

Guanosine 5'-diphosphate 3'-diphosphate (ppGpp): Positive effector for histidine operon transcription and general signal for amino-acid deficiency

(relaxed-stringent control/alarmones/*Salmonella typhimurium*/super-control/coupled *in vitro* protein synthesis)

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ABSTRACT Maximal expression of the histidine operon of *Salmonella typhimurium* in a coupled *in vitro* transcription-translation system is strongly dependent upon addition of guanosine 5'-diphosphate 3'-diphosphate (ppGpp). This requirement for ppGpp is exerted at the level of transcription through a mechanism distinct from the *his*-operon-specific regulatory mechanism. *In vivo* derepression of the *his* operon is markedly defective when histidine starvation is imposed on a *relA* mutant—unable to rapidly increase synthesis of ppGpp—growing in amino-acid-rich medium. Increased sensitivity of *relA* mutants to growth inhibition by a number of amino-acid analogs suggests that ppGpp is generally important in adjusting expression of amino-acid-producing systems. Analysis of these findings leads us to propose that ppGpp is a positive effector in a system that enables the cell to balance endogenous amino-acid production with environmental conditions of amino-acid availability, and to compensate efficiently for transient changes in these conditions. We propose a unifying theory of the role of ppGpp as the general signal molecule (alarmone) in a “super-control” which senses an amino-acid deficiency and redirects the cell's economy in response.

The “stringent phenomenon” in bacteria (1) has been primarily interpreted as being a regulatory mechanism for adjusting the rate of synthesis of stable RNA (i.e., rRNA and tRNA) with respect to the availability of amino acids for protein synthesis. Evidence has accumulated suggesting that the unusual nucleotide guanosine 5'-diphosphate 3'-diphosphate (ppGpp)—synthesized by the *relA* gene product on the ribosome as a function of tRNA charging (2)—acts as a negative effector in this regulation, although the precise mechanism is unclear. Comparison of *rel⁺* and *relA* bacterial strains has revealed that ppGpp also may negatively control other aspects of cellular metabolism: biosynthesis of lipids (3), nucleotides (4), and polyamines (5); and uptake of purines and pyrimidines (6, 7).

In this paper we show that ppGpp is also a positive effector for transcription of the histidine operon of *Salmonella typhimurium*, and that it apparently positively regulates production of other amino acids.

MATERIALS AND METHODS

Bacterial Strains. Isogenic pairs of *S. typhimurium* strains were employed for the purpose indicated: TA471 (*his*Δ*OGDCBH2253 hisT1504*) and TA705 (*his*Δ*OGDCBH2253 hisT1504 relA1*)—preparation of *in vitro* protein synthesizing extracts; TA2383 (*hisG46 dhuA1*) and TA2384 (*hisG46 dhuA1 relA1*)—*in vivo his* operon dere-

pression experiments; and TA1995 (*dhuA1*) and TA1996 (*dhuA1 relA1*)—amino-acid analog studies. The *rel-1* mutation (8) has been designated a *relA* mutation (9) on the basis of genetic mapping, phenotype, and biochemical characterization (unpublished experiments). The *dhuA1* mutation (10) was used to facilitate strain construction and is not pertinent to these studies. The following *Escherichia coli* lysogens (11) were used as source of template DNA: TA1933 [*his-6607 Str^R (φ80 h imm^λ cI857 susS7, φ80 h dhisO⁺ imm^λ cI857 susS7)*]; and TA1940 [*his-6607 Str^R (φ80 h imm^λ cI857 susS7, φ80 h dhis01242 imm^λ cI857 susS7)*]. The intact *his* operon contained in each of the transducing phages originated from *S. typhimurium* genetic material (12).

Conditions for *In Vitro* Protein Synthesis. Except where indicated, 50 μl reaction mixtures contained: Tris-acetate, 53 mM (pH 8.0); potassium acetate, 58 mM; ammonium acetate, 27 mM; magnesium acetate, 10 mM; calcium acetate, 5 mM; dithiothreitol, 1.3 mM; 20 amino acids, 0.2 mM each; bulk tRNA, 0.5 mg/ml; ATP, 2 mM; CTP, GTP, UTP, 0.5 mM each; phosphoenolpyruvate, 20 mM; folic acid, 30 μg/ml; polyethylene glycol 6000, 35 mg/ml; *p*-toluenesulfonyl fluoride, 30 μg/ml; template DNA, 150 μg/ml; S-30 protein, 3.5 mg/ml. Reaction mixtures, minus S-30 protein, were incubated 3 min at 37° before the protein was added to initiate synthesis. Incubation at 37° was continued for 70 min and stopped by addition to the histidinol dehydrogenase assay mixture.

Concentrations given for magnesium acetate, calcium acetate, tRNA, and S-30 protein are only approximate and are determined for each protein synthesizing extract prepared. Analysis of optimal conditions for *in vitro* protein synthesis, and methods for preparing *Salmonella* protein-synthesizing extracts and template DNA, are contained in a separate report*.

Assay Methods and Reagents were: histidinol dehydrogenase (L-histidinol:NAD oxidoreductase, EC 1.1.1.23) ref. 13; histidinol phosphate phosphatase (L-histidinolphosphate phosphohydrolase, EC 3.1.3.15) ref. 14; 6-phosphogluconate dehydrogenase (6-phospho-D-gluconate:NADP oxidoreductase, EC 1.1.1.43) ref. 15; and ppGpp ref. 16. Total *in vitro* protein synthesis was measured as incorporation of a mixture of 13 ¹⁴C-labeled amino acids (Schwarz Bioresearch) into hot-acid-insoluble material*. Bulk tRNA was isolated (17) from *S. typhimurium* strain TA253 (*hisT1504*). ppGpp was prepared by Cashel's procedure (18).

Abbreviations: ppGpp, guanosine 5'-diphosphate 3'-diphosphate; pppGpp, guanosine 5'-triphosphate 3'-diphosphate.

* S. W. Artz, J. R. Broach, and B. N. Ames, in preparation.

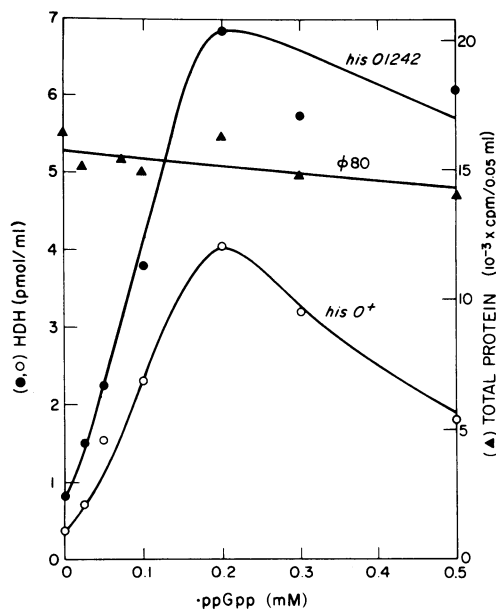


FIG. 1. Effect of ppGpp on *in vitro* histidinol dehydrogenase (HDH) synthesis (●, $\phi 80hisO1242$ DNA; ○, $\phi 80hisO^+$ DNA) and on total *in vitro* protein synthesis (▲, $\phi 80$ DNA). The S-30 extract was from TA705 (*relA*).

RESULTS

ppGpp is required for maximal *in vitro* *his* operon expression

We utilize an *in vitro* coupled protein synthesizing system which contains an S-30 cell extract, *his* operon DNA, and other components necessary for transcription and translation. *De novo* synthesis of histidinol dehydrogenase [product of the second structural gene (*hisD*) of the operon] serves as a measure of coupled *his* operon expression. Fig. 1 shows that *his* operon expression is strongly dependent upon addition of ppGpp when S-30 extract from a *relA* mutant is used: a stimulation of about 10-fold is observed. Functional specificity for this stimulation is indicated by lack of stimulation by ppGpp of total protein synthesis when phage $\phi 80$ DNA is used as template (Fig. 1). In addition, other guanine nucleotides tested (GDP, cyclic GMP, or excess GTP—each at 0.1 mM) are without significant effect.

In contrast, when S-30 extract from an isogenic *rel+* strain is used, the requirement for ppGpp to obtain maximal *in vitro* *his* operon expression is diminished (less than 2-fold stimulation) (Table 1). This lesser stimulation by ppGpp with *rel+* extracts is consistently observed, as is the marked

Table 1. Synthesis of ppGpp and pppGpp during coupled *in vitro* protein synthesis

S-30	Conditions*	ppGpp nmol/ml	pppGpp nmol/ml	HDH pmol/ml
<i>rel+</i>	Complete	18	22	1.04
<i>rel+</i>	- Amino acids	30	37	<0.01
<i>rel+</i>	+ 0.2 mM ppGpp			1.52
<i>relA</i>	Complete	3.5	1.5	0.70
<i>relA</i>	- Amino acids	3.0	1.2	<0.01
<i>relA</i>	+ 0.2 mM ppGpp			6.90

* Assay conditions ("complete") were as described in *Materials and Methods* with $\phi 80hisO1242$ DNA as template. Samples were analyzed for ppGpp and pppGpp (16) ($5 \mu\text{Ci}$ of [$\alpha\text{-}^{32}\text{P}$]GTP was added to each $50 \mu\text{l}$ reaction mixture) or histidinol dehydrogenase (HDH) (13) after 15 or 70 min, respectively.

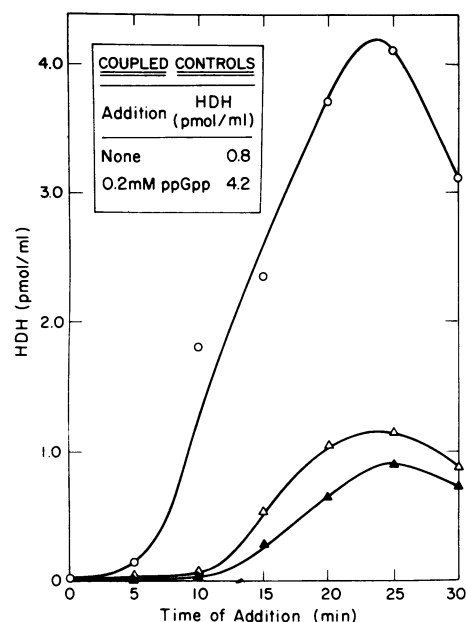


FIG. 2. Effect of ppGpp on uncoupled *in vitro* protein synthesis with S-30 from TA705 (*relA*) and $\phi 80hisO1242$ DNA. Amino acids were deleted from initial reaction mixtures and then added, along with $20 \mu\text{g/ml}$ of rifamycin SV to inhibit further transcription initiations (and with or without 0.2 mM ppGpp) at the times indicated (in $2 \mu\text{l}$), and incubation was continued for a total of 70 min. Symbols: ○, ppGpp added at zero time; Δ, no ppGpp added; ▲, ppGpp added at time indicated. Inset: Coupled control. Amino acids were added at zero time without rifamycin (and with or without ppGpp).

stimulation with *relA* extracts, even though independently prepared S-30 extracts may vary considerably in protein-synthesizing activity. The lesser dependence of *his* operon expression on added ppGpp with S-30 extracts from the *rel+* strain is accounted for by the ability of such extracts to produce and accumulate ppGpp during coupled protein synthesis while S-30 extracts from the *relA* mutant fail to do so (Table 1).

Requirement for ppGpp is independent of *his* operon-specific controlling elements

Fig. 1 demonstrates that ppGpp stimulates *in vitro* expression of *hisO1242* DNA about as much as wild-type (*hisO+*) *his* operon DNA. The *hisO1242* mutation deletes the "attenuator" site in the *hisO* region of the *his* operon and results in complete loss of the operon-specific control mechanism (19). We conclude, therefore, that the ppGpp requirement is exerted at a site other than the attenuator site, and that the regulatory function of this molecule is not part of the *his*-operon-specific control mechanism. This situation parallels that observed for catabolite-sensitive operons, in that cAMP regulation is effected through a mechanism distinct from operon-specific regulatory mechanisms (20).

ppGpp acts at the level of *his* operon transcription

In vitro uncoupling experiments (19, 21) provide evidence that the requirement for ppGpp in *his* operon expression is exerted at the level of transcription (Fig. 2). That is, when transcription and translation are dissociated, ppGpp stimulates *his* operon expression only when added while transcription occurs. Quantitative comparison between stimulation of transcription (Fig. 2) and stimulation of coupled synthesis in the same experiment (inset to Fig. 2) indicates that the ef-

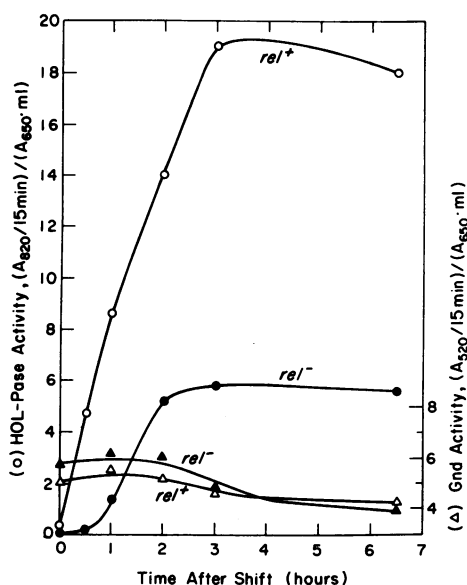


FIG. 3. Derepression of the *his* operon in *relA* and *rel+* strains starved for histidine in an amino-acid-rich medium. Strains TA2383 and TA2384 growing in minimal medium (22) containing 0.4% glucose and 20 amino acids (0.1 mM each) were centrifuged, washed, and resuspended at time zero in the same medium but with 1 mM histidinol in place of histidine. Samples were taken at intervals, and assayed for histidinol phosphate phosphatase (HOL-Pase) (O,●) and 6-phosphogluconate dehydrogenase (Gnd) (Δ,▲). Open symbols, TA2383 (*rel+*); closed symbols, TA2384 (*relA*).

fect of ppGpp can be accounted for entirely as a transcriptional requirement.

Derepression of the *his* operon *in vivo* is defective in a *relA* mutant

Derepression of histidinol phosphate phosphatase (product of the *hisB* gene), measured in isogenic *relA* and *rel+* derivatives of *S. typhimurium*, is shown in Fig. 3. Histidine limitation was imposed in media containing 19 amino acids, but

Table 2. Growth inhibition of *rel+* and *relA* strains by amino-acid analogs

Analog	mg	Zone of inhibition (mm)			
		NB		MIN	
		<i>rel+</i>	<i>relA</i>	<i>rel+</i>	<i>relA</i>
2-Thiazolealanine	0.17	15vt	22st	16c	18c
β-Chloroalanine	0.2	13t	21c	16c	20c
Serine hydroxamate	0.2	16vt	19st	18t	20c
2,2-Difluorosuccinate	1.0	7c	13st	8c	8c
Norvaline	0.4	<6	21c	23st	30st
Indolepropionate	0.4	<6	16t	9st	22st

Cultures of TA1995 (*rel+*) and TA1996 (*relA*) were grown overnight in nutrient broth (NB) or minimal + 0.4% glucose (22) (MIN) media; 0.1 ml was added to 2 ml of top agar (0.6%) and poured on a minimal-glucose plate. The indicated amount (in 20 μl) of each analog was placed on a filter paper disc (6 mm diameter) in the center of the plate. After 24 hr incubation at 37° the diameter of the zone of inhibition was scored and its turbidity evaluated (c, clear; st, slightly turbid; t, turbid; vt, very turbid). The amino acids reversing inhibition by these analogs were respectively: histidine; isoleucine + leucine + valine; serine; aspartate; isoleucine; tryptophan. We have not listed a number of analogs showing lesser differential inhibition and many analogs that inhibited both strains equally.

lacking histidine. Derepression of the *his* operon is markedly defective in the *relA* mutant under these conditions, both the rate and extent of derepression being affected. Growth rates were virtually identical for *relA* and *rel+* strains, with a doubling time of 40 min before, and 75 min during, derepression. A similar experiment (data not shown) in a minimal-glucose medium showed, in contrast, that the *relA* strain derepressed the *his* operon almost as much as the *rel+* strain (which showed normal derepression to about the *hisO1242* level). The importance of an amino-acid-rich medium for observing the derepression defect will be discussed. As part of these experiments we also measured ppGpp levels: in minimal-glucose medium the *rel+*, *relA1*, and *hisO1242* strains all contained about 0.1 mM ppGpp, and this level did not increase on starvation of the *relA1* mutant for histidine.

To assess nonspecific effects of the *relA* mutation, as reported for a particular *relA* mutation in *E. coli* (i.e., translation miscoding, ref. 23), the level of a constitutive enzyme—6-phosphogluconate dehydrogenase (15)—was measured in the same experiment (Fig. 3). Specific activity of this enzyme varies somewhat during histidine starvation, but the *relA* mutation does not apparently contribute to these changes.

relA mutation increases sensitivity to inhibition by amino-acid analogs

The growth inhibition caused by analogs of different amino acids is shown (Table 2) to be greater for a *relA* mutant than for an isogenic *rel+* strain. Increased sensitivity of the *relA* mutant to the various analogs is manifest both as a larger diameter zone of inhibition and a greater severity of inhibition within the zone (expressed as "turbidity"). The differential inhibition is enhanced by growing the bacteria in amino-acid-rich medium prior to exposure to the analog in minimal-glucose medium; i.e., subjecting them to a "downshift." Differential inhibition caused by the histidine analog thiazolealanine and the effect of a downshift are consistent with the results on histidine regulation presented above. The implications of all these results, for a general amino-acid control mechanism involving ppGpp, are discussed below.

DISCUSSION

Together with recent observations (19), evidence presented in this paper indicates that expression of the *his* operon of *S. typhimurium* is governed by at least two independent mechanisms—an operon-specific "activator-attenuator" mechanism (19) and a mechanism involving ppGpp as a positive effector. In the following discussion we will consider: first, how the two mechanisms combine to regulate synthesis of *his* enzymes; and second, the pleiotropic function of the ppGpp mechanism in cellular adaptation to physiological conditions of amino-acid imbalance and deficiency.

ppGpp and Histidine Regulation. In strains growing in minimal-glucose medium, expression of the *his* operon varies from a repressed level of 1 (reference level) to a fully derepressed level of about 12, whether derepression is elicited genetically (e.g., by the *hisO1242* constitutive mutation) or physiologically (e.g., by histidine starvation of a *hisO+* strain). It is in this range of expression that the *his* operon-specific "activator-attenuator" mechanism functions (19). However, the operon can be repressed below the level of 1 (to about 0.25) in strains growing in a rich medium containing an excess of all the amino acids (24). We conclude that this range is regulated by a mechanism involving the cellular concentration of ppGpp. Under growth conditions

of intermediate amino-acid supply the two independent mechanisms together become important in adjusting the synthesis of histidine biosynthetic enzymes. The combined effect is to integrate the production of histidine with the supply of, and demand for, all amino acids. These conclusions are based on the following evidence and interpretations.

(i) The concentration of ppGpp required for maximal *in vitro* expression of DNA template containing either the *hisO*⁺ or *hisO1242* regulatory region is 0.1–0.2 mM (Fig. 1). This is also the ppGpp level found in strains of *S. typhimurium*, containing either the *hisO*⁺ or *hisO1242* control region, growing in minimal-glucose medium (about 0.1 mM). In addition, a *relA* mutant can derepress the *his* operon normally when growing in minimal-glucose medium, conditions under which the ppGpp level of about 0.1 mM can be maintained, but not increased. Thus a level of about 0.1–0.2 mM ppGpp is sufficient for maximal *his* operon expression. ppGpp must therefore be of physiological importance in *his* operon regulation at levels *below* those maintained in cells growing in minimal-glucose medium, as is the case for cells growing in amino-acid-rich medium (25, 26). An important corollary of this conclusion is that the *in vitro* system closely mimics the *in vivo* situation with respect to the range of ppGpp concentrations effective in *his* operon regulation.

(ii) There is good correlation between variation in ppGpp levels and histidine enzyme levels as a function of external amino-acid supply. Synthesis of ppGpp on the ribosome is regulated by the charging of total tRNA species (2) as a function of intracellular concentrations of the 20 amino acids. In amino-acid-rich medium ppGpp is reduced to a level of about 30% that found in cells growing in minimal-glucose medium (25). Steady-state levels of histidine enzymes vary in parallel: the levels in either wild-type or *hisO1242* strains growing in amino-acid-rich media are 20–50% of those found in cells growing in minimal-glucose medium (ref. 24; unpublished results).

(iii) Derepression of the *his* operon is defective in *relA* mutants when histidine limitation is imposed during growth in amino-acid-rich medium (Fig. 3), but not in minimal-glucose medium. *relA* mutants, although unable to increase ppGpp levels rapidly in response to amino-acid starvation, are nevertheless able to maintain steady-state levels of ppGpp near those found in *rel*⁺ strains (26), apparently owing to “leakiness” of all *relA* mutations examined (2). Thus, a *relA* mutant growing in minimal-glucose medium has a ppGpp level high enough to allow nearly maximum derepression of the *his* operon as a consequence of normal functioning of the operon-specific “activator-attenuator” mechanism, which is independent of ppGpp. A *relA* mutant growing in amino-acid-rich medium, however, cannot rapidly increase the ppGpp level to that necessary for maximal expression. Derepression occurs under these conditions as a result of functioning of the operon-specific mechanism, but the rate is slower and the extent of derepression decreased about 3-fold because of lack of function of the ppGpp mechanism (Fig. 3).

(iv) The level of ppGpp drops precipitously (20- to 40-fold) and then gradually readjusts to a new intermediate steady-state level when *E. coli* is subjected to an amino-acid “shift-up” (25). Our *in vitro* results (Figs. 1 and 2) suggest that synthesis of *his* operon mRNA would decline dramatically during this transient shift period. Thus rapid shifts in ppGpp levels, over a wide range of concentrations, can pro-

vide an efficient mechanism for adjusting steady-state levels of histidine enzymes.

Together these arguments support the idea that the variation in histidine enzyme levels that occurs with changes in the exogenous supply of all amino acids is under direct control of the intracellular concentration of ppGpp. We propose that ppGpp is a component of a sensing mechanism for adjusting the synthesis of histidine biosynthetic enzymes with respect to the need for histidine *relative* to the supply of all of the amino acids in the external environment. The operon-specific regulatory mechanism (19) responds to the need for histidine specifically; the ppGpp regulatory mechanism enables the cell to sense how the supply of histidine compares with the supply of all amino acids. In conjunction, the two systems adjust the rate of synthesis of histidine biosynthetic enzymes so that production of histidine reflects the rate necessary for optimal utilization of this amino acid in cell growth.

ppGpp and General Amino-Acid Control. The analysis we have given for the role of ppGpp in histidine regulation predicts a similar positive-control function of this molecule in regulating production of the whole family of amino acids. Comparison of the effects of amino-acid analogs on growth of *rel*⁺ and *relA* strains is consistent with a general amino-acid control involving ppGpp. Analogs that result in decreased growth of *relA* mutants (Table 2) are those which, either directly or indirectly, interfere with tRNA charging. Such analogs therefore elicit elevated ppGpp synthesis in *rel*⁺ strains while *relA* mutants are unable to respond in this way. Furthermore, decreased growth of *relA* mutants is most apparent when cells are pregrown in amino-acid-rich medium and subsequently exposed to analogs in minimal-glucose medium. These observations suggest that *relA* mutants are more sensitive to certain amino-acid analogs because of a general inability to adjust amino-acid production owing to the defect in ppGpp synthesis.

Repression by complex media has been observed for several amino-acid biosynthetic pathways (e.g., 27–29), and these observations are consistent with a ppGpp general control system for amino acids. This phenomenon has been extensively studied in the case of tryptophan regulation (29), and it is significant that ppGpp stimulates *trp* operon expression in a coupled *in vitro* system (30). As discussed for histidine, the observed *in vivo* differences between steady-state enzyme levels are relatively small (2- to 4-fold), and the major regulation by ppGpp probably occurs during transient shift conditions.

ppGpp regulation likely influences amino-acid producing and utilizing systems in addition to biosynthetic pathways. Induction of protease activity during amino-acid starvation is defective in *relA* mutants of *E. coli* (31). Other possibilities that have not been investigated include amino-acid transport systems, and peptide permease and utilization systems. It also seems likely that the various systems involved in amino-acid production will fall into a hierarchy of control. That is, different systems may respond to different threshold levels of ppGpp as determined by the severity of amino-acid deficiency. Thus, whereas maximal expression of the histidine biosynthetic system appears not to require the very high levels of ppGpp that transiently occur on severe amino-acid starvation, other types of amino-acid producing systems may be sensitive primarily to such high levels. Evidence for a hierarchy of control among the systems regulated by cAMP has been discussed (ref. 32; M. D. Alper and B. N. Ames, in preparation).

Alarmones. We view ppGpp as a signal molecule which indicates to the cell an imbalance or deficiency in amino-acid supply. It thus is analogous in function to cAMP which indicates to the cell a deficiency in "fuel" availability. This type of signal molecule, which we call an alarmones, and which we will discuss in more detail elsewhere (B. N. Ames and S. W. Artz, in preparation), serves to reorient the cell's economy in response to stress in a particular area of metabolism. ppGpp is produced primarily in response to growth-rate limitation caused by amino-acid stress, and acts to correct this in various ways: inhibition of ribosomal and other macromolecular syntheses, activation of transcription of amino-acid biosynthetic operons, activation of protease activity, inhibition of some transport systems not concerned with amino acids, etc. It has been reported that ppGpp also stimulates *in vitro* expression of some catabolic operons (30, 33, 34). This may reflect a need to increase fuel utilization, specifically for amino-acid biosynthesis, when amino acids are not available—or are suddenly removed—from the environment.

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