

An Unusual Correlation between ppGpp Pool Size and Rate of Ribosome Synthesis during Partial Pyrimidine Starvation of *Escherichia coli*

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Escherichia coli was exposed to partial pyrimidine starvation by feeding a *pyrBI* strain orotate as the only pyrimidine source. Subsequently, differential rates of synthesis of rRNA and of a few ribosome-associated proteins as well as the pool sizes of nucleoside triphosphates and ppGpp were measured. As the orotate concentration in the medium was reduced, the growth rate decreased and the pools of pyrimidine nucleotides, particularly UTP, declined. We did not observe the normal inverse relation between concentration of ppGpp and growth rate; rather, we observed that the ppGpp pool was low at slow growth rates. Upshifts in growth rate were made by adding uracil to a culture growing slowly on orotate. Downshifts could be provoked by adding aspartate plus glutamate to a culture growing at a high concentration of orotate. Following the upshift, both the rates of synthesis of the ribosomal components and the pool of ppGpp increased rapidly, while they all decreased after the downshift. These results are discussed in relation to the role of ppGpp in the growth rate control and the stringent response.

Enteric bacteria contain more ribosomes per unit of mass during rapid growth than during slow growth (43). Although the mechanism by which the growth rate regulation occurs remains unknown, it seems to be intimately connected with the stringent response and involves the pool of ppGpp (2, 42). This stringent response requires a functional *relA* gene (45) and affects the synthesis of all stable RNAs and several mRNAs. It is elicited during nutritional downshifts and is provoked both by the removal of amino acid supplements and by changes in the carbon energy supply (7, 21). Mutants defective in the stringent response, i.e., relaxed mutants (45), do not accumulate ppGpp during either downshifts or starvation for amino acids (6, 7). These observations, together with the finding of an inverse correlation between the rate of rRNA synthesis and the size of the pool of ppGpp under different growth conditions (2, 13, 41), led to the hypothesis that the intracellular ppGpp pool, which is determined by the number of ribosomes with no charged amino acid tRNA (15, 23, 33, 38), controls the level of ribosome synthesis (4).

While studying pyrimidine metabolism in *Escherichia coli*, we observed that both the ppGpp pool and the growth rate decline when cells are starved of pyrimidine (35). Given the above hypothesis, this suggested the untenable possibility that cells contain numerous ribosomes while lacking precursors for RNA synthesis, which is in fact in contrast to the previous finding that *E. coli* was shown to contain less RNA per unit of weight when grown under conditions of pyrimidine limitation (11). We have reinvestigated this phenomenon by measuring the rates of synthesis of rRNA and a few ribosome-associated proteins and the size of the ppGpp pool under different conditions of steady-state partial pyrimidine starvation and during shifts between these different growth conditions. We hoped that the results might contribute to the

discussion of whether ppGpp regulates (2, 14, 16) stable RNA synthesis in *E. coli* as a function of the growth rate.

MATERIALS AND METHODS

Bacterial strains and phages. NF321 is *E. coli* B AS19 *leu pyrB5 rel⁺* and was previously described (13). Strains CSH45 $\Delta lac thi trpR$ (λ cI857 S7) (29) and CP172 *thr leu thi lacY ilvC* (λ dilv5; λ b515 b519 nin5 cI857 S7) (22) were used to isolate the phages λ dilv5 containing the hybrid *rrnX* operon and λ cI857 S7 for hybridization purposes.

Preparation of hybridization filters. Lytic replication of the temperate phages was induced by a temperature shock, and phage particles were isolated and separated by isopycnic banding in CsCl gradients (29). The denatured DNA was isolated by extracting twice with phenol and then twice with chloroform. It was precipitated with ethanol, resuspended, and dialyzed against 0.03 M sodium citrate (pH 7)–0.3 M NaCl. The DNA was immobilized on 9-cm nitrocellulose filters (BA85; Schleicher and Schuell) which were cut into small circles (11-mm diameter) before use.

Bacterial growth. The cells were grown with shaking at 37°C in the Tris-buffered medium of Edlin and Maaløe (12) at a reduced phosphate concentration (0.3 mM). The medium was supplemented with a pyrimidine source, i.e., either uracil (20 μ g/ml) or orotate at the indicated concentration, leucine (20 μ g/ml), thiamine (1 μ g/ml), and glucose (1 mg/ml). Cell growth was monitored by measuring the optical density at 436 nm (OD₄₃₆) on an Eppendorf photometer.

Pools of nucleoside triphosphates and ppGpp. The cultures were labeled with ³²P (30 μ Ci/ml, 90 Ci/mol) for 1 to 2 generations before harvesting to ensure constant specific activity during the experiment. To extract the nucleotides, 200 μ l of culture was mixed with 40 μ l of 4 M HCOOH and left in an ice bath for 30 min. Cell debris was removed by centrifugation. Nucleotides were separated by two-dimensional chromatography on polyethyleneimine-impregnated

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cellulose thin-layer plates (19), and 1.2 M KH_2PO_4 (pH 3.4) was used as the solvent for chromatography in the first dimension instead of 0.85 M KH_2PO_4 (pH 3.4) in order to displace the ppGpp farther away from the application start point while still being able to separate UTP.

Measurement of the rates of RNA, DNA, and protein accumulation. (i) **Labeling.** The traditional labeling procedure using radioactive uracil could not be used to determine the rate of stable RNA accumulation because it perturbs the pyrimidine starvation. Instead, the cells were labeled at low specific activity with ^{32}P (ca. 30 mCi/mol) for about one-half a generation before starting the experiment and with [^3H]leucine (0.3 $\mu\text{Ci/ml}$) to measure protein synthesis.

(ii) **Measurements of precipitates.** To determine the accumulation of nucleic acids, 0.5-ml aliquots (designated sample A) of the culture were precipitated on an ice bath with 6.7% (wt/vol, final concentration) trichloroacetic acid (TCA) for 0.5 to 1.5 h. The precipitate was collected on a glass-fiber filter (Whatman GF/C), washed several times with 5% TCA and once with 96% ethanol, and dried before determining radioactivity by scintillation counting. Simultaneously with sample A other 0.5-ml aliquots (designated sample B) of the culture were mixed with NaOH (0.33 M, final concentration), incubated at 37°C for 2 h to hydrolyze the RNA, and subsequently precipitated with TCA (8%, final concentration) and treated as described for sample A. The radioactivity contained in sample B was taken to represent primarily the amount of DNA made during the labeling period. The difference between TCA-precipitable radioactivity before and after hydrolysis in NaOH, i.e., the difference in radioactivity between samples A and B, was taken to represent the amount of accumulated RNA.

(iii) **Measurements of hydrolysates.** In addition to the procedure described above, the RNA content was also sometimes determined by measuring the UV absorbance of the alkaline hydrolysates and by quantitation of the individual nucleotides after separation by ion-exchange chromatography on polyethyleneimine-impregnated cellulose thin-layer plates. Thus, 1.0-ml samples of the ^{32}P -labeled cultures were harvested by centrifugation at 4°C and resuspended in 1.0 ml of the basal salt medium. Then, 0.1 ml of 5 M perchloric acid was added to each sample; after 0.5 to 1.5 h of incubation in an ice bath, the precipitates were collected by centrifugation and washed, first in 1.0 ml of 0.5 M perchloric acid and subsequently in 1.0 ml of 80% ethanol. After being dried in a vacuum centrifuge, the pellets were suspended in 0.1 ml of 0.5 M KOH and incubated at 37°C overnight. In the morning, 20 μl of 5 M perchloric acid was added to each sample, and after 0.5 to 1.5 h of incubation in an ice bath, the precipitated potassium perchlorate as well as unhydrolyzed material was removed by centrifugation. The extracts were neutralized by addition of 75 μl of potassium hydroxide (0.5 M) to each 100- μl supernatant, and the precipitated salt was removed by centrifugation. The nucleotides in the extracts were quantitated by measuring the A_{260} and by application of 10- μl aliquots to polyethyleneimine-impregnated cellulose thin-layer plates to separate the nucleotides by chromatography in 0.5 M sodium formate–0.5 M formic acid (pH 3.4) (36). Their positions were determined by inspection of the chromatograms in UV light and by autoradiography. The spots were cut out, and the radioactivity was determined by liquid scintillation counting.

Measurements of the relative rate of synthesis of specific proteins. The relative rates of synthesis of specific proteins were measured as the ratio of the rate of [^{35}S]methionine incorporation in the specific proteins separated by two-

dimensional gel electrophoresis (32, 34) during a pulse to that in total protein in the same pulse. In order to correct for recovery during preparation, the culture samples were mixed with aliquots of a reference culture, labeled with [^3H]lysine for 30 min, and chased with unlabeled lysine for 2 min. The relative rate of synthesis of a protein (α) is given as the dimensionless ratio $(^{35}\text{S}_\alpha/^{35}\text{H}_\alpha)/(^{35}\text{S}_{\text{total}}/^{35}\text{H}_{\text{total}})$ (37). For steady-state measurements, the culture grown with uracil (20 $\mu\text{g/ml}$) served as a reference and was labeled with 20 μCi of [^3H]lysine (87 Ci/mmol) per ml. Samples (1 ml) of the experimental cultures in steady-state growth were labeled for 2 min with carrier-free [^{35}S]methionine (4 $\mu\text{Ci/ml}$, 1,130 Ci/mmol), chased for 2 min with unlabeled methionine, cooled in an ice bath, mixed with an equal portion of the reference culture, and prepared for two-dimensional gel electrophoresis and counting as previously described (34). The ratio of ^{35}S to ^3H in total protein was found by precipitating duplicate samples with TCA (6.7%) on glass-fiber filters. For the shift measurements, the preshift culture was labeled with [^3H]lysine (20 $\mu\text{Ci/ml}$) for 30 min and chased with unlabeled lysine for 2 min. Then 1-ml aliquots were taken at the indicated times and pulse-labeled for 30 s with carrier-free [^{35}S]methionine. The samples were then chased for 1 min with cold methionine, placed on an ice bath, and treated as described above.

Pulse-labeling of rRNA synthesis. The cells were grown in low-phosphate glucose minimal medium supplemented with adenine (5 $\mu\text{g/ml}$) and either 50 μg of orotate per ml or 20 μg of uracil per ml and prelabeled with 20 mCi of ^{32}P per mol for 2 generations. The cultures were then subjected to [^3H]adenine (final specific activity, 4,400 Ci/mol) pulses for the indicated times and harvested by pipetting into a 100°C warm sodium dodecyl sulfate (SDS)-lysis mixture (5) and kept at 100°C for 2 min. To determine the incorporation of [^3H]adenine in rRNA during the pulse period, the RNA was extracted and hybridized to λ DNA as previously described (8) except for the use of 0.3 M NaCl–0.025% SDS–0.03 M sodium citrate (pH 7) as the hybridization buffer. Each hybridization was carried out in triplicate. Each vial contained one nitrocellulose filter coated with λ *dilv5* (*rrnX*) DNA and another filter with λ *cI857 S7* (control DNA) and 5 to 50 μl of purified RNA. The difference between ^3H -radioactivity hybridizing to λ *ilv5* DNA and λ *cI857* DNA was taken to represent rRNA. The ratio of ^3H to ^{32}P in total purified RNA was determined by pipetting 10 μl of the purified RNA onto glass-fiber filters in duplicate. The filters were washed several times with 5% TCA and once with 96% ethanol, dried, and counted.

RESULTS

Choice of growth conditions. Preliminary experiments were performed to establish conditions under which a strain of *E. coli* would grow exponentially while exposed to different levels of partial pyrimidine starvation. Strain NF321 was selected with a mutation in the *pyrBI* operon, which encodes the aspartate transcarbamylase subunits and requires pyrimidines for growth (13). Although uracil is normally used to satisfy this nutritional requirement, it could not be used in these experiments, because reducing the uracil concentration lowers the growth yield but not the growth rate (3). Therefore, we selected orotate, which is an intermediate in the pyrimidine biosynthetic pathway and causes partial pyrimidine starvation when applied as an external pyrimidine source (11, 46, 48). As expected, NF321 grew exponentially but with increasing doubling time as the orotate con-

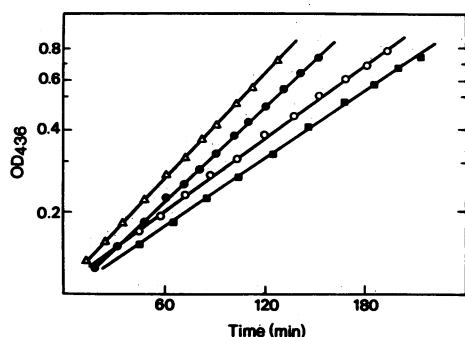


FIG. 1. Growth of NF321 on different pyrimidine sources: uracil at 20 µg/ml (Δ) or orotate at 200 (\bullet), 75 (\circ), or 50 (\blacksquare) µg/ml.

centration was reduced from 200 to 50 µg/ml (Fig. 1). Moreover, orotate became a very poor pyrimidine source when Casamino acids were added to the medium. By testing combinations of amino acids, we were able to show that aspartate plus glutamate or asparagine was responsible for this growth inhibition (data not shown), presumably because orotate is taken up by the transport system for these amino acids. Thus, it was possible to perform upshifts by adding uracil to a culture that was growing slowly on a low concentration of orotate and downshifts by adding aspartate and glutamate to a culture growing in the presence of a high concentration of orotate.

rRNA synthesis in pyrimidine-limited steady states. NF321 was grown exponentially in the presence of either uracil or different orotate concentrations (Fig. 1) and labeled with ^{32}P and ^3H leucine. Aliquots of the cultures were taken at different times in order to measure the rate of RNA, DNA, and protein accumulation per unit mass, as described in Materials and Methods. The results are listed together with the pool levels of nucleoside triphosphates and ppGpp in Table 1. They show that the RNA content increases with increasing growth rate, while the content of DNA remains constant and the total protein concentration decreases slightly. This is the expected pattern of macromolecular biosynthesis as a function of the growth rate of *E. coli* (4) and was also observed previously for pyrimidine starvation conditions (11). However, in contrast to earlier findings, the pool of ppGpp became larger when the growth rate increased in response to increasing orotate concentration. Thus, cells containing the most rRNA also contained the most ppGpp. Previous studies of the ppGpp pool, which all manipulated the carbon source or the amino acid supply (2, 4, 13), have shown an inverse relationship between the size of the ppGpp pool and the growth rate and rRNA levels. Our experiments establish that there is a positive correlation between the

TABLE 2. Relative rates of rRNA synthesis measured by pulse-labeling^a

Pyrimidine source (concn) (µg/ml)	Doubling time (min)	$\frac{(^3\text{H}/^{32}\text{P})_{\text{rRNA}}}{(^3\text{H}/^{32}\text{P})_{\text{total RNA}}}$ at:	
		1.5 min	30 min
Uracil (20)	43	0.24	0.51
Orotate (50)	79	0.16	0.51

^a Strain NF321 was grown in the minimal medium containing 0.3 mM inorganic phosphate supplemented with thiamine (1 µg/ml), leucine (20 µg/ml), glucose (1 mg/ml), adenine (5 µg/ml), and the pyrimidine source as indicated. The cultures were background labeled with ^{32}P (5 µCi/ml) for 2 generations and pulse-labeled (time = 0) with 200 µCi (i.e., 4,400 Ci/mol, final specific activity) of ^3H adenine per ml. (Adenine was included in the growth medium prior to the pulse, because addition of even very small concentrations of adenine dramatically reduces the pool of 5-phosphoribosyl-1-pyrophosphate [1]. This, in turn, disturbs the orotate phosphoribosyltransferase reaction [44] upon which our experiments are based. The inclusion of adenine made it difficult for us to make very short pulses [the 1.5-min pulses represented only 630 hybridized ^3H cpm in the presence of uracil and 270 hybridized ^3H cpm in the presence of orotate], but on the other hand, we have not perturbed the steady states.) The ratio of ^3H to ^{32}P was determined in the total RNA and in RNA hybridized to λ *rnnX* DNA. The observation that at 30 min only 51% of the RNA hybridized to the λ *rnnX* DNA indicates that tRNA and mRNA do not hybridize to the probe and that the hybridization reactions were not complete.

content of ribosomal components and the ppGpp pool under conditions of pyrimidine limitation. It was still possible, however, that the ppGpp pool controlled the rate of stable RNA synthesis in our experiments but that the hypothetical excess RNA was rapidly degraded. In order to test this possibility, the bacteria were pre-labeled with ^{32}P of a low specific activity while growing on either uracil (20 µg/ml) or orotate (50 µg/ml). Then pulses of ^3H adenine were given, and the RNA was extracted at different intervals. The ratio of ^3H to ^{32}P was determined for both total RNA and rRNA (defined as RNA hybridizing to the *rnnX* operon on λ DNA [10]). The results are presented in Table 2. The limiting value of the composite ratio $(^3\text{H}/^{32}\text{P})_{\text{rRNA}}/(^3\text{H}/^{32}\text{P})_{\text{total RNA}}$, at pulse times approaching time zero, is a measure of the fraction of transcribing RNA polymerases engaged in formation of rRNA chains, and as seen from Table 2, this fraction was lower in cells grown in orotate than in those grown in uracil. We conclude that ppGpp alone does not regulate the steady-state synthesis of rRNA during partial pyrimidine starvation.

Pyrimidine upshift. The growth rate increased shortly after uracil (20 µg/ml) was added to a culture of NF321 growing on orotate (50 µg/ml) (Fig. 2A). The pools of CTP and UTP increased abruptly, while the purine nucleotide pools fell in response to the relief from pyrimidine starvation (Fig. 2B). The intracellular concentration of ppGpp reached a new and

TABLE 1. Contents of RNA and protein in pyrimidine-limited steady states^a

Pyrimidine source ^b	Doubling time (min)	µg of RNA/OD ₄₃₆ U	µg of DNA/OD ₄₃₆ U	µg of protein/OD ₄₃₆ U	α EF-Tu	α S1	ppGpp	UTP	CTP	ATP	GTP
Ura 20	46	52	7.8	115	0.74	0.22	44	450	280	1,320	580
Oro 200	51	45	7.8	109	0.75	0.22	26	250	150	1,510	800
Oro 75	65	40	7.2	109	0.66	0.13	23	110	150	1,720	970
Oro 50	73	37	7.7	111	0.61	0.11	15	33	86	2,080	1,080

^a α (EF-Tu or S1) means the differential rate of synthesis of that particular protein relative to the rate of total protein synthesis in arbitrary units for each protein. α was determined by growing the cells with ^3H lysine and pulse-labeling for 2 min with ^{35}S methionine. The nucleotide pools are expressed as picomoles per OD₄₃₆ unit.

^b Ura, Uracil; Oro, orotate. Numbers indicate the concentrations (in micrograms per milliliter) used.

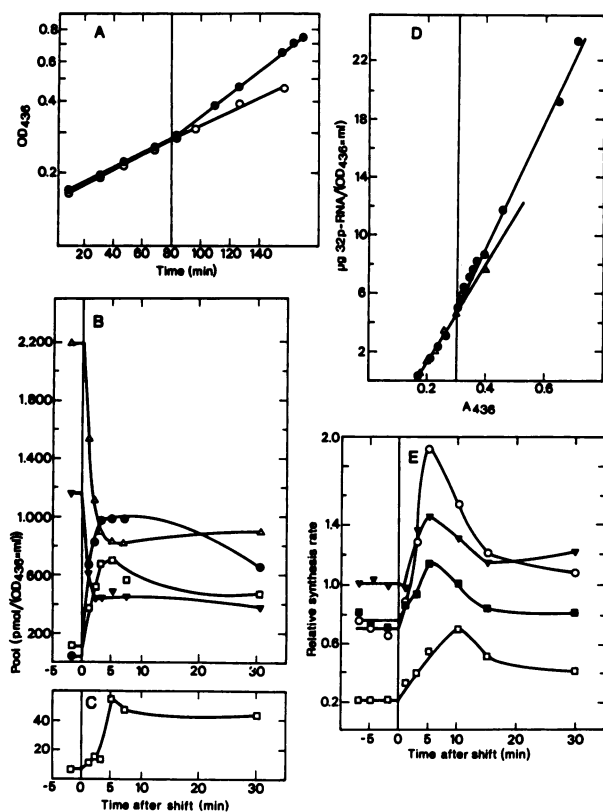


FIG. 2. Effects of a pyrimidine upshift on NF321. The strain was grown exponentially with 50 μg of orotate per ml as the pyrimidine source. At time zero, uracil was added at a concentration of 20 $\mu\text{g}/\text{ml}$. The different panels show different aspects of the resulting response. (A) Growth response as monitored by measuring OD_{436} . Symbols: \bullet , shifted culture; \circ , unshifted culture. (B) Response in the pools of nucleoside triphosphates ATP (Δ), GTP (\blacktriangledown), UTP (\bullet), and CTP (\square). (C) Response in the pool of ppGpp. (D) RNA accumulation measured as TCA-precipitable, NaOH-labile radioactivity in shifted culture (\bullet) and unshifted culture (Δ). (E) Synthesis rate of different proteins relative to the total rate of protein synthesis of EF-Tu (\blacksquare), EF-G (\blacktriangledown), ribosomal protein S1 (\square), and β -subunit of ATPase (\circ).

higher steady-state level within 5 min of the upshift (Fig. 2C), and the rate of stable RNA accumulation increased more than the growth rate (Fig. 2D). The synthesis rates of ribosomal protein S1 and elongation factor EF-G (measured by 30-s pulses with [^{35}S]methionine) increased considerably relative to the rate of total protein synthesis. Thus, the pyrimidine upshift was normal with respect to the synthesis of ribosomal components but abnormal with respect to the ppGpp pool.

Pyrimidine downshift. When the culture was growing with 200 μg of orotate per ml as the pyrimidine source in the presence of aspartic acid and glutamic acid (100 $\mu\text{g}/\text{ml}$ each), the generation time was ca. 200 min and the pools of pyrimidine nucleotides (particularly UTP) were extremely low. This indicated that the two amino acids inhibited the uptake of orotate, since they had no effect on the growth of the bacteria in the presence of uracil. We used this observation to create a downshift in the pyrimidine supply (Fig. 3).

Following the addition of aspartate and glutamate (100 $\mu\text{g}/\text{ml}$ each) to a culture growing on orotate (200 $\mu\text{g}/\text{ml}$), the growth rate decreased gradually and approached the steady-

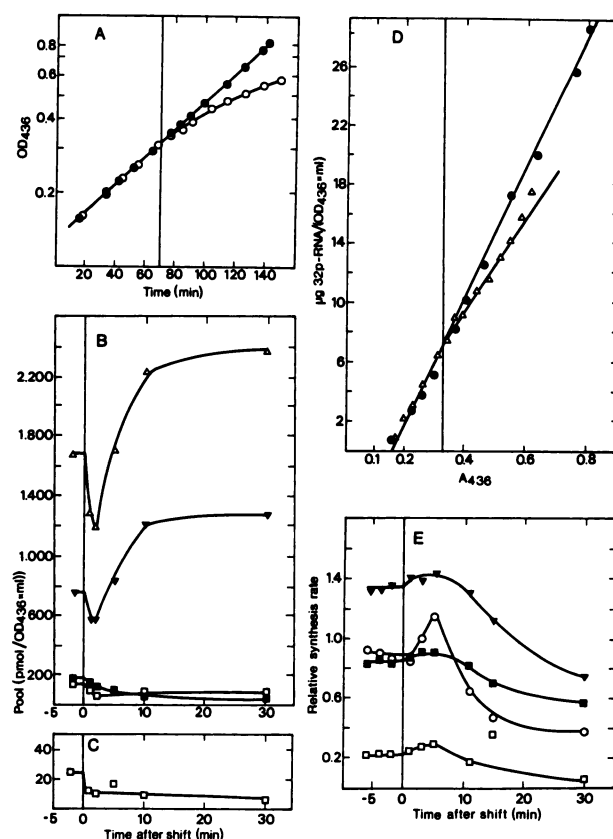


FIG. 3. Effects of a pyrimidine downshift on NF321. The strain was grown with 200 μg of orotate per ml as the pyrimidine source. At time zero, aspartate and glutamate (100 $\mu\text{g}/\text{ml}$ each) were added to inhibit orotate uptake. The different panels show different aspects of the resulting response. (A) Growth response as monitored by measuring OD_{436} . Symbols: \circ , shifted culture; \bullet , unshifted culture. (B) Response in the pools of nucleoside triphosphates ATP (Δ), GTP (\blacktriangledown), UTP (\blacksquare), and CTP (\square). (C) Response in the pool of ppGpp. (D) RNA accumulation measured as TCA-precipitable, NaOH-labile radioactivity in shifted culture (Δ) and unshifted culture (\bullet). (E) Synthesis rate of different proteins relative to the total rate of protein synthesis of EF-Tu (\blacksquare), EF-G (\blacktriangledown), ribosomal protein S1 (\square), and β -subunit of ATPase (\circ). When the RNA content was estimated by measuring the A_{260} of PCA-precipitable, KOH-labile components or after chromatographic separation of the nucleotides in the alkaline hydrolysate, the rate of RNA accumulation per OD_{436} unit was found to decrease 26 to 30% after addition of aspartate and glutamate.

state generation time of 200 min (Fig. 3A). The pools of UTP and CTP, already low in the presence of 200 μg of orotate per ml, decreased even further (Fig. 3B). The ppGpp pool behaved similarly with CTP and UTP (Fig. 3C). Shortly after addition of the two amino acids, the rates of synthesis of the ribosomal protein S1 and the elongation factors EF-Tu and EF-G decreased relative to the rate of total protein synthesis (Fig. 3E) and the differential rate of stable RNA accumulation fell (Fig. 3D). In Fig. 3D, it appears as if the differential rate of RNA synthesis accelerates again shortly after the downshift. However, this very reproducible phenomenon is an artifact of the TCA-NaOH extraction procedure at the very slow growth rate established after the shift, since the rate of accumulation of RNA, measured by determining the concentrations of nucleotides in the alkaline hydrolysates by

UV measurements and chromatography (see Materials and Methods), remained at 70 to 74% of the preshift value for extended periods of time (data not shown). The difference between the two methods is most likely due to the phospholipids, which may contribute considerably to the acid precipitable ^{32}P radioactivity at the very slow growth rates (31).

Thus, also in the pyrimidine downshift, we find the inverted correlation between the synthesis of ribosomal components and the levels of ppGpp.

DISCUSSION

We shall first discuss the possible reasons why the ppGpp pool increases as a function of the growth rate in these experiments and secondly discuss the relevance of the pools of nucleoside triphosphates and ppGpp for stable RNA synthesis.

Behavior of the ppGpp pool during pyrimidine starvation. We have established that the basal level of ppGpp increases as a function of the growth rate when *E. coli* is exposed to partial pyrimidine starvation. This finding apparently opposes the previously established inverse relationship between the size of the ppGpp pool and the growth rate (2, 13). Although this linear relationship was not seen before, it may not be surprising at all, first because the RelA-factor dependent ppGpp synthesis takes place on starving ribosomes possessing an occupied P site and an uncharged tRNA in the A site (15, 23, 33) and second because the condition of pyrimidine starvation is likely to limit the functional activity of the ribosomes, and hence their rate of charged tRNA consumption, by causing a limitation in the availability of mRNA.

The condition of pyrimidine limitation is really a starvation for the building blocks for RNA synthesis, i.e., it involves a shortage for UTP and CTP, whose concentrations become very low under these growth conditions. A priori, this may be expected to limit ribosome function in two, not mutually exclusive, ways.

First, there is the possibility that the shortage of UTP and CTP causes the cells to make less mRNA relative to rRNA and tRNAs, thereby resembling a short exposure to rifampin, which leads to a fall in the basal ppGpp pool (17a) and to a progressive reduction in the accumulation of ppGpp following induction of amino acid starvation (26), which is explained by the lack of new RNA chain initiation in the presence of rifampin and the decay of the preexisting mRNAs. However, this alternative is in conflict with the presented data of this paper, since the rate of stable RNA formation decreased relative to total RNA synthesis during pyrimidine starvation. The second alternative is that ribosome function is limited by the low pools of CTP and UTP during pyrimidine starvation, since this growth condition may reduce the RNA chain growth rate because the elongating RNA polymerase has high K_m s for the nucleotide substrates, particularly for UTP (18, 25). This alternative is more consistent with the available evidence, since growth in the presence of low pyrimidine nucleotide pools gives rise to a considerable derepression of the synthesis of both aspartate transcarbamylase, which is encoded by the *pyrBI* operon, and orotate phosphoribosyltransferase, which is encoded by the *pyrE* gene. This rationale is relevant in this context, because the expression of both these genes is regulated by attenuation and because transcriptional readthrough past the attenuators requires that the ribosomes follow very closely after the RNA polymerase in the attenuator region (3, 9, 35, 39, 40, 47). Thus, the derepression of

pyrBI and *pyrE* gene expression supports the theory that the coupling between transcription and translation becomes tighter during pyrimidine starvation and hence that progression of RNA chain elongation limits the functional activity of the ribosomes under these conditions.

The changes of the basal level of ppGpp may involve both RelA-dependent and RelA-independent pathways for ppGpp synthesis (7, 16, 28, 41). In our experiments, the RelA-independent pathways may have contributed significantly, since the fall in the basal ppGpp pool was also seen with *relA* mutants of *E. coli* K-12 exposed to limitation in the pyrimidine supply (35) and with a *relA* mutant of *E. coli* B (47a). Furthermore, the response was seen during several years of studies of pyrimidine gene regulation in *Salmonella typhimurium* (17a), although in these studies the ppGpp pool was not quantitated accurately.

Regardless of the reason for the decreasing ppGpp pools under pyrimidine starvation, we decided that this observation might contribute to the discussion of whether the basal ppGpp pool contributes to the control of stable RNA synthesis. On one hand, Baracchini and Bremer (2) have claimed that both the growth rate control and the inhibition of stable RNA synthesis during amino acid starvation (i.e., the stringent response) are mediated by ppGpp; on the other hand, Gaal and Gourse (14) have found that the *relA spoT* mutants of *E. coli*, making no ppGpp at all, are able to regulate the RNA synthesis as a function of the growth rate, while being unable to exert the stop of stable RNA synthesis in response to amino acid starvation.

Effects of the ppGpp pool on stable RNA synthesis during partial pyrimidine starvation. We consistently found that during steady-state growth or during pyrimidine up- and downshifts the relative rate of stable RNA synthesis was linearly correlated with the ppGpp pool, rather than being inversely correlated with this pool, as was found previously in numerous cases (2, 7, 13). Also, the synthesis rates for a few proteins associated with the ribosomes were found to be linearly rather than inversely correlated with the size of the ppGpp pool during pyrimidine starvation, indicating that this growth condition generally causes a fall in the expression of stringently controlled genes normally believed to be repressed by high ppGpp pools.

The most straightforward conclusion that could be drawn from these results is that the basal-level ppGpp pool does not regulate the synthesis of stable RNA and that it also does not regulate transcription initiation at the so-called stringently controlled promoters as a function of the growth rate, much in line with the conclusion made by Gaal and Gourse (14). However, this conclusion seems premature, since it leaves the question of whether the well-established inverse correlation between the ppGpp pool and the rate of stable RNA synthesis is purely fortuitous, reflecting a regulation mechanism which does not mean anything, or whether the ppGpp pool has entirely different functions, such as regulating the metabolism only by allosteric control of enzyme activity. Also, it was never disputed that the ppGpp pool plays a role in the stop of stable RNA synthesis during amino acid shortage, since all *relA* mutants are unable to perform this sudden shift (14, 45). We find it difficult to accept that the ppGpp pool should play a role in the regulation of gene expression when the concentration is very high and play no role at all when present at low concentrations, particularly since it was shown that changes in the steady-state ppGpp pool do indeed influence the frequency of transcription initiation at the *rrnB* promoter (16, 41) and since the basal-level concentrations of ppGpp in vivo are similar to those

concentrations of the compound which influence the transcription process *in vitro* (24, 25).

The difficulty in sorting out this problem may well be that the cell possesses several ways of achieving the goal of regulating the rate of stable RNA synthesis as a function of the growth rate. Each of these mechanisms may predominate at selected growth conditions, and each of them might be deleted without totally abolishing the regulation.

Our results suggest that reductions in the pyrimidine nucleoside triphosphate pools downregulate stable RNA synthesis, either directly or indirectly. On the other hand, it also seems certain that a large pool of ppGpp downregulates the synthesis of stable RNA, when the cells lack a required amino acid (6, 7) or when the carbon supply suddenly becomes suboptimal (21, 30). In line with the ideas of the late Ole Maaløe (17, 27), we have recently proposed that the stable rRNA synthesis is determined at least in part by competition between different promoters for a limited amount of free RNA polymerase and that the stringently controlled promoters (e.g., the promoters of the stable RNA operons) are generally difficult to saturate with RNA polymerase (20). We argued that both the repression of biosynthetic gene expression and changes in the RNA chain elongation rate might contribute to adjusting the amount of free RNA polymerase in the cells as a function of the medium (20). Since both low concentrations of the nucleoside triphosphates (18, 24) and high concentrations of ppGpp (24) reduce the RNA chain elongation rate *in vitro*, it seems reasonable that all growth conditions which produce either of these nucleotide pool effects *in vivo* (e.g., pyrimidine starvation or amino acid starvation) may lead to a sequestering of RNA polymerase in the chain elongation phase and thus reduce the relative rate of stable RNA synthesis (20). The experimental results reported here are consistent with this hypothesis, but the proof is still lacking.

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