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Mutations in the *spoT* Gene of *Salmonella typhimurium*: Effects on *his* Operon Expression

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The *spoT* gene of *Salmonella typhimurium* has been identified. Mutations in *spoT* map between *gltC* and *pyrE* at 79 min. The *spoT1* mutant has elevated levels of guanosine 5'-diphosphate-3'-diphosphate (ppGpp) during steady-state growth and exhibits a slower than normal decay of ppGpp after reversal of amino acid starvation. The *spoT1* mutation elevates *his* operon expression but is distinct from known *his* regulatory mutations. Elevated *his* operon expression in *spoT* mutants causes resistance to the histidine analogs, 1,2,4-triazole-3-alanine and 3-amino-1,2,4-triazole. These properties of *spoT* mutants allowed us to identify and characterize additional *spoT* mutants. Approximately 40% of these mutants are temperature sensitive for growth on minimal medium, suggesting that the *spoT* function is essential or that excessive accumulation of ppGpp is lethal.

In vitro and in vivo studies have indicated that the *his* operon of *Salmonella typhimurium* is positively regulated by guanosine 5'-diphosphate-3'-diphosphate (ppGpp) (31, 36, 37). Stephens et al. (31) have demonstrated that ppGpp is required for maximal in vitro expression by a mechanism which is independent of attenuator control. They showed that ppGpp stimulates transcription in this assay. They have also demonstrated that derepression of *his* operon expression in vivo is defective in a *relA* mutant (23) grown in amino acid-supplemented medium. Growth in rich medium reduces expression of the *his* operon relative to cells growing in minimal glucose medium (2). This has been termed metabolic regulation. Winkler et al. (36) proposed that the concentration of ppGpp in cells growing in amino acid-rich medium is not high enough to allow maximal *his* operon expression. It follows that mutations affecting the metabolism of ppGpp in *S. typhimurium* might be identified by virtue of phenotypic effects on *his* operon expression.

As a first step towards a more complete analysis of the genes involved in ppGpp metabolism, we isolated mutations in the *spoT* gene of *S. typhimurium*. Mutants of *Escherichia coli* with lesions in the *spoT* gene exhibit elevated intracellular levels of ppGpp, defective degradation of ppGpp, and slow growth (12, 17, 18–20). These phenotypes can be explained by the fact that the *spoT* gene encodes an 80,000-dalton protein capable of hydrolyzing ppGpp to guanosine 5'-diphosphate and pyrophosphate (3, 14, 32). The slow-growth phenotype is attributed to ppGpp being a pleiotropic inhibitor of gene expression (7). We demonstrate here that *spoT* mutations in *S. typhimurium* have a similar phenotype and map location. The effects of the *spoT1* mutation on ppGpp metabolism and *his* operon expression during exponential growth are examined. In addition, *spoT* mutants with severe growth defects are described.

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MATERIALS AND METHODS

Bacteria, media, and growth conditions. The bacterial strains used here were all derivatives of *S. typhimurium* LT2. The genotypes of the bacterial strains used are shown in Table 1. Vogel and Bonner E medium containing 0.2% glucose was used as a minimal medium (35). L-Amino acids and other nutritional supplements were added at the concentrations recommended by Davis et al. (10). Difco nutrient broth (0.8%) with 0.5% (wt/vol) NaCl added was used as complex medium (NB). Tetracycline was used at 10 and 25 μg/ml in E and NB media, respectively. When used as a sole carbon source, sodium glutamate was added to E medium lacking glucose and citrate at a final concentration of 0.2% (wt/vol). Modified low-phosphate MOPS minimal medium (24) with $^{32}P_i$ (20 to 100 μCi/ml) added was used to label cells for ppGpp assays as described by Bochner and Ames (5). All incubations were carried out at 37°C unless otherwise noted.

Chemicals. Antibiotics, carbon sources, amino acids, 1,2,4-triazole-3-alanine (TRA), and 3-amino-1,2,4-triazole (AT) were obtained from Sigma Chemical Co. $^{32}P_i$ was obtained from ICN Pharmaceuticals Inc. Nutrient broth, nutrient agar, and agar were obtained from Difco Laboratories. All other chemicals and reagents were obtained from sources cited in the references.

Genetic techniques. Transduction crosses were performed by using P22 (HT 105/1, *int-201*) as described by Maloy and Roth (22). Insertions of Tn*10* near the *spoT* gene were isolated by selecting *pyrE*$^+$ transductions of TR6672 (*pyrE125*) using a pool of Tn*10* insertion mutants as the donor. Isogenic pairs of wild-type and mutant alleles of *relA*, *spoT*, and *hisO* were obtained as siblings in transductional crosses in which the drug resistance phenotype of a linked transposon was selected. GltC$^+$ strains (containing *gltC115*1) were distinguished from GltC$^-$ (wild-type) strains by their ability to utilize sodium glutamate as a sole carbon source. The inheritance of *spoT* alleles was detected by using either serine-methionine-glycine (SMG) or AT plates (see below). The inheritance of *hisU*$^+$ or *hisU1820*

TABLE 1. Bacterial strains[a]

Strain	Genotype
JL 1045	*pyrG1602 cod-2 cdd-1* (J. Ingraham)
TA2437	*zga-607*::Tn*10 relA1 dhuA1*
TA2798	*zga-607*::Tn*10 relA1 spoT1 dhuA1*
TA2799	*spoT1*
TR3379	*relA*$^+$ (isogenic to TR3381)
TR3381	*relA1*
TR6093 (BB42)	*gltC1151*
TR6478	*spoT*$^+$ (isogenic to TR6479)
TR6479	*spoT1*
TR6672	*pyrE125*
TR6673	*hisU1820*
TT7428	*relA21*::Tn*10 cdd-1 cod-2*
TT7429	*zhj-1036*::Tn*10* (Tn*10* 82% linked to *spoT*)
TT7431	*zhj-1025*::Tn*10 relA1 pyrE125* (Tn*10* 70% linked to *pyrE*)
TT7433	*zhj-1036*::Tn*10 spoT1 relA1*
TT7542	*relA21*::Tn*10*
TT7603	*zhj-1036*::Tn*10 spoT21 relA1*
TT8387	*zhj-1036*::Tn*10 spoT22 relA1*
TT8919	*zhj-1036*::Tn*10 spoT*$^+$ *hisOG1302*
TT8920	*zhj-1036*::Tn*10 spoT1 hisOG1302*
TT8980	*zhj-1036*::Tn*10 spoT23*
TT8981	*zhj-1036*::Tn*10 spoT24*
TT10029	*hisO1242*
TT10030	*his*$^+$ (isogenic to TT10029)
TT10031	*his*$^+$ *relA21*::Tn*10* (isogenic to TT10032)
TT10032	*hisO1242 relA21*::Tn*10*
TT10033	*spoT*$^+$ *zee-2*::Tn*10 hisO1242* (from TR6478)
TT10034	*spoT1 zee-2*::Tn*10 hisO1242* (from TR6479)
TT10035	*spoT*$^+$ *relA21*::Tn*10* (from TR6478)
TT10036	*spoT1 relA21*::Tn*10* (from TR6479)
TT10040	*zhj-1036*::Tn*10 spoT1 relA1*
TT10041	*zhj-1036*::Tn*10 spoT*$^+$ *relA1*

[a] Unless otherwise noted, all strains were constructed for this study.

alleles was scored as smooth and rough colonies, respectively, using E medium with 2% glucose as a carbon source.

Isolation of *relA*::Tn*10*. A Tn*10* insertion in the *relA* gene was isolated by transducing JL1045 to *pyrG*$^+$ using donor phage grown on a mixed pool of independent, randomly generated insertion mutations, as described by Davis et al. (10). Three hundred *pyrG*$^+$ transductants were screened for the SMG and AT sensitivity phenotypes typical of *relA* mutants (see below for definition). One mutant, TT7428, was shown to have a characteristic RelA phenotype with respect to ppGpp and RNA synthesis during amino acid starvation (data not shown). The Tet^r and AT sensitivity phenotypes of the *relA*::Tn*10* mutation are 100% linked by P22 transduction. Strain TT7542 was constructed by transducing LT2 to tetracycline resistance using TT7428 as the donor.

Isolation of *spoT1*. The *spoT1* mutation was obtained as a phenotypic suppressor of *relA1* by selecting a spontaneous mutant of strain TA2437 that could grow on glucose minimal medium containing serine and leucine (2 mM each). The *spoT1* mutation was then transduced from the original isolate, TA2798, into TR6672 (*pyrE125*), resulting in strain TA2799 (Table 1). The sibling pair TR6478, TR6479 (*spoT*$^+$ *spoT1*) was constructed by transducing TR6672 (*pyrE125*) to *pyrE*$^+$ using TA2799 as the donor. The presence or absence of the *spoT1* allele was confirmed by transducing TT7431 (*pyrE125 relA1*) to pyrE$^+$ and determining the co-inheritance of the AT resistance phenotype (see below).

Localized mutagenesis of *spoT*. Additional *spoT* alleles were obtained by localized mutagenesis of a P22 lysate using hydroxylamine as described by Davis et al. (10). TT7429 (*zhj-1036*::Tn*10 spoT*$^+$) was used as the donor, and TR3381 (*relA1*) was used as the recipient strain. Tetracycline-resistant transductants selected on NB plus tetracycline at 30°C were replica printed to AT plates at 30°C to detect *spoT* (AT-resistant) mutants (see below). Temperature-sensitive *spoT* mutants were isolated in a *relA*$^+$ background by transducing LT2 to tetracycline resistance using the mutagenized TT7429 donor phage lysate. Mutants unable to grow at 42°C on E minimal medium plates were isolated as described by Davis et al. (10).

Biochemical techniques. Histidinol dehydrogenase (EC 1.1.1.23) was assayed by the method of Ciesla et al. (8). $^{32}P_i$ labeling of nucleotides, formic acid extraction, and precipitation of excess $^{32}P_i$ were performed as described by Bochner and Ames (5). Basal levels of ppGpp were determined by using cells growing in MOPS media containing $^{32}P_i$ (100 μC/ml). Cells were rapidly harvested at an optical density of 0.3 at 600 nm. Extracted samples (20 μl) were applied to polyethyleneimine cellulose sheets (Brinkmann Instruments, Inc.), and chromatography was performed in 1.5 M KH_2PO_4, pH 3.4 (6). The radioactive spots were visualized by autoradiography after overnight exposures. The ppGpp spots, along with blank spots above and below the ppGpp samples, were traced, excised, and counted in 10 ml of distilled H_2O in a liquid scintillation counter. Blank samples were averaged and subtracted from ppGpp samples. Amino acid starvation was induced by the addition of AT to a final concentration of 40 mM. Growth inhibition by AT was reversed by the addition of histidine (2 mM).

Determination of sensitivity to SMG. Alfoldi et al. (1) found that the growth of *E. coli relA* strains is inhibited by the presence of serine, leucine, or methionine in glucose minimal medium. Uzan and Danchin (33) have found that the sensitivity of *E. coli relA* strains to the amino acids serine, methionine, and glycine is increased in a synergistic manner when all three amino acids are present. Stephens has determined that the *relA1* mutant of *S. typhimurium* is sensitive to serine and leucine in combination, but not separately (John Stephens, Ph.D. dissertation, University of California, Berkeley, 1976). To detect the amino acid sensitivity of *S. typhimurium relA* strains unequivocally using the replica-printing method, we supplemented E-glucose medium with serine, methionine, glycine, and leucine (100 μg/ml each), adenine and thymine (50 μg/ml each), and calcium pantothenate (1 μg/ml). This SMG medium is similar to the complete SMG medium of Uzan and Danchin (33), but with leucine added. Growth inhibition of *S. typhimurium relA* strains is complete using our SMG medium, and the presence of a *relA* mutation can be easily detected by replica printing. Growth inhibition of *relA* strains on all serine-containing media is completely reversed by isoleucine.

Determination of resistance to AT. The principal effect of AT is a blockage of histidine biosynthesis due to inhibition of the imidazole glycerol phosphate-dehydratase activity of the *hisB* enzyme (15). However, at high concentrations it also interferes with purine metabolism (15). To study the histidine-

specific effects of AT, we circumvented the secondary toxicities by including adenine, thiamine, and methionine in E medium containing AT. To cause a lowering of ppGpp basal levels, 19 amino acids (no histidine) were also included in the medium in addition to adenine and thiamine. Thiamine and methionine were added to all media to reverse the inhibitory effects of adenine. Unless otherwise indicated, AT was used at a final concentration of 15 mM. When *relA* and *spoT* strains are scored using AT medium, it is not critical how cells are pregrown, how the plates are inoculated, or what temperature is used; this is in striking contrast to the sensitivity of the SMG phenotype to these variables (33).

Determination of TRA resistance. Overnight cultures (NB, 37°C) were washed and resuspended in an equal volume of 0.15 M NaCl. Washed cells (0.1 ml) were added to the top agar and plated. Both top and bottom agar media were identical to standard AT plates (see above) except that AT was present at a final concentration of 0.8 mM. TRA (20 μl of an 80 mM solution) was applied in a 6-mm filter disk. When many colonies were tested for TRA resistance, the cells were applied as radial streaks (26). The addition of histidine completely eliminates all effects of TRA.

RESULTS

The first *spoT* mutation of *S. typhimurium*, *spoT1*, was isolated as described above. The selection was based on similar selections used to obtain *spoT* mutants in *E. coli* (25) and on the fact that serine and leucine inhibit the growth of *S. typhimurium* strains containing the *relA1* mutation (23; Stephens, Ph.D. dissertation). We establish here that the *spoT1* mutation of *S. typhimurium* is analogous to *spoT* mutations of *E. coli*.

The *Salmonella spoT* mutation is located between *pyrE* and *gltC*. The *spoT* gene of *E. coli* is located between *pyrE* and *gltC* at 82 min (16). Figure 1 depicts the gene order and P22 cotransduction linkages of these genes in *S. typhimurium*. In addition, a Tn*10* insertion near *spoT* (*zhj-1036*::Tn*10*) was isolated as described above. Our results indicate that the *spoT1* locus is closely linked to this transposon as well as to *gltC1151* (in TR6093). The probable gene order deduced from two-factor P22 transductional crosses was confirmed by three-factor crosses (28; data not shown). The rare classes of recombinants in these experiments were consistent with the gene orders *pyrE-spoT-gltC* (experiment 1) and *pyrE–zhj-1036*::Tn*10–spoT* (experiment 2). Thus, the genetic location of the *spoT1* mutation of *S. typhimurium* is analogous to that of *spoT* mutations in *E. coli*.

The *spoT* locus is distinct from the *hisU* locus. Mutation *hisU1820*, which maps at 81 min, confers resistance to TRA and has been proposed to exhibit relaxed control of RNA synthesis during carbon and energy shiftdown (4, 9). Conceivably, these phenotypes could result from a lesion in the *spoT* gene. However, we found that the *hisU1820* mutation (in TR6673) is unlinked by P22 transduction to either *pyrE* (0 of 548 transductants) or *zhj-1036*::Tn*10* (0 of 1,037 transductants). Other *hisU* alleles are likewise unlinked (data not shown). Davidson et al. have also found that *hisU1820* does not affect ppGpp metabolism (9).

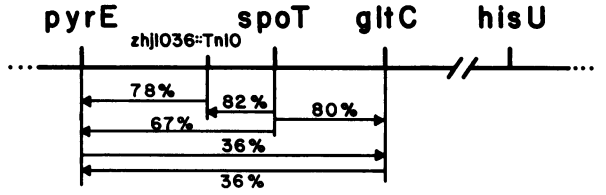

FIG. 1. Transductional mapping of the *spoT* region of *S. typhimurium*. Arrowheads denote selected markers.

TABLE 2. Effect of *spoT1* on ppGpp levels

Strain	ppGpp level (pmol/OD_{600}) in growth medium[a]	
	Minimal[b]	Minimal + amino acids[c]
hisO$^+$		
spoT$^+$ TR6478	9.5 (50)[d]	2.6 (32)
spoT1 TR6478	29.7 (65)	10.7 (40)
hisO1242		
spoT$^+$ TT10033	10.3 (60)	5.7 (35)
spoT1 TT10034	51.5 (74)	13.9 (42)
hisOG1302[e]		
spoT$^+$ TT8919	14.2 (43)	5.3 (28)
spoT1 TT8920	45.7 (52)	17.8 (38)

[a] Intracellular levels of ppGpp in growing cells were determined as described in the text. All cultures were harvested at OD_{600} = 0.3. The specific activity of $^{32}P_i$ was 500 μCi/μmol (P_i = 0.2 mM).
[b] Minimal medium was MOPS plus 0.2% glucose.
[c] MOPS-glucose medium was supplemented with 18 amino acids (no histidine or glutamine).
[d] Culture doubling times (in minutes) are given in parentheses.
[e] TT8919 and TT8920 are His$^-$, so histidine was added to the cultures.

Effects of the *Salmonella spoT1* mutation on ppGpp metabolism. To verify the assignment of *spoT* to this mutation, we examined its effects on ppGpp metabolism. The archetypical *E. coli spoT1* mutation exhibits several characteristic defects in ppGpp metabolism as compared to *spoT*$^+$ strains: (i) elevated basal levels of ppGpp associated with a slower growth rate, (ii) higher induced level of ppGpp during amino acid deprivation, (iii) slower decay of ppGpp when amino acid-starved cells are resupplemented with amino acids, and (iv) failure to accumulate guanosine 5′-triphosphate-3′-diphosphate (pppGpp) during amino acid starvation (12, 17, 18–20).

The *S. typhimurium spoT1* mutation caused identical effects on ppGpp metabolism. The basal levels of ppGpp in various *S. typhimurium* strains were measured in glucose minimal medium and amino acid-supplemented glucose minimal medium (Table 2). In each of three pairs of strains, isogenic except for the *spoT* allele, the presence of the *spoT1* mutation caused elevated basal levels of ppGpp and reduced growth rates in both media.

The *spoT1* mutation caused ppGpp to reach a higher induced level than a *spoT*$^+$ isogenic strain during amino acid starvation. Figure 2 shows the effects on ppGpp levels of histidine starvation in both *relA*$^+$ *spoT1* (TR6479) and *relA*$^+$ *spoT*$^+$ (TR6478) strains. The level of ppGpp in the *spoT1* strain rose to more than four times the level of ppGpp in the *spoT*$^+$ strain.

The mutant ppGppase present in the *spoT1* strain had a reduced activity as evidenced by an in vivo decay rate slower than that of the wild-type enzyme. Figure 2 depicts the decay of ppGpp after the reversal of histidine starvation. The half-life of ppGpp decay was dramatically lengthened in the *spoT1* strain relative to the *spoT*$^+$ strain. The *spoT*$^+$ strain immediately resumed growth, whereas the *spoT1* strain resumed growth only after the 15 min required for ppGpp level to return to basal levels (data not shown).

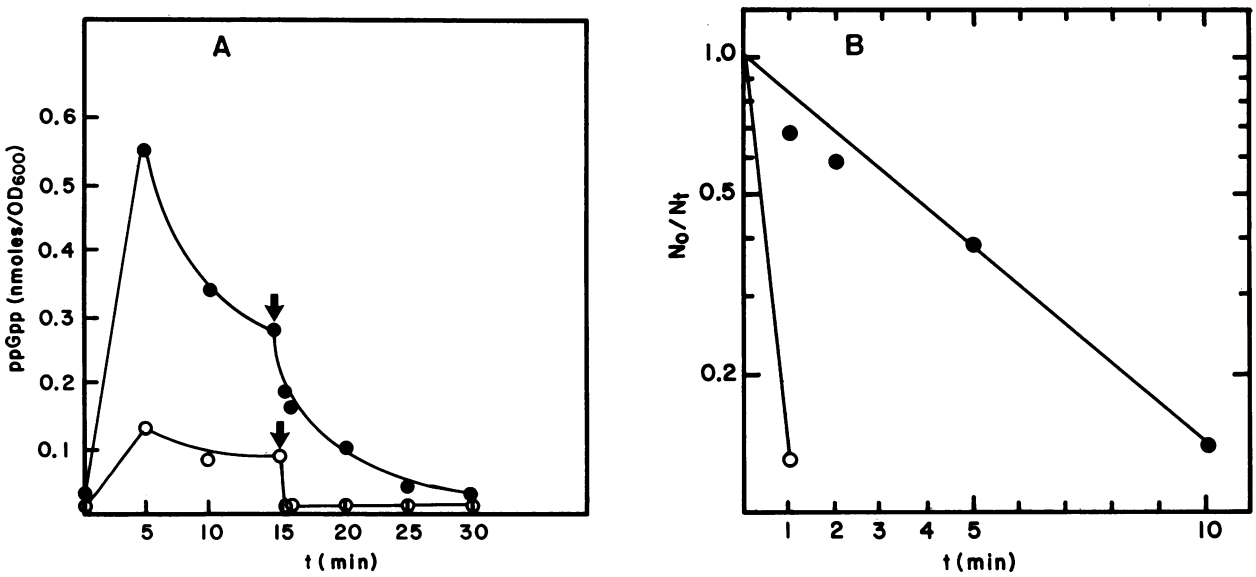

FIG. 2. (A) Accumulation and decay of ppGpp during histidine starvation and resupplementation. Cultures of TR6478 (*spoT*$^+$) and TR6479 (*spoT1*) were labeled with $^{32}P_i$ (30 μCi/ml, 0.2 mM P_i) as described in the text. MOPS medium contained 0.2% glucose and 19 amino acids (no histidine). Histidine starvation was induced by the addition of AT (40 mM) at $t = 0$ ($OD_{600} = 0.3$). At $t = 15$ min (arrows), histidine (2 mM) was added to the cultures. At various times 0.1-ml samples were treated with formic acid, and the excess $^{32}P_i$ was precipitated as described in the text. Portions (10 μl) were subjected to one-dimensional polyethyleneimine cellulose chromatography as described in the text. (B) Exponential decay rates of ppGpp at various times after histidine resupplementation. Symbols: ○, strain TR6478; ●, strain TR6479. Quantitative ppGpp values were obtained as described in the text.

Assuming that histidine resupplementation turns off ppGpp synthesis, the rates of ppGpp turnover can be estimated as first-order decay rates. The inset of Fig. 2 depicts N_t/N_0 as a function of time. N_t is the intracellular concentration of ppGpp t minutes after histidine addition, and N_0 is the concentration of ppGpp at time 0, just before histidine addition. The half-life of ppGpp was <0.3 min for the *spoT*$^+$ strain. The ppGpp half-life for the *spoT1* strain was 3.3 min, reflecting an in vivo decay rate >10-fold slower than that of the *spoT*$^+$ strain. The effect of the *S. typhimurium spoT1* mutation on the ppGpp decay rate was quantitatively similar to that of the *E. coli spoT1* mutation and is consistent with the finding that the *spoT* gene product is a ppGppase (3, 14, 32).

We found that pppGpp does not appreciably accumulate during amino acid starvation of the *spoT1* mutant, in marked contrast to an isogenic *spoT*$^+$ strain (data not shown). We conclude that the *spoT1* mutation of *S. typhimurium* provokes alterations in ppGpp metabolism completely analogous to those described for classical *E. coli spoT* mutants, and we can justifiably refer to the locus as *spoT*.

Use of histidine analogs to prevent growth of *relA* mutants. Previously, *relA* mutants have been scored genetically by their inability to grow on SMG media (33) or serine and leucine (Stephens, Ph.D. dissertation). We found that a combination of all four amino acids was most effective (see above). Since lowered ppGpp levels reduce the expression of the *his* operon, it should also be possible to identify strains carrying *relA* by their sensitivity to histidine antimetabolites. This expectation was tested and found to be correct by Stephens et al. (31).

To devise a phenotype for scoring and selecting mutants affecting ppGpp levels, we tested the effect of AT (an inhibitor of histidine biosynthesis) on the growth of strains isogenic except for the *relA* locus. In the presence of 20 mM AT, the growth of a *relA* strain, TR3381 (*relA1*) or TT7542 (*relA*::Tn*10*), was distinctly slower than that of an isogenic *relA*$^+$ strain. Supplementation of minimal medium with 19 amino acids (no histidine) exacerbated this property of the *relA1* strain as judged by a greater degree of growth inhibition and by the enhanced sensitivity of the *relA* strains to AT by a factor of approximately 10, reducing the MIC from 20 to ~2 mM AT.

The effect of 19 amino acids is presumed to be due to a lowering of basal ppGpp levels such that the *his* operon is no longer fully saturated for its ppGpp effect (31, 36; Table 2). Cells with decreased expression of the *his* operon are extremely sensitive to AT. In *relA*$^+$ but not *relA* strains this extreme sensitivity can be overcome by ppGpp synthesis induced by starvation. If this general idea is correct, one would expect that in a *relA* strain any mutation which increases ppGpp levels should relieve sensitivity to AT. This prediction was tested.

Suppression of the sensitivity of *relA* strains to AT by *spoT1*. Table 3 shows that the sensitivity to AT of *relA* strains was reversed by *spoT1*. In the *relA1* background, presence of a *spoT1* allele permitted the strain to grow at 25 mM AT, whereas the *relA* mutant alone was sensitive to as little as 5 mM AT; we suggest that resistance is due to increased ppGpp levels that stimulate *his* operon expression. Presumably, the *relA*::Tn*10* insertion used here destroys all *relA* gene product activity. This suggests that *spoT* causes the accumulation of ppGpp synthesized by a *relA*-independent pathway.

Table 3 also shows that *spoT1* suppressed the growth impairment of *relA1* and *relA21*::Tn*10* mutants on SMG medium, as it did in the serine-leucine medium used to select it. This probably reflects a positive effect of ppGpp on isoleucine biosynthesis, since SMG medium induces isoleucine starvation of *relA* strains (34). The growth inhibi-

TABLE 3. Suppression of sensitivity to AT of *relA1* and *relA21*::Tn*10* by *spoT1*

Relevant genotype[a]	Growth medium[b]			
	Minimal[c]	AT (5–25 mM)	AT (40 mM)	SMG
relA$^+$ *spoT*$^+$ (TR3379, TR6478)	+	+	−	+
relA1 spoT$^+$ (TR3381, TT10041)	+	−	−	−
relA21::Tn*10 spoT*$^+$ (TT10035)	+	−	−	−
relA1 spoT1 (TT10040)	+	+	−	+
relA21::Tn*10 spoT1* (TT10036)	+	+	−	+

[a] See Table 1 for complete genotype and sibling relationships.
[b] Positive notation indicates good patch growth at 37°C after replica plating from NB master.
[c] Minimal medium was E plus 0.2% glucose.

tion of *S. typhimurium relA* strains on SMG plates was completely reversed by the addition of isoleucine but not by the addition of histidine.

The correction of both sensitivity to AT and sensitivity to SMG by *spoT1* is consistent with *spoT1* increasing ppGpp pools and not causing a specific effect on either of the two amino acid pathways.

Suppression of the sensitivity of *relA* strains to AT by *hisO1242*. To verify that enhanced *his* operon expression is required to suppress the sensitivity to AT of *relA* strains, we determined the effects of a *his* mutation (Δ*hisO1242*) which abolishes the *his* attenuator but retains the *his* promoter and results in constitutive high-level expression of the *his* operon. Table 4 shows that in strains isogenic except for the *relA* and *hisO* alleles, the Δ*hisO1242* mutation suppressed the AT-sensitive phenotype of *relA21*::Tn*10* at all but the highest AT concentration (25 mM). In contrast, the Δ*hisO1242* mutation had no effect on the sensitivity of *relA21*::Tn*10* to SMG. We conclude that enhanced *his* operon expression (most clearly shown by attenuator deletion) partially suppresses the sensitivity of *relA* strains to AT. It follows that *spoT1* suppresses the sensitivity of *relA* strains to AT by enhancing *his* operon expression.

The histidine biosynthetic enzymes are elevated in *spoT1* mutants. The *spoT1* mutation caused elevated basal levels of ppGpp in cells growing in amino acid-supplemented minimal medium (Table 2). Considering the data of Winkler et al. (36, 37) and the supportive evidence presented above, we expected that the *spoT1* mutation would cause an elevation of the histidine biosynthetic enzymes during steady-state growth in the presence of amino acids. We monitored histidinol dehydrogenase (EC 1.1.1.23) activity, the final step in histidine biosynthesis, as a measure of *his* operon expression. Histidinol dehydrogenase is the product of the second gene in the operon, *hisD*, and thus should be less susceptible to transcriptional polar effects and unaffected by the activities of the downstream promoters, P2 and P3 (29).

TABLE 5. Effect of *spoT1* on histidinol dehydrogenase enzyme activity

Strain	Histidinol dehydrogenase enzyme activity (cpm ml^{-1} h^{-1} OD_{650}^{-1}) in growth medium[a]			
	Minimal	Minimal + histidine	Minimal + amino acids[b]	Minimal + amino acids + histidine
hisO$^+$				
spoT$^+$ TR6478	1.85 (50)[c]	2.00 (47)	0.95 (33)	0.44 (36)
spoT1 TR6479	1.97 (66)	2.98 (63)	1.70 (42)	1.02 (40)
hisO1242				
spoT$^+$ TT10033	27.31 (48)	24.0 (48)	12.5 (34)	9.9 (36)
spoT1 TT10034	31.8 (66)	35.3 (63)	23.0 (44)	18.8 (42)
hisOG1302[d]				
spoT$^+$ TT8919	ND[e]	18.6 (48)	ND	17.4 (36)
spoT1 TT8920	ND	17.3 (69)	ND	18.7 (45)

[a] Histidinol dehydrogenase was assayed as described in the text.
[b] E-glucose minimal medium was supplemented with 18 amino acids (no histidine or glutamine).
[c] Culture doubling times (in minutes) are given in parentheses.
[d] TT8919 and TT8920 are His$^-$, so histidine was added to the cultures.
[e] ND, Not determined.

The data presented in Table 5 confirm our prediction. Histidinol dehydrogenase activity was elevated approximately twofold in *spoT1* strains, but only when the cells were grown in amino acid-supplemented medium. This effect can be demonstrated with both *hisO*$^+$ and Δ*hisO1242* (attenuator-deleted) strains, indicating that the ppGpp effect is on the *his* promoter and not on the attenuation mechanism (see below). Growth of either *spoT*$^+$ or *spoT1* strains in glucose minimal medium elevated both intracellular ppGpp levels (Table 2) and *hisD* enzyme activity (Table 5) relative to the levels measured in cells grown in amino acid-supplemented medium. The *spoT1* mutation caused cells grown in amino acid-enriched medium to resemble cells grown in glucose minimal medium with respect to *hisD* enzyme levels, ppGpp levels, and growth rate (Tables 2 and 5). Our observations are consistent with the increased sensitivity to AT (see

TABLE 4. Partial suppression of sensitivity to AT of *relA21*::Tn*10* by *hisO1242*

Relevant genotype[a]	Growth medium[b]					
	Minimal	AT (5–25 mM)	AT (30 mM)	AT (40 mM)	AT (80 mM)	SMG
relA$^+$ *hisO*$^+$ (TT10030)	+	+	+	−	−	+
relA$^+$ *hisO1242* (TT10029)	+	+	+	+	−	+
relA21::Tn*10 hisO*$^+$ (TT10031)	+	−	−	−	−	−
relA21::Tn*10 hisO1242* (TT10032)	+	+	−	−	−	−

[a] See Table 1 for complete genotypes and sibling relationships.
[b] See the text for the composition of growth media. Positive notation indicates good patch growth at 37°C after replica plating from NB master.

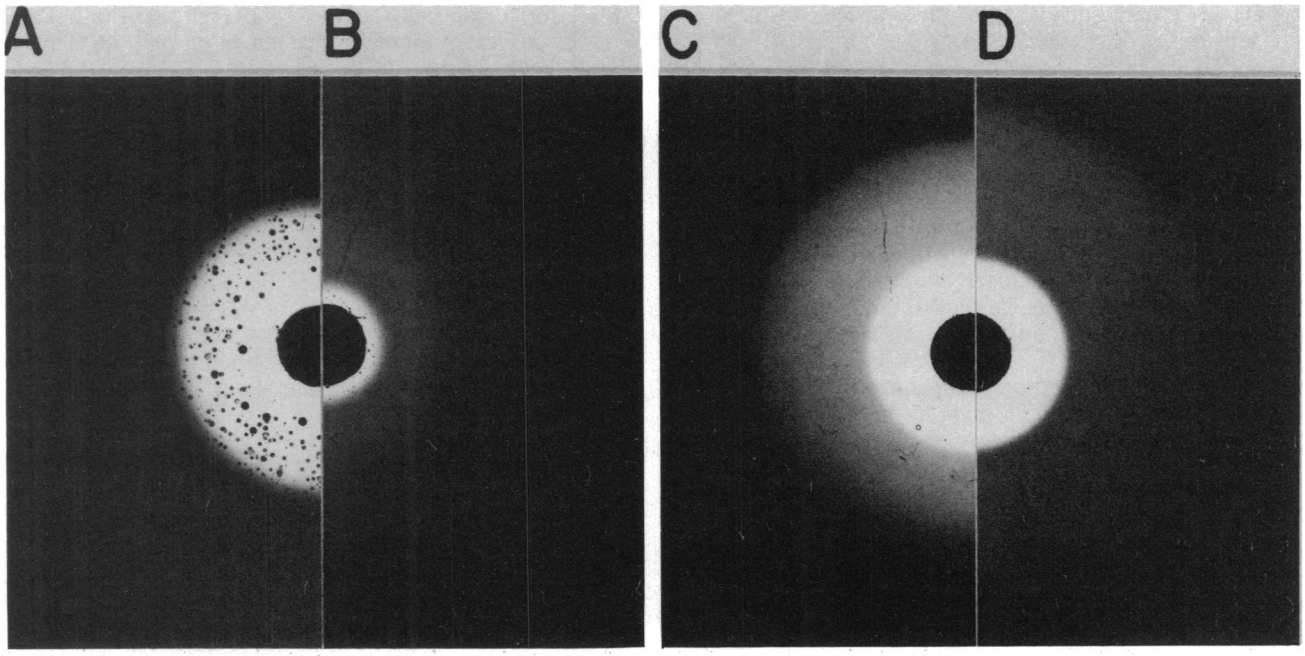

FIG. 3. Inhibition of growth by TRA. The TRA assay is described in the text. Plates were incubated at 37°C for 24 h. Panels: A and B, strains TR6478 (*spoT*$^+$) and TR6479 (*spoT1*), respectively (19 amino acids, no histidine); C and D, strains TR6478 and TR6479, respectively (no amino acids added).

above) and TRA (see below) caused by the inclusion of amino acids other than histidine in the test media.

Histidinol dehydrogenase assays of the Δ*hisOG1302* derivatives support the idea that ppGpp-mediated stimulation occurs at the level of recognition of the *his* promoter. The Δ*hisOG1302* mutation is a deletion that fuses the *his* operon to an upstream foreign promoter, eliminating both histidine-specific and metabolic regulation of the intact *his* genes (1, 36). In contrast to the *hisO*$^+$ and Δ*hisO1242* strains, cells containing the Δ*hisOG1302* mutation did not respond to either growth in minimal medium or the presence of the *spoT1* mutation by elevating *hisD* enzyme levels. As shown in Table 2, the presence of the *spoT1* mutation or growth in minimal medium still resulted in lowered growth rates and elevated ppGpp levels in strains containing Δ*hisOG1302*. Thus, the Δ*hisOG1302* mutation does not affect ppGpp levels, but rather alters the response of the *his* operon to elevated ppGpp levels. The simplest explanation of these results is that the foreign promoter of Δ*hisOG1302* is nonresponsive to ppGpp levels, whereas the *his* P1 promoter is activated by ppGpp (see below).

The *spoT1* mutation confers resistance to TRA. The histidine analog TRA causes repression of *his* operon expression (21). This analog has been used to isolate resistant mutants that have an elevated basal level of the *his* biosynthetic enzymes (27). The *spoT1* mutation elevated basal levels of the histidine biosynthetic enzymes when cells were grown in amino acid-supplemented minimal medium (see above). Thus, *spoT1* strains should be resistant to TRA when grown in the presence of amino acids. TRA repressed the *his* operon, but wild-type cells had adequate basal levels of the *his* enzymes to grow in the presence of TRA. However, when cells were grown in the presence of amino acids without histidine (which lowers the ppGpp basal level and shuts down *his* operon expression) and a low concentration of AT (0.8 mM) (which inhibits *hisB* enzyme activity), the *hisB* enzyme activity was so low that derepression was required for growth. Under these conditions, growth of wild-type cells was sensitive to the false repressor TRA. This sensitivity could be reversed by mutations that derepress the *his* operon, i.e., *hisO*, *hisT*, *hisR*, and *hisU* (data not shown). The twofold derepression of the *his* operon caused by *spoT1* (see above) was also sufficient to cause resistance to TRA.

Figure 3 depicts the TRA-resistant phenotype of a *spoT1 relA*$^+$ strain and the TRA-sensitive phenotype of a *spoT*$^+$ *relA*$^+$ strain. Growth of the *spoT*$^+$ strain was inhibited within a zone 11 mm from the edge of the filter disk (Fig. 3, panel A); growth of the *spoT1* strain was only inhibited in a zone 2 mm from the edge of the filter disk (panel B). Spontaneous TRA-resistant mutants can be seen within the zone of inhibition of the *spoT*$^+$ strain. Most of these TRA-resistant mutants exhibited the rough colony morphology characteristic of *his* regulatory mutants (27). The TRA resistance phenotype of *spoT1* is consistent with the elevation of the basal level of histidine synthetic enzymes by a mutation that increases basal ppGpp levels.

The effect of omitting the amino acids from the TRA test medium is depicted in panels C (*spoT*$^+$) and D (*spoT1*) of Fig. 3. The zone of growth inhibition extends 5 mm from the edge of the disk in both cases. Thus, on medium lacking amino acids, *spoT1* confers very little resistance to TRA and has very little effect on *his* operon expression (see above). When *his* constitutive mutants were originally selected the minimal medium used lacked amino acids (4, 27). This explains why *spoT* mutants were not previously recovered as *his* regulatory mutations.

Positive selection of *spoT* mutations. Since *spoT1* suppresses the sensitivity of a *relA* mutant to AT, it should be possible to select new *spoT* alleles by selecting AT-resistant mutants in a *relA* parent strain. This was tested by plating TT7542 (*relA21*::Tn*10*) on AT medium at 30°C and selecting spontaneous survivors; these arose at a frequency of 10^{-6}. Of 20 independent AT-resistant isolates, 15 were found to

contain *spoT* mutations. One of these also had a temperature-sensitive growth phenotype.

Isolation of additional *spoT* alleles by localized mutagenesis. New *spoT* alleles were isolated by localized mutagenesis of the *spoT* locus with hydroxylamine. Mutations in the *spoT* gene were detected by their ability to confer resistance to AT on the *relA* parent as discussed earlier. Approximately 20,000 derivatives of TR3381 (*relA1*) were screened for resistance to AT to obtain 103 independent *spoT* mutants. Subsequent transduction experiments showed that the AT resistance phenotype is closely linked to *zhj-1036*::Tn*10* (near *spoT*) in each mutant. Of the 103 mutants, 40 grew well at 30 and 37°C but were unable to grow on minimal glucose (E medium) plates at 42°C. Of the remaining 63 mutants, 12 grew very slowly at all three temperatures, producing tiny colonies. The remaining 51 mutants did not have severe growth defects. The temperature sensitivity of the 40 *spoT*(Ts) mutants was shown to be closely linked to *zhj-1036*::Tn*10* and associated with the AT resistance phenotype in backcrosses.

One slow-growing *spoT* mutant and one temperature-sensitive *spoT* mutant from this mutant screening were selected for further analysis. One of the 12 slow-growing mutants, TT8387 (*spoT22 relA1*), was chosen because it showed a low spontaneous reversion frequency to fast growth. The original *S. typhimurium spoT1* mutation also displayed a slow-growth phenotype. The slow-growth phenotype of *spoT* mutants was easily detected as a small colony size during transduction experiments. The small-colony phenotypes of *spoT1* and *spoT22* are associated with the AT resistance phenotypes, indicating that both phenotypes are probably due to single mutations in the *spoT* gene. With *zhj-1036*::Tn*10* as the selected marker, 165 of 200 Tet^r transductants of TR3381 (*relA1*) were small AT-resistant colonies when TT7433 (*zhj-1036::Tn10 spoT1 relA1*) was used as the donor. When TT8387 (*zhj-1036*::Tn*10 spoT22 relA1*) was used as the donor, 131 of 166 Tet^r transductants were small, AT-resistant colonies. The remainder of the transductants in both crosses were all large, AT-sensitive colonies. These results indicate that small colony size is a reliable phenotype associated with some *spoT* mutations that can be utilized for mapping purposes.

As an addition to the spectrum of *spoT* mutations already described, we isolated two more *spoT* mutations as temperature-sensitive mutations in a *relA*$^+$ genetic background, as described above. Since *relA*$^+$ strains are already AT resistant, *spoT* mutants could not be identified by using AT plates as in the previous selection. Two independent, hydroxylamine-induced *spoT*(Ts) *relA*$^+$ mutants were obtained after screening Tet^r transductants for temperature sensitivity. The two mutants, TT8980 [*spoT23*(Ts)] and TT8981 [*spoT24*(Ts)], have similar phenotypes. Similar to the other *spoT* mutations, *spoT23*(Ts) and *spoT24*(Ts) confer resistance to TRA on wild-type strains at 37°C. These mutants also grow slowly, a property that results in a small colony phenotype. We transduced LT2 to Tet^r using TT8921 [*spoT24*(Ts) *zhj-1036*::Tn*10*] as the donor to determine whether all of these phenotypes are due to a mutation in the *spoT* gene. The TRA-resistant, temperature-sensitive, and small-colony phenotypes associated with *spoT24*(Ts) were inseparable (data not shown), indicating that all three phenotypes are probably due to a single mutation in the *spoT* gene.

Since the *spoT24*(Ts) mutation was isolated in a *relA*$^+$ background, we wanted to know whether it was capable of suppressing the AT sensitivity phenotype of *relA1*. Therefore, we transduced the *spoT24*(Ts) mutation into TR3381 (*relA1*) selecting for Tet^r encoded by a Tn*10* element near *spoT* in the donor. Inheritance of *spoT24*(Ts) did indeed confer resistance to AT on the *relA1* recipient. Interestingly, the *relA1* allele suppressed both the temperature-sensitive and the small-colony phenotypes of *spoT24*(Ts). Reintroduction of the *relA*$^+$ allele restores temperature sensitivity and slow growth at permissive temperatures. This ability of a *relA* mutation to improve the growth of *spoT* mutants has also been observed in *E. coli* (20). Presumably, a reduction in ppGpp synthesis can reduce the accumulation of ppGpp and the concomitant ill effects of this accumulation. This is consistent with the fact that the *relA1* mutation reduces the basal level of ppGpp by 30% as compared to wild-type cells (Kenneth E. Rudd, unpublished data).

DISCUSSION

Mutations in the *spoT* gene of *S. typhimurium* are completely analogous to *E. coli spoT* mutations with respect to map location and physiological defects. The *spoT* gene encodes a ppGppase, and *spoT* mutants have elevated basal levels of ppGpp. Using isogenic *spoT*$^+$ and *spoT1* mutants, we were able to correlate elevated ppGpp levels with elevated *hisD* enzyme levels during conditions of steady-state growth. Mutations in the *spoT* gene also increase resistance to AT and TRA, two histidine antimetabolites.

At several points in this study it was noted that the effects of *spoT* on the *his* operon were more marked in medium containing 19 amino acids (no histidine). Presumably, this amino acid supplementation decreases the basal ppGpp concentration to a level significantly below saturation for its stimulatory effect on *his* expression. This decreases *his* operon expression and increases sensitivity to AT and TRA. The *spoT1* mutation elevates ppGpp back up to saturating levels and restores maximal *his* expression.

The effects of ppGpp on *his* operon expression provide new methods for the genetic manipulation of ppGpp metabolism. Growth of *relA* cells on AT is completely inhibited, regardless of cell density, growth temperature, or the medium in which cells to be tested are pregrown. Both *relA*$^+$ and *spoT1* transductants of *relA* strains can be selected directly on AT plates (K. E. Rudd and J. R. Roth, unpublished data). Spontaneous AT-resistant revertants of *relA* have been isolated and should include new mutants affecting either ppGpp levels or *his* enzyme levels (Rudd and Roth, unpublished data). Previously, SMG medium has been used in a similar way to identify *relA* strains, which grow slowly or not at all on this medium. Although SMG medium apparently induces starvation for isoleucine, the reason for this starvation is not known. The use of both media, however, provides an effective means of identifying mutational effects on ppGpp levels in that any mutation affecting ppGpp should be detectable on both media, whereas regulatory mutations specific to isoleucine or histidine will affect only one growth response. The phenotypic effects of *spoT1* and Δ*hisO1242* on *relA* strains illustrate this point (Tables 3 and 4).

The histidine analog TRA has been used previously to isolate *S. typhimurium* mutants with increased basal levels of the histidine biosynthetic enzymes (27), but no *spoT* mutations were identified. Here we report that *spoT* mutations of *S. typhimurium* confer TRA resistance to wild-type cells, but only in the presence of amino acids. Apparently, this enriched medium reduces ppGpp levels below the level that is required to saturate the *his* operon, and the *spoT* mutation can restore ppGpp levels. The fact that earlier

mutant hunts were not done on this medium explains why *spoT* mutants were not selected.

Mutations in the *relA* gene are thought to reduce *his* operon expression due to effects of lowered ppGpp on *his* promoter function. Superficially, this idea is contradicted by the observation that Δ*hisO1242* (an attenuator deletion) appears to suppress the defect of the *relA* mutant in *his* expression, at least under some conditions (Table 4). A closer look at the this phenomenon, however, reveals that *relA* effects on *his* expression are detectable even in a Δ*hisO1242* derivative, but the range of the effect is shifted. As seen in Table 4, in *hisO*$^+$ strains a concentration of 5 mM AT distinguishes *relA*$^+$ from *relA* strains. In a Δ*hisO1242* background 30 mM AT is required to distinguish *relA*$^+$ and *relA* strains, but they are still distinguishable. Apparently, at all levels of promoter function a substantial fraction of transcripts stop at the attenuator even in cells starved for histidine. Thus, even though the Δ*hisO1242* mutation increases *his* operon expression, a saturating level of intracellular ppGpp is still required to obtain maximal operon expression (Tables 2 and 5) and therefore maximal resistance to AT.

The Δ*hisOG1302* fusion eliminates the stimulation of *his* expression caused by either growth in minimal medium or by the presence of the *spoT1* allele (Table 5). This fusion removes the *his* promoter, the leader region, and part of the *hisG* gene. This renders the cell His$^-$, requiring the presence of exogenous histidine in all growth media. Addition of histidine to amino acid-supplemented medium reduces the *hisD* enzyme levels of TR6478 (*spoT*$^+$) and TR6479 (*spoT1*), but does not affect the stimulatory effect of *spoT1* (Table 5). Thus, the failure to see an effect of ppGpp on *his* expression in strains containing the Δ*hisOG1302* mutation cannot be due to the presence of exogenous histidine. Likewise, deletion of the leader region cannot explain the failure to respond to ppGpp because strains with the Δ*hisO1242* (attenuator-deleted) mutation (TT10033 and TT10034) respond normally (Table 5). Previous experiments have demonstrated that ppGpp control of *his* expression is unaffected by the deletion of *hisG* (36). Thus, the new promoter element fused to the *his* gene by Δ*hisOG1302* is most likely responsible for the loss of ppGpp control. Translation of the *hisD* mRNA should be unaffected by the Δ*hisOG1302* fusion. Therefore, the *spoT1*-mediated stimulation of *his* operon expression is not a translational effect, which is consistent with in vitro results (31).

The effect of *E. coli spoT1* on the expression of an F′ *his S. typhimurium* operon has been examined previously by Winkler et al. (37). Although they observed effects similar to those reported here when using the Δ*hisO1242* operon, they failed to see a stimulatory effect using the *hisO*$^+$ operon. This failure may have been due to either the episomal nature of the *his* operon or to differences between *E. coli* and *S. typhimurium*.

We have isolated many new *spoT* mutants in *S. typhimurium*. Among the mutants are strains with temperature-sensitive lethal and slow-growth phenotypes. This suggests that *spoT* is an essential gene. Currently available evidence also strongly suggests that the *spoT* gene of *E. coli* is essential for growth (3). This requirement for the *spoT* gene product can be demonstrated even in *relA* strains. This is probably due to alternative routes of ppGpp synthesis. These alternative routes apparently generate the near-normal ppGpp basal levels that are found in *relA* mutants of both *E. coli* (13) and *S. typhimurium* (31; unpublished data).

It has been proposed that the *relA1* mutation of *E. coli* arose as a spontaneous suppressor of the naturally occurring *spoT1* mutation (20). Our observations support that contention. In fact, during the isolation of *spoT*(Ts) mutants in a *relA*$^+$ background, two *spoT*(Ts) mutations were obtained that had also acquired spontaneous *relA* mutations, essentially mimicking the proposed evolution of the *E. coli relA1* mutation. We obtained intragenic and extragenic suppressors of the temperature-sensitive and slow-growth phenotypes of *spoT relA* double mutants (K. E. Rudd and J. R. Roth, manuscript in preparation). These should enable us to identify other genes, notably *relS* (11), involved in ppGpp synthesis. These mutants should include strains with reduced basal levels of ppGpp, allowing us to correlate ppGpp levels and *his* expression over a wider range than we demonstrated here.

The results presented in this study strongly support the idea that ppGpp is a positive regulator of *his* operon expression in vivo. This regulation probably takes place at the level of transcription initiation and might be part of a system of general amino acid control. The stimulation of *his* enzyme synthesis is independent of histidine-specific regulation mediated through the attenuator. Inclusion of amino acids other than histidine in the growth medium reduces *his* operon expression. It is not known how many other biosynthetic operons are stimulated by ppGpp, but a similar control of isoleucine biosynthesis has been demonstrated (34). It has also been suggested that methionine biosynthesis is positively regulated by ppGpp (30). The use of amino acid analogs has provided evidence that serine, aspartate, and tryptophan biosynthesis may also respond to increased levels of ppGpp (31). Possibly, this control has evolved as a way to coordinately regulate the various amino acid biosynthetic operons in response to general amino acid availability.

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