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Regulation of Membrane Phospholipid Synthesis by the *relA* Gene: Dependence on ppGpp Levels†

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ABSTRACT: A series of experiments using a pair of isogenic *rel*$^+$ strains of *Escherichia coli* differing only in the *spoT* locus has demonstrated a quantitative correlation between the inhibition of phospholipid synthesis and the intracellular level of ppGpp. The conditions examined were (1) amino acid starvation; (2) release from amino acid starvation; and (3) balanced growth. We also have been shown the presence of a third gene (in addition to *relA* and *spoT*) concerned with ppGpp metabolism and have found that the level of ppGpp during amino acid starvation is unaffected by an increase in the dosage of the *relA* gene.

The synthesis of membrane phospholipids as well as the synthesis of stable RNA species is regulated by the *relA* locus of *Escherichia coli* (Sokawa et al., 1968; Merlie and Pizer, 1973; Polakis et al., 1973; Nunn and Cronan, 1974, 1976; Nunn et al., 1975). Amino acid starvation of stringent (*rel*$^+$) strains results in a two- to four-fold decrease in the rate of phospholipid synthesis, whereas relaxed (*relA*$^-$) strains synthesize phospholipids normally during amino acid starvation.

In vivo, control has been shown to be exerted at the levels of both fatty acid synthesis and phospholipid synthesis (Nunn and Cronan, 1974, 1976). The molecular mechanism causing the decreased rate of lipid synthesis is thought to involve the inhibition of fatty acid and phospholipid biosynthetic enzymes by guanosine 3′,5′-bis(diphosphate) (ppGpp).

Cashel and Gallant (1969) showed that this unusual nucleotide accumulates during amino acid starvation of *rel*$^+$ (but not *relA*) strains (see review by Cashel, 1975). The case for the involvement of these nucleotides in *relA* gene control of lipid synthesis is based on both in vivo and in vitro experiments. Merlie and Pizer (1973) showed an inverse relationship between the rate of phospholipid synthesis and the presence of ppGpp during the onset and release of stringency. However, these data were not sufficiently detailed to allow a quantitative comparison between the ppGpp concentration and the rate of phospholipid synthesis. The in vitro experiments showed that ppGpp inhibits certain lipid biosynthetic enzymes (Merlie and Pizer, 1973; Polakis et al., 1973; Lueking and Goldfine, 1975; Ray and Cronan, 1975).

Although these experiments strongly suggest a role for ppGpp in the inhibition of lipid synthesis, the interpretation of these data is not completely straightforward. First, the enzymatic experiments are comprised by the knowledge that such enzyme inhibition data can be misleading. Enzymes are known in both the phospholipid (Merlie and Pizer, 1973) and nucleotide (Erlich et al., 1975) synthetic pathways which can be inhibited by ppGpp but which do not appear to play a role in stringent control.

The in vivo experiments of Merlie and Pizer (1973) do not greatly strengthen the enzymatic studies. These authors fell short of demonstrating a quantitative relationship between the level of ppGpp and the rate of phospholipid synthesis and thus the present case for involvement of ppGpp in the stringent control of lipid synthesis is not compelling.

Our previous experiments (Nunn and Cronan, 1974, 1976) were designed to clarify interpretation of the enzyme inhibition data. The present paper demonstrates a quantitative relationship between the intracellular levels of ppGpp and the rate of membrane phospholipid synthesis. In this work we used *spoT*$^-$ strains of *E. coli,* which are deficient in the turnover of ppGpp (Laffler and Gallant, 1974, 1975; Stamminger and Lazzarini, 1974). We also have examined the effects of dosage of the *relA* gene on ppGpp content, RNA synthesis, and phospholipid synthesis during the stringent response.

Experimental Procedures

Bacterial Strains. The relevant phenotypes of the *E. coli* K12 strains are given in Table I. Several of these strains are constructed in the course of this work. The genetic procedures we used for strain construction have been described previously (Nunn and Cronan, 1974; Cronan, Silbert and Wulff, 1972).

Strains WY3 and WY4 are *relA*$^+$ strains derived from strain NF161 which are isogenic excepting the *spoT* locus. Strain NF161 was converted to *str*^{R} and then converted to a *met*$^+$, *pyrE*$^-$ strain via conjugation with strain WN18. This recombinant strain was then transduced to *pyrE*$^+$ with P1 phage grown on strain WN30 (*spoT*$^+$). Strain WY4 is a strain which acquired the *spoT*$^+$ allele whereas strain WY3 is a *pyrE*$^+$ recombinant which remained *spoT*$^-$.

Strains WN30 and WN31 are a similarly constructed set of isogenic *spoT* strains derived from strain AT2538 by transduction to *pyrE*$^+$ with phage grown on NF161. Strain WN33 is an *arg*$^+$ (by transduction) *thyA*$^-$ (by trimethoprim

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TABLE I: Bacterial Strains.

Strain	*relA*	*spoT*	Other Relevant Markers[a]	Source
NF161	+	−	*metB,argA*	N. Fiil strain
NF162	−	−	*metB,argA*	N. Fiil strain
WY3	+	+	*argA*	NF161; see text
WY4	+	−	*argA*	NF161; see text
WN30	+	+	*argE,his,proA,thr,leu*	AT2538; see text
WN31	+	−	*argE,his,proA,thr,leu*	AT2538; see text
WN32	+	+	*his,proA,thr,leu*	WN30; see text
WN33	+	+	*his,proA,thr,leu,thyA*	WN32; see text
WN34	+	+	*his,proA,thr,leu,argA*	WN33; see text
WN35	−	+	*his,proA,thr,leu,argA*	WN33; see text
WN36	+	+	*his,proA,thr,leu,nalA,recA,argA*	WN34; see text
WN37	−	+	*his,proA,thr,leu,nalA,recA,argA*	WN35; see text
WN38	−/+	+	F143*recA*/WN36	WN36; see text
WN39	−/−	+	F143*recA*/WN37	WN37; see text
WN40	+/+	+	F160/WN36	WN36; see text
WN41	+/−	+	F160/WN37	WN37; see text
WN18	−	−	*pyrE*	*met*$^+$ transductant of CS101-4U (Cronan and Bell, 1974)
KL163	−	−	*recA*$^-$,*nalA*$^-$,*thyA*$^-$	B. Low strain
CP78	+	+	*arg,his,thr,leu*	N. Fiil strain
CP79	−	+	*arg,his,thr,leu*	N. Fiil strain

[a] The Coli Genetic Stock Center, Yale University, has assigned the following complete genotypes to strains NF161 and AT2538: strain NF161; F$^-$, *metBl, argA52, spoT,* λ$^-$; strain AT2538; F$^-$, *argE3, his-4, thi-1, proA2, thr-1, pyrE60, leu-6, mtl-1, xyl-5, ara-14, galK2, lacY1, str-31,* λ$^-$, *supE44.*

selection) derivative of strain WN30. Strains WN34 and WN35 are *argA*$^-$ *spoT*$^+$ derivatives of WN33 which are isogenic excepting the *relA* locus. The *relA* lesion was introduced from strain NF162 as previously described (Nunn and Cronan, 1974). Strains WN36 and WN37 are *recAl* derivatives of strains WN34 and WN35 constructed by mating with strain KL163 for *nalA* recombinants which were then scored for inheritance of the *recA* phenotype by sensitivity to ultraviolet light (200 ergs/mm^2). The F′ factors, F143 *recA*$^-$ and F160, were introduced by selection of *argA*$^+$ recombinants. The presence of the F′ factors was also confirmed by mating with appropriate recipient strains. The inheritance of *relA*$^-$ and *spoT*$^-$ was scored by assay of RNA synthesis and of pppGpp formation, respectively (see below).

The Tris-buffered low phosphate (10^{-3} M) medium of Bell (1974) was the minimal medium used. It was supplemented with glucose (0.4%), thiamine (0.5 μg/ml), and 20 μg/ml of the required L-amino acids. Cultures were grown at 37 °C in gyrotory water bath shakers.

Phospholipid and RNA Synthesis. The incorporation of $^{32}PO_4$ and [^{3}H]uracil into phospholipid and RNA, respectively, was assayed as described by Nunn and Cronan (1974) and Nunn et al. (1975). The incorporation of [^{3}H]adenosine into RNA was assayed as described by Friesen and Fiil (1973).

Assay of ppGpp Concentrations. Cultures were grown on the appropriately supplemented low phosphate medium containing 200 μCi/ml $^{32}PO_4$ for at least one generation prior to the experiment. Samples (100 μl) of experimental cultures were added to 100 μl of cold 2 M formic acid which were then assayed on polyethyleniminecellulose thin-layer plates as described by Cashel et al. (1969).

The *spoT*$^-$ phenotype was identified by the absence of pppGpp on the chromatogram. In *spoT*$^+$ strains the ppGpp data reported also include the minor contribution of pppGpp.

Results

Phospholipid Synthesis, ppGpp Levels, and the Stringent Response. The *spoT*$^-$ mutation impairs the turnover of ppGpp (Laffler and Gallant, 1974; Stamminger and Lazzarini, 1974). Thus, when the stringent response is released (by termination of amino acid starvation), *spoT*$^-$ strains take 20–40 min to reduce ppGpp to its normal basal level, wheras *spoT*$^+$ strains require only about 1 min. This differential response allowed us to more definitively test the dependence of the rate of phospholipid synthesis on the intracellular ppGpp concentration. If the rate of membrane phospholipid synthesis is dependent on the intracellular concentration of ppGpp, then following the release of stringency, the normal rate of phospholipid synthesis should resume much more slowly in a *spoT*$^-$ strain than in a *spoT*$^+$ strain. However, a finding that synthesis resumed at similar times in both strains would be strong evidence that ppGpp is not the effector of the *relA* gene meditated control of phospholipid synthesis.

To perform this experiment, we first constructed a pair of isogenic (*spoT*$^+$ and *spoT*$^-$) strains from strain AT2538 (*spoT*$^+$, *rel*$^+$). In agreement with the results of others (Laffler and Gallant, 1974, 1975; Stamminger and Lazzarini, 1974), we found the *spoT*$^-$ strain grew much more slowly than its *spoT*$^+$ sibling (Table II). However, we also constructed a similar isogenic pair of strains from strain NF161. As shown in Table II, the *rel*$^+$ *spoT*$^-$ strain of this pair grew as well as the *relA*$^-$ *spoT*$^-$ strain. Due to these improved growth properties, these latter strains, WY3 and WY4, were used in most of our experiments.

We first examined the rates of phospholipid synthesis and ppGpp disappearance upon reversal of the stringent response. In the experiments presented in Figure 1, strains WY3 and WY4 were starved for isoleucine (by valine addition) until the stringent response was maximal. Starvation was then reversed by addition of isoleucine. As shown in Figure 1a, b, following isoleucine addition, the ppGpp content of strain WY4 (*spoT*$^+$) decreased with a half-life of about 20 s whereas the half-life of ppGpp disappearance in the *spoT*$^-$ strain, WY3, was about 10 min. In agreement with previous results (Laffler and Gallant, 1974, 1975; Stamminger and Lazzarini, 1974), the maximal level of ppGpp in the *spoT*$^-$ strain was several fold

TABLE II: Properties of Strains during Balanced Growth.[a]

Strain	*relA*	*spoT*	Doublings/ h	ppGpp + pppGpp nmol/10^9 cells	Rate of Net RNA Synthesis	Rate of Phospholipid Synthesis	μmol of Phospholipid/ mg of Protein
NF161	+	−	0.90	0.132	290	102	0.214
NF162	−	−	1.10	0.070	389	178	0.257
CP78	+	+	0.83	0.050	396	163	0.243
CP79	−	+	0.83	0.045	420	169	0.240
WY3	+	+	0.83	0.109		158	0.199
WY4	+	−	0.83	0.140		145	0.201
WN30	+	+	0.80	0.093		152	0.226
WN31	+	−	0.35	0.397		70	0.248

[a] The rates of RNA and phospholipid synthesis are cpm per 10^6 cells incorporated during a 5-min exposure to [^{3}H]uracil or $^{32}P_i$, respectively.

greater than in the *spoT*$^+$ strain. The data of Figure 1c,d show that phospholipid synthesis resumed much more slowly (20–25 min) in the *spoT*$^-$ strain (after amino acid restoration) than it did in the *spoT*$^+$ strain (2–4 min). In both cases, phospholipid synthesis resumed its normal rate only after the ppGpp content had declined to the normal basal level. Similar results were found when the rate of uracil incorporation into RNA was determined (Figure 2). Therefore, the rates of both phospholipid and net RNA synthesis appear to depend on the intracellular level of ppGpp.

It can also be seen in Figure 1c, d that the stringent response for phospholipid synthesis was two- to three-fold more severe in the *spoT*$^-$ strain than in the *spoT*$^+$ strain. This correlates well with the three-fold greater content of ppGpp found in the *spoT*$^-$ strain. It appears from the data in Figure 2 that the degree of stringency of net RNA synthesis may also be greater in the *spoT*$^-$ strain.

Phospholipid Synthesis and ppGpp Levels during Balanced Growth. In confirmation of the original results of Gallant et al. (1970), Sokawa et al. (1975) recently reported a close correlation between the rate of net RNA synthesis, the content of RNA, and the basal level of ppGpp under the conditions of balanced growth. The latter workers found that both the rate of RNA synthesis and the RNA content of strain NF161 (*relA*$^+$) were less than that of strain NF162 (*relA*$^-$). However, no such difference was observed between strains CP78 and CP79. Although the CP and NF strains are not closely related, Sokawa et al. (1975) attributed this difference to the fact that the NF strains are *spoT*$^-$ whereas the CP strains are *spoT*$^+$. It was suggested that the increased basal ppGpp level of the *relA*$^+$, *spoT*$^-$ strain inhibited RNA synthesis and thus resulted in the decreased RNA content of this strain (Sokawa et al., 1975).

As shown in Table II, we have confirmed the RNA synthetic data of Gallant et al. (1970) and Sokawa et al. (1975) and find a similar inverse correlation between the rate of phospholipid synthesis and ppGpp levels. The phospholipid–protein ratio of strain NF161 also is lower than in strains NF162, CP78, and CP79 (as expected from the relative synthetic rates).

We have also listed some results with isogenic sets of *relA*$^+$ *spoT* strains in Table II. Both *spoT*$^-$ strains have elevated ppGpp levels compared with their *spoT*$^+$ siblings. However, the ppGpp level in strain WN31 (which is descended from strain AT2538) is much higher than in strain WY4 (a derivative of strain NF161). This increased ppGpp content of strain WN31 correlates well with decreased rates of growth and phospholipid synthesis observed in the former strain. Strain WY4 grows and synthesizes phospholipid normally as expected from the low ppGpp content of this strain.

These results therefore suggest that a close correlation exists between the level of ppGpp and the rate of phospholipid synthesis during balanced growth.

Studies on Diploid relA Strains. We also attempted to increase the intracellular content of ppGpp without recourse to the *spoT*$^-$ mutation. The *relA* gene seems to code for stringent factor, a ribosome-bound enzymatic activity responsible for the formation of ppGpp (Haseltine et al., 1972; Block and Haseltine, 1973, 1975). It was reasoned that an increase in the dosage of the *relA*$^+$ gene might increase the amount of stringent factor in the cell and consequently result in an increase in the amount of ppGpp accumulated during the stringent response.

We therefore constructed strains of *E. coli* which possess two copies of the *relA*$^+$ gene (+/+), one of episomal location and the other located on the chromosome. This strain, WN40, was then compared with monoploid *relA*$^+$ and *relA*$^-$ strains and strains WN41 (+/−), WN38 (−/+), and WN39 (−/−). As shown in Table III, *relA*$^+$ is dominant over *relA*$^-$ in the accumulation of ppGpp and in its effect on phospholipid synthesis. In agreement with the results of Fiil (1969), we also find that *relA*$^+$ is dominant in its effect on RNA synthesis. However, WN40, the strain with two copies of the *relA*$^+$ gene was no more stringent in phospholipid or in RNA synthesis and accumulates no more ppGpp than strains possessing only a single functional *relA* gene.

It should be noted that the strains listed in Table III are *spoT*$^+$ strains. Similar results were obtained with a series of *spoT*$^-$ diploid strains descended from strains NF161 and NF162.

Discussion

The *spoT* lesion impairs the turnover of ppGpp, perhaps at the step of conversion to pppGpp (Laffler and Gallant, 1974; Stamminger and Lazzarini, 1974). We find that, upon release from stringency, the normal rate of phospholipid synthesis resumes immediately in a *spoT*$^+$ strain, whereas, in an otherwise isogenic *spoT*$^-$ strain, a prolonged delay is observed before the normal rate of synthesis is resumed (Figure 1). Furthermore, neither strain commences phospholipid synthesis at a normal rate until the intracellular ppGpp level has declined to < 0.3 nmol/10^9 cells. The rate of phospholipid synthesis also appears inversely proportional to the intracellular ppGpp levels during balanced growth (Table II). We have, thus, shown a very strong quantitative correlation between ppGpp concen-

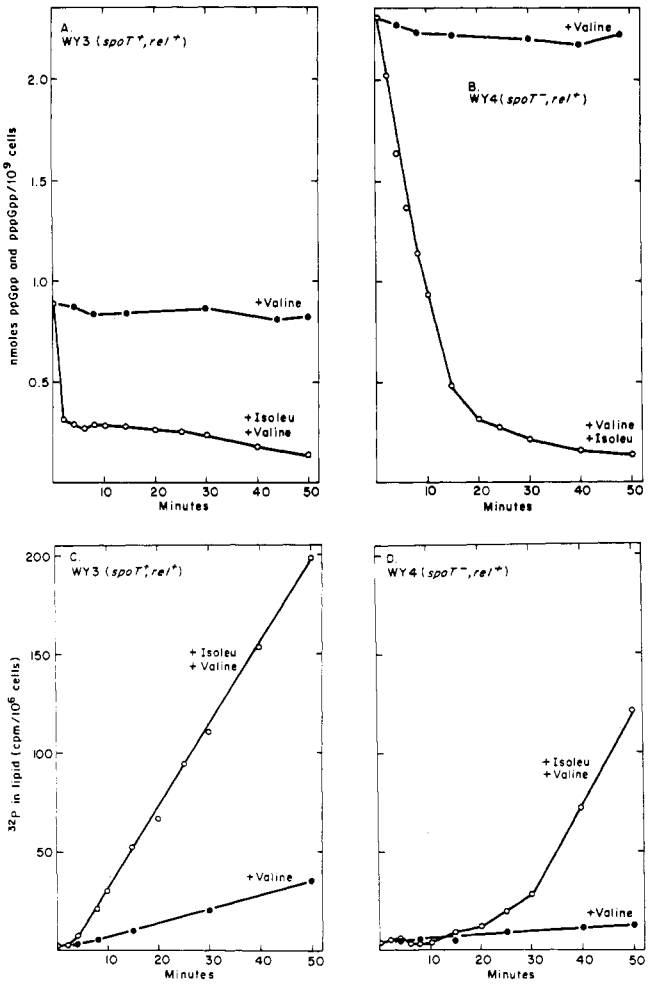

FIGURE 1: Resumption of lipid synthesis as a function of ppGpp levels. Exponentially growing cultures of (A) WY3 (*spoT*$^+$, *relA*$^+$) and (B) WY4 (*spoT*$^-$, *relA*$^+$) were divided into several portions. To measure ppGpp levels, $^{32}P_i$ (200 μCi per ml) was added to one portion of each culture for at least one doubling time before amino acid starvation. Upon reaching a cell density of 2.0–2.5 × 10^8 cells per ml, valine (500 μg per ml) was added to both the labeled and unlabeled portions of each culture. Fifteen minutes after valine-induced amino acid starvation, the labeled and unlabeled cultures were each divided into two equal portions. Isoleucine (100 μg per ml) was added to one portion of each of the labeled and unlabeled cultures (WY3 and WY4). The level of ppGpp in the labeled cultures was then measured as described by Cashel et al. (1969). Other portions of the original cultures were supplemented with 4 μCi per ml of $^{32}P_i$ (4 μCi per μmol) to measure phospholipid synthesis (C and D). At the indicated time intervals, 1.5-ml samples were removed from each culture and pipetted into chloroform–methanol (1:2), and the radioactivity in phospholipids was quantitated as described by Nunn and Cronan (1974). Cell density was monitored in a Klett colorimeter at 540 nm. Valine addition causes starvation for isoleucine in *E. coli* K12 strains (Ramakrishnan and Adelberg, 1965). The rates of phospholipid synthesis in cultures of WY3 and WY4 without valine were identical with the maximal rates of phospholipid synthesis observed following isoleucine addition. These data were omitted for clarity.

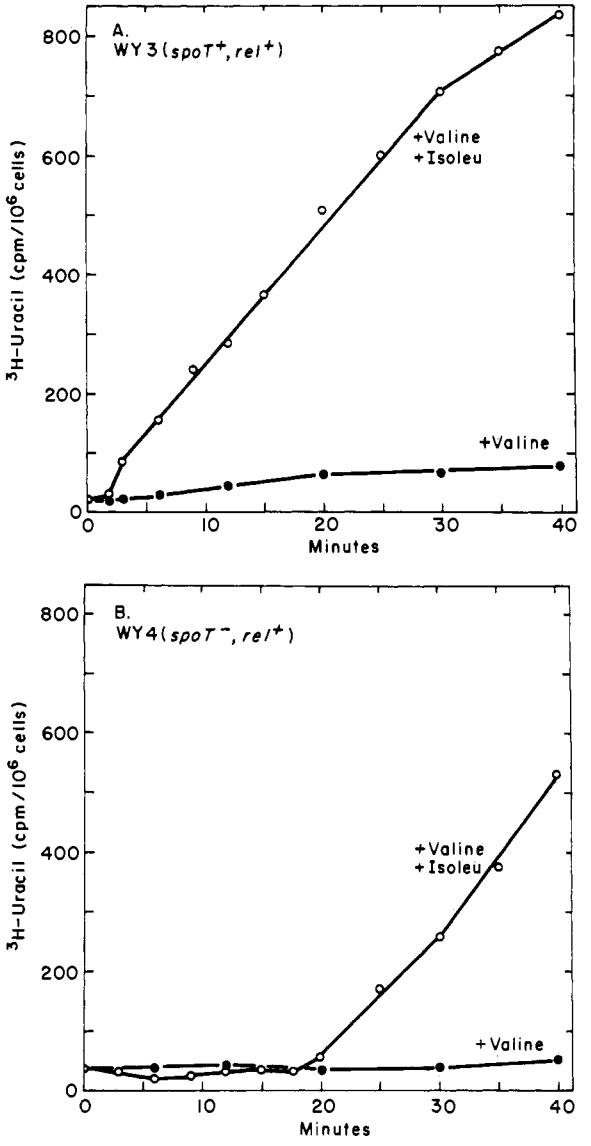

FIGURE 2: Resumption of RNA synthesis as a function of ppGpp levels. Cultures of WY3 (*spoT*$^+$, *relA*$^+$) and WY4 (*spoT*$^-$, *relA*$^+$) were treated as described in Figure 1, except that to assay for ribonucleic acid synthesis the cultures were supplemented with 1 μCi per ml [^{3}H]uracil (0.1 μCi per μg). Samples were removed at the indicated time intervals and assayed as described previously (Nunn et al., 1975).

tration and the rate of phospholipid synthesis. In fact from the data of Figure 1, a rough K_i of about 1 mM can be calculated for the inhibition of phospholipid synthesis in vivo by ppGpp. This figure is about 30% of the maximal intracellular ppGpp concentration found by us in an amino acid deprived *relA*$^+$ *spoT*$^-$ strain. These data, therefore, greatly strengthen the hypothesis that ppGpp is the effector of the stringent effect on phospholipid synthesis and make quite unlikely the hypothesis that stringency involves some as yet unrecognized regulatory mechanism which controls lipid synthesis.

Our finding that lipid synthesis is more stringent in a *spoT*$^-$ *relA*$^+$ strain than in an isogenic *spoT*$^+$ *relA*$^+$ strain (Figure 1) is noteworthy in view of the findings of Polakis et al. (1973) on ppGpp inhibition of acetyl-CoA carboxylase, the first step in fatty acid synthesis. These workers found that saturating levels of ppGpp (similar to the maximal levels observed in vivo) inhibited a homogeneous preparation of this enzyme by only 50–60%. Since, as shown in Figure 1, lipid synthesis is not refractory to the increased levels of ppGpp accumulated during the stringent response of a *spoT*$^-$ strain, acetyl-CoA carboxylase may not be a rate-limiting step in the stringent response.

The properties of *spoT*$^-$ *relA*$^+$ strains vary greatly depending on the genetic background of the strain. Strains derived from strain NF161 grow normally and have only slightly elevated ppGpp levels. In contrast, *spoT*$^-$ *relA*$^+$ strains derived from strain AT2538 grow very poorly and possess very high basal levels of ppGpp (Table II; Laffler and Gallant, 1974, 1975; Stamminger and Lazzarini, 1974). It, therefore, appears that strains NF161 and AT2538 differ in at least three genes

TABLE III: Stringency of Diploid Strains.[a]

Strain	Genotype	ppGpp + pppGpp nmol/10^9 cells	Percent of Control[b] Rate of Net RNA Synthesis[c]	Percent of Control[b] Rate of Phospholipid Synthesis[d]
WN36	*rel*$^+$	1.72	11.8	37
WN37	*relA*	0.07	104.0	98
WN38	*relA*/*rel*$^+$	1.49	14.0	34
WN40	*rel*$^+$/*rel*$^+$	1.58	15.5	40
WN39	*relA*/*relA*	0.09	96.0	94
WN41	*rel*$^+$/*relA*	1.28	13.8	42

[a] Upon reaching a cell density of 2×10^8 cells per ml, the cultures were divided into two equal portions. To one portion of each culture L-valine (500 μg per ml, final concentration) was added. The other portion of each culture did not receive valine. To assay for ppGpp concentration, 200 μCi per ml of [^{32}P]orthophosphate (200 μCi per μmol) was added to aliquots from each of the cultures (plus and minus valine). At various time intervals, samples (100 μl) of each of the labeled cultures were pipetted into tubes containing 100 μl of ice-cold 2 M formic acid. The ppGpp concentrations in these formic acid extracts were then determined as described by Cashel et al. (1969). To assay for ribonucleic acid synthesis, 1-ml aliquots were removed from each of the unlabeled cultures (plus and minus valine) and added to tubes containing 0.5 μCi of [^{3}H]-adenosine (134 μCi per μmol). After a 5-min incubation at 37 °C, 1.0 ml of 10% trichloroacetic acid was added. The samples were assayed for the amount of [^{3}H]adenosine in RNA as described by Friesen and Fiil (1973). In order to assay for phospholipid synthesis, 1-ml aliquots from each of the unlabeled cultures (plus and minus valine) were added to tubes containing 40 μCi of [^{32}P]orthophosphate (40 μCi per μmol). After a 5-min incubation, the samples were quenched by the addition of chloroform–methanol (1:2) and the radioactivity in phospholipids was quantitated as described by Nunn and Cronan (1974). [b] Data are presented for cells labeled 30 min after valine addition and are given as percent of the control minus valine. [c] The control cultures (minus valine) averaged about 375 cpm/10^6 cells. [d] The control cultures (minus valine) averaged about 600 cpm/10^6 cells.

concerned with ppGpp metabolism, *relA*, *spoT*, and a third character which regulates the basal levels of ppGpp during the balanced growth of *spoT*$^-$ strains. We are currently in the process of a genetic analysis of this third characteristic.

Laffler and Gallant (1974, 1975) have suggested a scenario to account for the maintenance of the *relA*$^-$ lesion in the 58–161 line of *E. coli* (of which strain NF161 is a member). They suggest this maintenance was due to the prior mutation to *spoT*$^-$ which occurred in this strain. They propose the *relA*$^-$ mutation would have been selected as a suppressor of the slow growth caused by the *spoT*$^-$ lesion in a *relA*$^+$ phenotype. However, our finding that a *spoT*$^+$ *relA*$^+$ strain derived from strain NF161 grows at the same rate as the isogenic *spoT*$^-$ strain suggests that another rationale for the appearance and maintenance of the original *relA*$^-$ lesion will have to be found.

Amino acid starved strains (either *spoT*$^+$ or *spoT*$^-$) which carry two copies of the *relA*$^+$ gene make the same amount of ppGpp as a monoploid *relA*$^+$ strain. Therefore, the amount of ppGpp accumulated is dependent on factors other than gene dosage. Although the assay of Haseltine et al. (1972) is not strictly quantitative, we found that ribosomes from a *relA*$^+$/*relA*$^+$ strain had no more stringent factor activity than ribosomes from the monoploid *relA*$^+$ strain (Nunn, unpublished data). Therefore, the amount of stringent factor may be controlled at the transcriptional or translational levels. Block and Haseltine (1975) have calculated that the ratio of ribosomes to molecules of stringent factor is about 500. It, therefore, does not seem probable that the amount of functional stringent factor is limited by the supply of ribosomes.

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