracycline (20 μg/ml). Drug-sensitive colonies were picked and purified for further analysis.

RESULTS

Isolation of λd*spoT* phages. λ-transducing phages carrying the *spoT* gene were isolated by the method of Schrenk and Weisberg (37). Since *pyrE* is 70% cotransducible with *spoT* (20), isolation of λd*pyrE* specialized transducing phage was used as the first step in obtaining phages carrying the *spoT* gene. λd*pyrE*-transducing phages were isolated from heat-induced λcI857S7 lysogens of strain NF637. This strain is Δ*att*λ, and therefore all λ prophages are at secondary attachment sites. Presumably within the population of lysogens, prophages are present at many different secondary attachment sites on the host chromosome. The lysate prepared from this culture was used to infect strain JF650 *pyrE*. Fifteen independent lysogens yielding λd*pyrE* specialized transducing phages were found; phage from five of these were also capable of transducing the *spoT* gene.

The five strains, JF447, JF450, JF451, JF452, and JF454, lysogenic for λd*pyrE spoT* were heat induced, and the phage were purified by CsCl gradient centrifugation. Phage from the two bands of each gradient were used to transduce strain NF930 *pyrE spoT* to distinguish transducing phage from helper. It was found that the lower band from strains JF447, JF450, and JF452, and the upper band from JF454 consisted

of the transducing phage. Strain JF451 yielded only one band, a mixture of transducing and helper phages.

DNA prepared from each of the purified transducing phages was digested with several restriction endonucleases. Figure 1 shows the maps derived from these digestions.

Cloning the *spoT* and *pyrE* genes on pBR322. λd*pyrE spoT*454 was used for cloning both *spoT* and *pyrE* genes on pBR322, since it is the smallest of the five λd*pyrE spoT* phages. Plasmid pBR322 DNA and the phage DNA were digested with *Pst*I endonuclease and joined by T4 DNA ligase. The ligated DNA was used to transform strain NF930 *pyrE spoT1*, selecting for tetracycline resistance (Tc^r). One clone, strain JF1599, was found to harbor a recombinant plasmid carrying the *pyrE*$^+$ allele.

A series of experiments was carried out to compare the metabolism of ppGpp and pppGpp in the parental *spoT*$^+$ strain and the strain carrying the *spoT*$^+$ allele on the plasmid (JF1599). As can be seen in Fig. 2A and 3, the classical *spoT*$^+$ strain, NF929, accumulated both pppGpp and ppGpp during isoleucine starvation. The amount of these nucleotides decreased quickly to basal level after the addition of isoleucine to relieve the starvation; the half-life of ppGpp decay in this strain is 0.34 min. Fig. 2B and 3 show that the parameters of guanosine nucleotide metabolism in strain JF1599, with the *spoT*$^+$ allele on the multicopy plasmid pGA1, are very similar to strain NF929, showing that the *spoT*$^+$ allele is carried by pGA1. When pGA1 was cured from JF1599, the cured strain regained the *spoT1* phenotype (unpublished data). Thus, we conclude that the *spoT*$^+$ allele in JF1599 resides on the plasmid. This was confirmed by transforming strain NF930 *spoT* with CCC DNA derived from strain JF1599; the transformants were phenotypically *spoT*$^+$. These results prove that plasmid pGA1 carries the *spoT*$^+$ allele.

Figure 4 shows the restriction endonuclease digestion patterns for plasmid pGA1, several of its derivatives, and λd*pyrE spoT*454 from which it was derived. Restriction maps based on these data are shown in Fig. 5.

In vitro construction of derivatives of pGA1. To establish the position of the *pyrE* and *spoT* genes, several derivatives of pGA1 were constructed in vitro. Plasmid pGA2 was constructed as follows. CCC DNA of pGA1 was

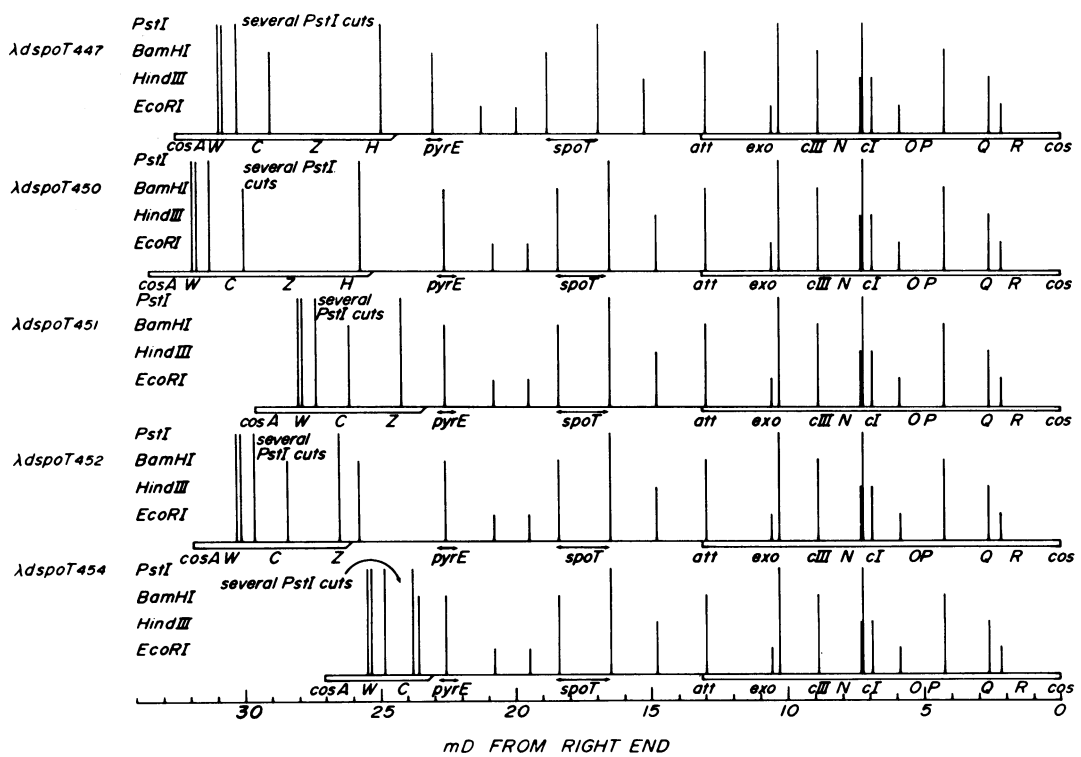

FIG. 1. *Restriction endonuclease maps of λdpyrE spoT. Maps were constructed by analyzing restriction enzyme fragments on 0.7% agarose gels. The positions of the pyrE and spoT genes were deduced from the plasmids whose structure is shown in Fig. 6. The double horizontal line represents phage DNA. mD, megadaltons.*

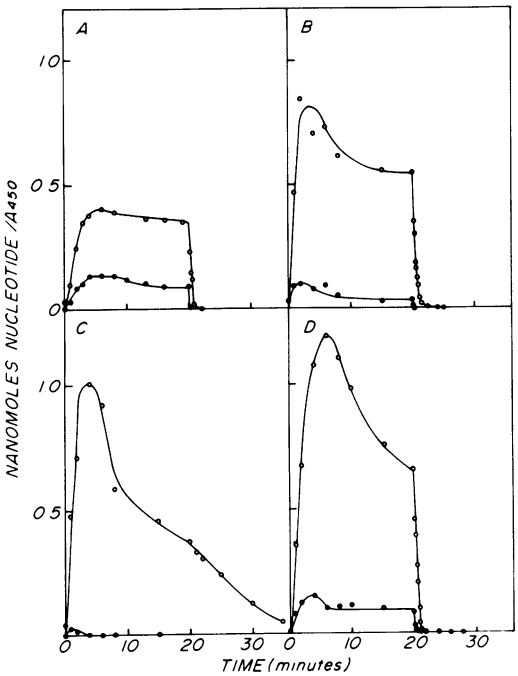

FIG. 2. *Accumulation of ppGpp and pppGpp. At zero time, L-valine (500 μg/ml) was added to each culture. At 20 min, L-isoleucine (500 μg/ml) was added to relieve the starvation. (A) Strain NF929, spoT⁺; (B) strain JF1599 (pGA1 spoT⁺); (C) strain JF1600 (pGA2 ΔspoT); (D) strain JF1601 (pGA3 spoT⁺). Symbols: ○, ppGpp; ●, pppGpp.*

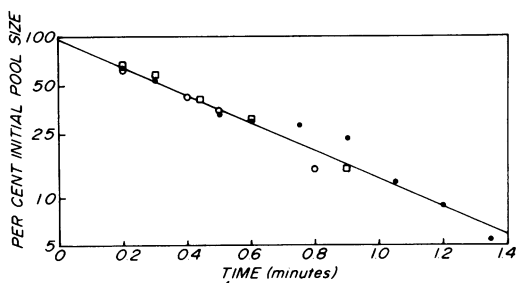

FIG. 3. *Decay of ppGpp pool after reversal of starvation. Symbols: □, strain NF929; ●, strain JF1599; ○, strain JF1601.*

digested in vitro with *Eco*RI, ligated, and then used to transform strain NF930, selecting for tetracycline resistance. CCC DNA was recovered from a culture grown from one of the transformed colonies. When analyzed by restriction endonuclease digestion and agarose gel electrophoresis, this plasmid (pGA2) was shown to have retained only the 5.2-megadalton (Mdal) *Eco*RI fragment of the parental pGA1. This is demonstrated by the restriction endonuclease digestion pattern of Fig. 4, lane 8 and is diagrammed in Fig. 5. The appropriate tests showed that pGA2 carried a functional *pyr*E gene, but not the *spoT* gene.

Plasmid pGA3 was constructed in a similar way, except that it employs partial digestion of pGA1 with *Bam*HI, followed by ligation and transformation of strain NF930. The structure of pGA3 is indicated in Fig. 4, lane 9 and in Fig. 5. This plasmid contained only the *spoT* gene. The evidence for this is shown in Fig. 2, where it can be seen that strain JF1601, carrying pGA3 (Fig. 2D), accumulated ppGpp upon amino acid starvation, whereas strain JF1600, carrying pGA2 (Fig. 2C) did not. The decay rate of ppGpp in strain JF1601 (pGA3) is similar to the *spoT*⁺ strains (Fig. 3).

As a confirmation of the structure of these plasmids pGA5, a third derivative of pGA1 was constructed. In this case, the 4.2-Mdal *Bam*HI fragment of pGA1 was cloned in the *Bam*HI site of pBR322. pGA5 (Fig. 5) was shown by the appropriate tests to contain neither the *pyrE* nor the *spoT* genes.

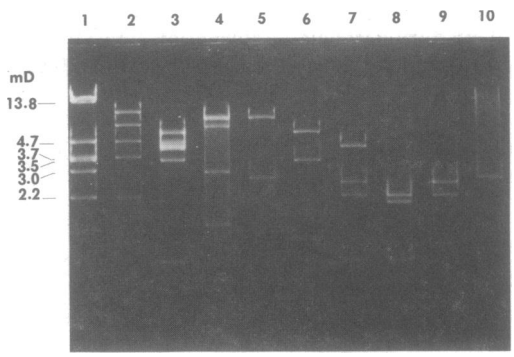

FIG. 4. *Restriction endonuclease digestion patterns of phages and plasmids. Molecular weight standards were EcoRI-generated fragments of bacteriophage λ. Lanes: (1) λcI857S7 digested with EcoRI; (2) λdpyrE spoT454 digested with EcoRI showing six fragments (8.9, 6.3, 4.7, 3.7, 2.2, and 1.3 Mdal [mD]); (3) λdpyrE spoT454 digested with BamHI showing seven fragments (5.4, 4.6, 4.3, 4.2, 4.1, 3.5, and 1.0 mDal). Three bands (4.3, 4.2, and 4.1 Mdal) are not clearly separated; (4) λdpyrE spoT454 digested with PstI. Four bands (7.3, 6.2, 3.0, and 1.6 Mdal) bigger than 0.7 Mdal are clearly shown, whereas several additional bands smaller than 0.7 Mdal are not clear. The band at 7.3 Mdal is composed of two fragments; (5) pGA1 digested with PstI showing the plasmid constructed from pBR322 (2.6 Mdal, lower band) and 7.3 Mdal PstI fragment of λdpyrE spoT454 (upper band); (6) pGA1 digested with EcoRI showing three fragments (5.2, 3.4, and 1.3 Mdal); (7) pGA1 digested with BamHI showing four fragments (4.2, 2.5, 2.2, and 1.0 Mdal); (8) pGA2 digested with BamHI showing three fragments (2.2, 2.0, and 1.0 Mdal); (9) pGA3 digested with BamHI showing two fragments (2.5 and 2.2 Mdal); (10) pBR322 digested with BamHI showing one fragment (2.6 Mdal).*

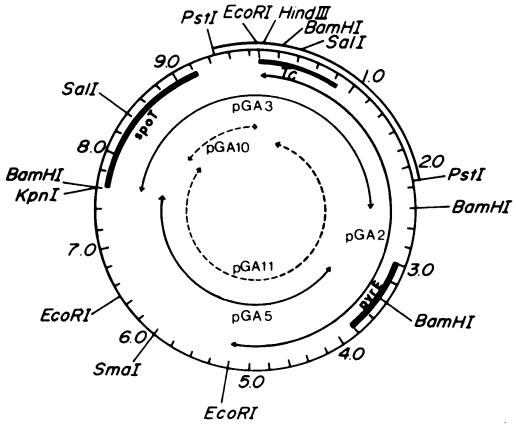

FIG. 5. *Restriction endonuclease maps of pGA1 and derivatives constructed in vivo. Plasmids pGA2, pGA3, and pGA5 are indicated by the solid double-pointed arrows. pGA10 and pGA11 are indicated by broken double-pointed arrows. The double lines on pGA1 indicate pBR322. Each division on the map indicates 0.1 Mdal. Gene positions are indicated by the black bars.*

Two additional plasmids were constructed in vitro from pGA1. First, to construct pGA11 (Fig. 5), pGA1 was treated with *Sal*I, which cleaves it in two fragments of 8.05 Mdal and 1.85 Mdal. The 8.05-Mdal fragment, carrying the origin of plasmid replication and the *pyrE* gene, was ligated and used to transform strain NF930. A determination of the accumulation of pppGpp upon amino acid starvation of the strain carrying pGA11 showed that the *spoT* allele was not expressed from this plasmid. Second, to construct pGA10, plasmid pGA1 DNA was subjected to a double digestion with *Eco*RI and *Sal*I. DNA fragments were ligated to plasmid pBR322, previously digested with *Eco*RI and *Sal*I. Transformants carrying this plasmid (pGA10; Fig. 5) did not express the *spoT*$^+$ gene.

A comparison of the structure of these five derivative plasmids indicates that the position of the *spoT* and *pyrE* genes is as shown in Fig. 5.

Tn*3* insertion in the *spoT* gene. The position of the *spoT* gene inferred from the restriction endonuclease analysis presented above was verified by the selection of Tn*3* insertions by the method of Kretschmer and Cohen (18). Recall that strain JF1599 harbors plasmid pGA1, which carries Tc^r. This strain was transformed with pSC304, which carries the Tn*3* transposon (Ap^r [18]). Transformed clones, which were resistant to both Ap and Tc, were grown overnight at 30°C in L broth liquid medium containing ampicillin and tetracycline. This culture was diluted 100-fold in fresh L broth containing the same drugs and was incubated overnight at 42°C. CCC DNA from single-colony isolates of this culture was prepared and analyzed. Because the replication of pSC304 is temperature sensitive, any clones growing at 42°C in the presence of both drugs were expected to contain plasmids which were recombinants of pGA1 and pSC304 (16, 18). However, in the initial attempt it was found that most of the colonies carried both parental plasmids. To increase the probability of obtaining pGA1::Tn*3* plasmids, all the colonies from the transformation described above were pooled; CCC DNA was isolated after culture growth at 42°C and plasmid amplification. This pooled plasmid DNA was used to transform strain NF930; clones resistant to both Ap and Tc were selected at 42°C. Several of these clones were found to be *spoT* but *pyrE*$^+$, indicating a Tn*3* insertion in the *spoT* gene. Tn*3* has a single *Bam*HI site 0.9 Mdal from one end of the 3.2-Mdal Tn*3* sequence, and three *Pst*I sites (36, 40). Restriction endonuclease digestion with *Bam*HI and *Pst*I showed an additional *Bam*HI site and three new *Pst*I sites in four isolates of pGA1:: Tn*3*. The insertion in all four occurred at or near the same site. This class of plasmids is called pGA13 (Fig. 6).

Plasmid pGA8 was isolated after transposition of Tn*3* from pSC304 to pGA3, in the same manner as described above. It was found that the insertion occurred at or near the same position as in pGA13, but in the opposite orientation (Fig. 6). Since the polar effect of Tn*3* on distal gene(s) depends on its orientation (36), inactivation of the *spoT* gene by the Tn*3* insertion might be due to such an effect, rather than direct insertion in the gene. However, since the two independent insertions are in opposite orienta-

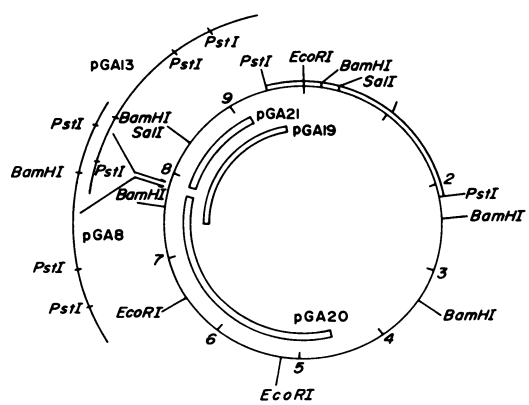

FIG. 6. *Restriction endonuclease maps of insertion and deletion plasmids. The double lines on pGA1 indicate pBR322. Insertion positions are shown by arrows. Deletions of pGA1 are indicated by double lines in the inner circles.*

tion, at least one insertion probably occurs in the *spoT* gene.

Deletion plasmids. The evidence from the in vitro constructions and Tn*3* insertions presented in the previous sections strongly suggests that the *spoT* gene occupies the position indicated in Fig. 5. As a final confirmation of this three independent, spontaneous deletion plasmids were obtained in vivo. Two of these, pGA19 and pGA20, were derived from pGA1 by screening *spoT* derivatives of strain 1599, and the third, pGA21, was derived from pGA13 by screening for Ap sensitivity (i.e., loss of Tn*3*). The endonuclease digestion patterns of these plasmids (Fig. 6) showed three different deletions: (i) 2.2 Mdal, which removes the 1.85-Mdal *Bam*HI *Pst*I fragment in pGA19; (ii) 3.4 Mdal, which removes the 1.1-Mdal *Eco*RI-*Bam*HI, and 1.3-Mdal *Eco*RI fragments in pGA20; and (iii) 1.2 Mdal, which removes the *Sal*I site in pGA21. All these plasmids were *pyrE*$^+$ but did not express the *spoT*$^+$ allele.

Identification of *spoT* gene product. It has been shown that the expression of genes carried by plasmids can often be detected in minicells (24, 35). *E. coli* minicells carrying various plasmids were purified and radioactively labeled with [^{35}S]methionine. The minicells were lysed and subjected to electrophoresis on a 12.5% SDS-polyacrylamide gel (Fig. 7). Recall that only pGA1 and pGA3 are phenotypically *spoT*$^+$ (see above). Therefore, the protein band(s) which appear in minicells containing these two plasmids, but which are absent in the minicells carrying all other plasmids, should represent the *spoT* gene product. Lane 3 of Fig. 7 shows that there are at least seven extra proteins synthesized in minicells carrying pGA1 in addition to the background of proteins synthesized in the minicell itself (Fig. 7, lane 1) and in minicells containing pBR322 (Fig. 7, lane 2). Since the *spoT*$^+$ allele is not expressed from pGA2 or pGA5, proteins B, C, D, and F (Fig. 7, lanes 4 and 6) can be ruled out as possible *spoT* gene products. Of the remaining bands, E and G can be ruled out because they are synthesized by minicells carrying pGA13 (Fig. 7, lane 9), which has an insertion in the *spoT* gene. Protein A is not synthesized in minicells carrying any of the insertion or in vivo deletion plasmid mutants. Therefore we conclude that protein A (Mr, 80,000 daltons) is likely to be the *spoT* gene product, assuming that all proteins encoded by the plasmids are synthesized in minicells. This conclusion is strengthened by data to be presented below.

Since there is a possibility that the *spoT* gene product does not contain methionine, plasmid-containing minicells were labeled with ^{14}C-amino acid mixture. An SDS protein gel of these cell extracts showed that no additional protein which was larger than 10×10^3 could be detected (unpublished data). As a confirmation of the above results, portions of the samples labeled with [^{35}S]methionine were analyzed by the two-dimensional gel system of O'Farrell (30). These showed that only protein A was expressed by both pGA1 and pGA3 but not by the other plasmids (unpublished data).

Purification of ppGpp-ase. We attempted to purify the *spoT* gene product by making use of its possible increased expression in strain JF1599, bearing pGA1. Figure 8 shows ppGpp-degrading activity in crude extracts of various strains. The *spoT*$^+$ strain, NF929, showed an activity of 0.92 U of protein per mg (one unit is defined as conversion of 1 nmol of ppGpp per min at 37°C); this is about five times higher than that of the *spoT* strain, NF930. The crude extract of the lysogen of λd*pyr*E *spoT*447, strain JF447, had an activity of 2.6 U/mg, and that of the strain JF1599, containing pGA1, showed 8.6

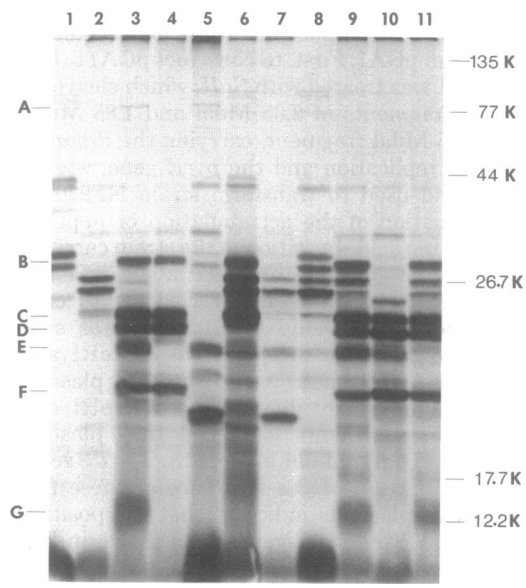

FIG. 7. *Polypeptides synthesized by plasmids in purified minicells. [^{35}S]methionine-labeled proteins were separated by 12.5% SDS-polyacrylamide gel at 70 V. Lanes: (1) minicells only; (2) pBR322; (3) pGA1; (4) pGA2; (5) pGA3; (6) pGA5; (7) pGA8; (8) pGA10; (9) pGA13; (10) pGA20; (11) pGA21. The peptides A to G are specified by genes on the bacterial portion of pGA1. The protein markers used are RNA polymerase subunit β (135,000), elongation factor Tu (44,000), ribosomal protein L1 (26,700), ribosomal protein L10 (17,700), and ribosomal protein L12 (12,200) synthesized in UV-killed host cells by λdrif^{d}18 (34), and stringent factor (77,000) purified as described earlier (33).*

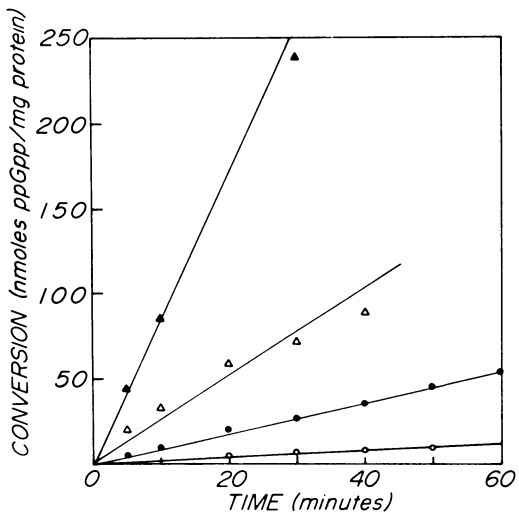

FIG. 8. *In vitro ppGpp-degrading activity in various strains. The crude extracts were incubated with radioactive ppGpp in assay buffer. At intervals, samples were withdrawn, and the fraction of ppGpp converted to GDP, P_i, and PP_i was determined as described in the text. Symbols: ○, strain NF930 spoT; ●, strain NF929 spoT$^+$; △, strain JF447 λdpyrE spoT; ▲, strain JF1599 spoT$^+$ on pGA1.*

U/mg. The values for strain JF1599 are 9- and 45-fold higher, respectively, than for the *spoT*$^+$ and *spoT* strains. Thus, the strain carrying the *spoT*$^+$ allele cloned on the plasmid ought to be very useful for purifying the gene product.

A crude extract of strain JF1599 was prepared and fractionated through Bio-Gel P-300 as described in Materials and Methods. The elution profile from the Bio-Gel P-300 column is shown in Fig. 9A, where three peaks of ppGpp-degrading activity can be seen. Incubation of pp$G^{32}p$ with fractions of peak I produced mainly radioactive pyrophosphate. There was almost no detectable pyrophosphatase activity in this peak. Thus, the activity in peak I most likely degrades pp$G^{32}p$ to GDP plus $^{32}PP_i$.

Peak II contained an activity which degraded pp$G^{32}p$ to $^{32}P_i$. There are two possible pathways for obtaining $^{32}P_i$ from pp$G^{32}p$: (i) Cleavage of ^{32}Pi from the 3′-pyrophosphate of pp$G^{32}p$ to produce ppGp plus $^{32}P_i$; or (ii) Cleavage of $^{32}PP_i$ from the 3′-pyrophosphate of pp$G^{32}p$ (peak I activity) followed by pyrophosphatase due to peak II activity. The radioactive product of pp$G^{32}p$ produced by peak III activity was neither P_i nor PP_i as identified on PEI-cellulose plates developed in 1.5 M potassium phosphate (pH 3.4). The product migrated between GTP and ATP and might be p$G^{32}p$.

Extracts from strain NF929, JF1600 (pGA2), and JF1601 (pGA3) were fractionated through the Bio-Gel P-300 steps. These showed that the activities of peaks II and III differed by no more than twofold. However, the activities of peak I in the strains NF929, JF1600, and JF1601 extracts were 7- to 16-fold lower than that of the strain JF1599 (Table 3), suggesting that only peak I contains genuine *spoT* gene product activity (pppGpp-ase). If the activity of peaks II and III of the Bio-Gel P-300 column is not due to the *spoT* gene product, there probably are other enzymes which degrade ppGpp via different pathways, although we cannot say whether these are active in vivo.

Figure 10 shows that the amount of the 80,000 molecular weight protein in each fraction from the Bio-Gel P-300 column correlates well with

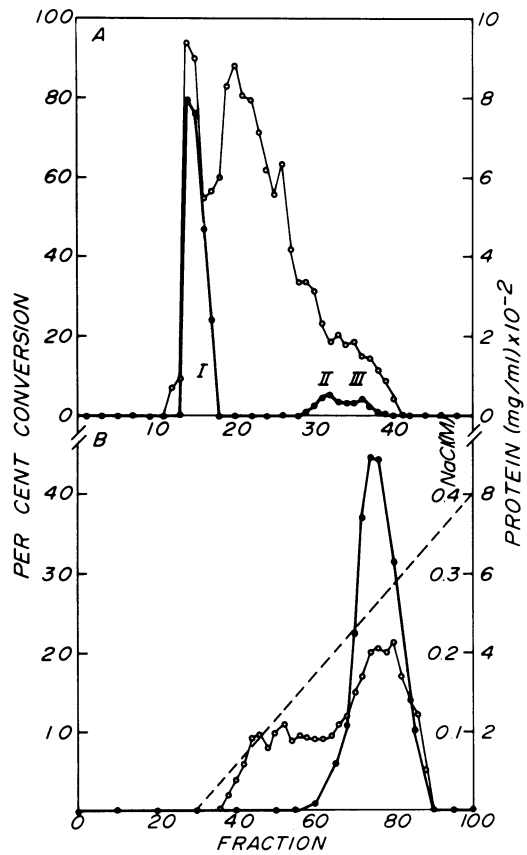

FIG. 9. *Purification of ppGpp-ase. (A) Gel filtration of S100 extract of strain JF1599. Three activity peaks were detected in the eluate of the Bio-Gel P-300 column as described in the text. Protein concentration and ppGpp degrading activity shown in the figure were obtained from tenfold-diluted samples. (B) Chromatography of peak I from A on DEAE-cellulose eluted with 200 ml of 0 to 400 mM linear gradient. Symbols: ○, protein concentration; ●, ppGpp-degrading activity; broken line, NaCl concentration.*

TABLE 3. *Comparison of ppGpp-degrading activity*[a]

Strain	Sp act (U/mg)		
	Peak I	Peak II	Peak III
NF929	3.8	15.6	12.5
JF1599	61.7	19.8	12.9
JF1600	7.0	36.9	13.5
JF1601	9.3	31.9	12.6

[a] S100 extract from each strain was prepared as described in the text and purified through a Bio-Gel P-300 column. The extracts from each strain contained three activity peaks. The most active fraction in each peak was used to compare the activity in various strains.

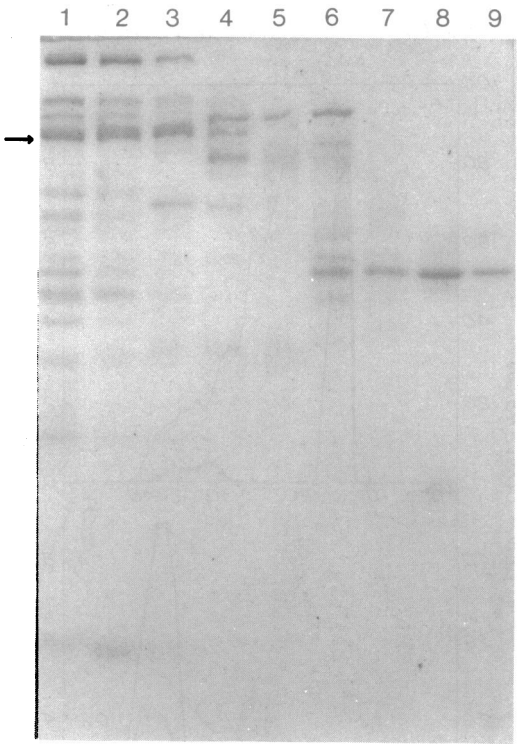

FIG. 10. *SDS-polyacrylamide gel of fractions of the Bio-Gel P-300 column from Fig. 9A. The position of radioactive ppGpp-ase determined in Fig. 7 is indicated by the arrow. Lanes: (1) fraction 14; (2) fraction 15; (3) fraction 16; (4) fraction 18; (5) fraction 20; (6) fraction 22; (7) fraction 24; (8) fraction 26; (9) fraction 28.*

in vitro ppGpp-ase activity. This result substantiates the suggestion that the 80,000 molecular weight protein, which was identified as the *spoT* gene product (Fig. 7), corresponds to ppGpp-ase.

To further purify ppGpp-ase, fractions of peak I from the P-300 column were pooled and chromatographed in DEAE-cellulose column as described in Materials and Methods (Fig. 9B). The peak of ppGpp-ase activity was eluted at 0.25 M NaCl. Incubation of ppGp^{32}p with fractions of this peak produced only $^{32}PP_i$, demonstrating that the fractions were free of pyrophosphatase activity. However, the specific activity was decreased by this step, probably due to loss of total activity. A summary of the purification is presented in Table 4.

Some properties of the partially purified ppGpp-ase were determined, using fraction 14 of the Bio-Gel P-300 column. (i) The enzyme activity was strongly stimulated by addition of Mn^{2+} as reported previously (17, 39). At the optimum concentration (0.8 mM), the activity was stimulated tenfold. (ii) Although maximum activity was observed with 150 mM NaCl, salt concentration was not an important factor. At optimum salt concentration, the activity was stimulated only about 10%. (iii) Uncharged tRNA was inhibitory. The activity was inhibited at least twofold by the addition of either $tRNA^{total}$ or $tRNA^{phe}$ at 2.5 mg/ml. This effect is the reverse of what is observed with the ppGpp-synthesizing enzyme, ATP:GTP pyrophosphate transferase (stringent factor), which is stimulated by uncharged tRNA (15). (iv) RNase had no effect on the activity; therefore, the enzyme does not require free RNA for activity. (v) The K_m value of ppGpp-ase was estimated at 0.55 mM, a value higher than that of most enzymes.

DISCUSSION

In this work we have studied the *spoT* gene by cloning it on λ phage and then on a multicopy plasmid. By constructing and selecting various deletion and insertion plasmids, we were able to localize the position of the *spoT* and *pyrE* genes. The *spoT* structural gene was found to be in a 1.85-Mdal *Bam*HI-*Pst*I fragment which is 4.2 Mdal from the position of the *pyrE* gene. Since the size of the *pyrE* gene product is about 20,000 daltons (unpublished data), the size of the gene is about 0.4 Mdal. Therefore we infer that the distance between the two genes is about 4 Mdal.

A strain carrying the *spoT* gene cloned on a plasmid showed about tenfold greater ppGpp-

TABLE 4. *Purification of ppGpp-ase*

Stage	Protein (mg/ml)	Sp act (U/mg)	Purification (fold)
Crude extract	42	8.6	1
S100	22	37.0	4.3
Bio-Gel P-300 column fraction 14	0.94	61.8	7.2
DEAE-cellulose column fraction 73	0.040	17.8	2.1

ase activity in vitro than a normal *spoT*$^+$ strain. However, the in vivo decay rate of ppGpp in cells with this plasmid was not increased, even though the cells contain at least a tenfold higher amount of the gene product. Therefore ppGpp-ase is not limiting in the wild-type (*spoT*$^+$) cell.

In vitro assay of the fractions separated by P-300 gel filtration and DEAE-cellulose chromatography showed that the *spoT* gene codes for ppGpp-ase, which cleaves pyrophosphate from the 3'-pyrophosphate of ppGpp. This scheme was proposed by Heinemeyer and Richter (17) and Sy (39). However, these workers were unable to detect pyrophosphate as a product of this reaction. The crude extract purified by them might have contained pyrophosphatase which degrades the pyrophosphate to inorganic phosphate. In addition to ppGpp-ase activity, there are two other activities which also degrade ppGpp. The first might be a 3'-phosphatase which degrades ppGpp to guanosine 5'-pyrophosphate, 3'-phosphate (ppGp). The second activity might be a 5'-phosphatase which cleaves inorganic phosphate from ppGpp to produce guanosine 5'-phosphate, 3'-diphosphate (pGpp). Both ppGp and pGpp may be unstable and could be further degraded. The existence of ppGp (C. C. Pao and J. Gallant, personal communication) in *E. coli* cells may be due to this activity.

We have estimated that ppGpp-ase is a protein of molecular weight 80,000. This protein was not expressed by any of the insertion or deletion mutants in the *spoT* gene. The amount of 80,000-dalton protein in each fraction of the Bio-Gel P-300 column correlates well with in vitro ppGpp-ase activity. This result supports the suggestion that the *spoT* gene product is the 80,000 molecular weight protein.

To obtain some indication whether the *spoT* gene is essential in *E. coli*, we have tried by P1 transduction to cross the deletion of the *spoT*, which is carried on a plasmid, to the chromosome. In this cross the plasmid (pGA13) carried *pyrE*$^+$ Δ*spoT* and the chromosome carried *pyrE* *spoT*$^+$.

We obtained several hundred *pyrE*$^+$ recombinants, but none was *spoT* (unpublished data). This might indicate that the *spoT* gene is essential.

Both *spoT* and *pyrE* gene products are pyrophosphatases (25). The *dut* gene (1), which is within 0.5 Mdal of the *pyrE* gene on the opposite side from the *spoT* gene (unpublished data), also codes for a pyrophosphatase. It is interesting to note that the genes for these three pyrophosphatase activities are closely linked.

As observed earlier (17, 39; Justesen, Ph.D. thesis, 1978) the *spoT* gene product is not a ribosomal protein and is found mainly in the cytoplasm. Furthermore, our results show that ppGpp-ase activity is not affected by RNase but is inhibited by tRNA. Therefore, it can be concluded that ppGpp is degraded in the cytoplasm by a mechanism which is quite different from that for pppGpp synthesis.

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