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Mutations That Render the Promoter of the Histidine Operon of *Salmonella typhimurium* Insensitive to Nutrient-Rich Medium Repression and Amino Acid Downshift

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The effects of mutations in the promoter of the histidine operon of *Salmonella typhimurium* were examined in vivo. The wild-type chromosomal copy of the *his* promoter was replaced with mutations in the −10 hexamer sequence and in the region between the −10 hexamer and the transcriptional start point—termed the discriminator sequence. The substitutions were performed with a phage M13 allele replacement system. Expression of the *his* operon is known to correlate with levels of guanosine 5′,3′-bispyrophosphate (ppGpp) in vivo. Strains containing either the wild-type *his* promoter or *his* promoter mutations were grown in both nutrient-rich and minimal media under steady-state conditions known to alter intracellular levels of ppGpp in a predictable way. The effect of the presence or absence of the *his* attenuator was assessed under these conditions as well. Expression of the *his* operon was studied by measuring the differential rate of β-galactosidase synthesis with a *his-lac* transcriptional fusion. Regulation of the *his* operon in the promoter mutants was also studied under conditions of a transient amino acid downshift induced by the addition of serine hydroxamate to cultures growing in nutrient-rich medium. These growth conditions cause elevated levels of ppGpp. The results provide physiological confirmation of previous evidence obtained with a coupled transcription-translation system in vitro which indicated that ppGpp regulates interaction of RNA polymerase at the *his* promoter. More specifically, the in vivo evidence shows that the region of the *his* promoter that includes the −10 hexamer and discriminator sequences is the target at which ppGpp stimulates transcription.

Expression of the histidine operon in the enteric bacteria *Salmonella typhimurium* and *Escherichia coli* is subject to two different control mechanisms; (i) an operon-specific attenuation mechanism and (ii) a global regulatory mechanism dependent on overall amino acid availability (1, 26). The attenuation mechanism is well characterized and is governed primarily by the levels of His-tRNA^{His} in the cell. When His-tRNA^{His} levels decline, *his* operon expression rises and vice versa. The global regulatory mechanism is less well understood, but the available evidence indicates that it is mediated by levels of the general signal molecule, or alarmone, guanosine 5′,3′-bispyrophosphate (also known as guanosine tetraphosphate or ppGpp). When levels of ppGpp rise, *his* operon expression is stimulated and vice versa.

The intracellular level of ppGpp depends on the activities of two enzymes that are the products of the *relA* and *spoT* genes (6, 29). The ribosome-associated RelA enzyme synthesizes ppGpp when uncharged tRNA occupies the ribosome's acceptor site (e.g., during amino acid downshift). The mechanism of regulation of the cytoplasmic SpoT enzyme is somewhat obscure (9, 19), but ppGpp accumulation as a function of SpoT activity appears to correlate inversely with the growth rate in response to the nutrient richness of the growth medium (5). In addition, SpoT synthesizing activity is inhibited during amino acid downshift concomitant with the activation of the RelA enzyme (13, 21).

Historically, the role of ppGpp as a regulatory molecule has been most closely associated with control of stable RNA (rRNA and tRNA) synthesis. Enteric bacteria use at least two regulatory systems, stringent control and growth rate-dependent control, to adjust stable RNA synthesis to amino acid availability and the steady-state growth rate, respectively. It is generally accepted that stringent control (shutoff of stable RNA synthesis during amino acid downshift) is mediated by ppGpp, although the mechanism is poorly understood. Whether or not ppGpp also mediates growth rate-dependent control is highly controversial (8, 11, 32).

Our point of view has been that the stimulatory effects of ppGpp on amino acid biosynthesis (and other things) are at least as important in the overall context of bacterial physiology as the inhibitory effects of ppGpp on stable RNA synthesis (and other things). The amino acid polyauxotrophic phenotype of a mutant (double null for *relA* and *spoT*) of *E. coli* (29) or *S. typhimurium* (31) that makes little or no ppGpp has provided strong support for this position. Although expression of many genes has been positively correlated with increased ppGpp levels (6), there has been surprisingly little detailed or mechanistic analysis in this area. To this end, we have been using the *his* operon as a model system to study how ppGpp stimulates gene expression in general (16, 17, 20–22).

Expression of the *his* operon has been correlated with ppGpp levels both in vivo (18, 21, 22, 27, 28) and with a DNA-dependent, coupled transcription-translation (S-30) system in vitro (17, 22). Experiments with the coupled system indicated that stimulation of *his* expression by ppGpp occurs at the level of transcription (22). Several mutant *his* promoters were constructed and tested for sensitivity to stimulation by ppGpp in the coupled system (17). Two of the mutations with an up promoter effect were found to render *his* expression nearly insensitive to ppGpp stimulation. Both mutations altered the region of the promoter melted by RNA polymerase in forming the open promoter complex. One of the mutations changed the sequence of the −10 hexamer, and the other mutation changed the region immediately downstream of the

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−10 hexamer, termed the discriminator sequence. The discriminator regions of stable RNA promoters have been implicated in stringent control and growth rate-dependent control and have a well-defined consensus sequence (GCGCCNC) (11, 23, 24). The wild-type *his* promoter discriminator is AT rich, as are the discriminators of some other putative ppGpp-stimulated promoters, but no obvious consensus sequence has been defined (17, 24).

The experiments described in this report were designed to address two important issues concerning the role of ppGpp in stimulating expression of the *his* operon. The first issue is whether promoter mutations in the −10 hexamer and discriminator regions found to alter ppGpp regulation in the coupled system in vitro have a corresponding physiological effect when tested in vivo. To address this issue, we recombined promoter mutations into the chromosomal *his* operon and tested their regulatory responses to medium richness during steady-state growth and to amino acid downshift conditions. The second issue has to do with the type of promoter mutation. As mentioned above, the two ppGpp-insensitive *his* promoter mutations analyzed in the coupled system both had an up promoter effect. It could be argued, therefore, that these mutations set *his* expression at a maximum level that masked the ability to observe any further stimulation by ppGpp (6). If this were true, then the target of ppGpp stimulation could be something other than transcription initiation. To address this issue, we constructed and analyzed a new discriminator mutation that has a down promoter effect.

Our results support the following major conclusions: (i) stimulation of *his* expression by ppGpp occurs at or near the *his* promoter, (ii) the *his* promoter region containing both the −10 hexamer and discriminator sequences is crucial for stimulation by ppGpp, and (iii) results obtained with the coupled system in vitro are physiologically relevant. Furthermore, the results support an independent finding (30) that the presence of a GC-rich consensus discriminator sequence is not sufficient for negative control by ppGpp. The *his* promoter with a GC-rich consensus discriminator was insensitive to stimulation by ppGpp but was not inhibited by it.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. The *S. typhimurium* strains used in this work were all derived from wild-type strain LT2 and are shown in Table 1. The nutrient-rich, complex medium used was LB medium (14) supplemented with 0.4% (wt/vol) glucose and 1× Vogel and Bonner salts (25). The minimal medium used was M9 (14) supplemented with 0.2% (wt/vol) glucose and 40 μg each of histidine hydrochloride and tryptophan per ml. All cultures were grown at 37°C with shaking. The media used for allele replacement (3) were 2× YT (14) containing 20 μg of chloramphenicol per ml to select integrants and nutrient agar (Difco) containing sodium deoxycholate (0.3%, wt/vol) to select segregants. MacConkey lactose agar was from Difco.

Construction of *his* promoter mutations. The *his* promoter mutations *hisGp3423*, *hisGp3424*, and *hisGp3425* were constructed in three successive steps by utilizing the *dut ung* method of oligonucleotide-directed mutagenesis (12). A kit for this purpose was obtained from Bio-Rad Laboratories. The procedure started with phage M13mp9::*his*5 (17), which carries a 3.1-kb fragment containing the beginning of the *S. typhimurium his* operon with a promoter mutation that converted the wild-type −10 hexamer to the consensus $E\sigma^{70}$ −10 hexamer (*hisGp3400*) (Fig. 1). Each mutation made served, in turn, to provide the DNA template for construction of the subsequent mutation. A stock of a uracil-incorporated, single-stranded DNA template was prepared at each step by using the phage to infect *dut ung* mutant strain RZ1032, growing the culture, and recovering progeny phage from the supernatant. An oligonucleotide primer containing the desired base changes was annealed to the template at a primer-template ratio of 5:1. Second-strand synthesis was performed in vitro by using phage T4 DNA polymerase and T4 DNA ligase, and the reaction products were used to transform dut^+ ung^+ strain MV1190. Several plaques were picked and purified. Double-stranded, replicative-form DNA was obtained and analyzed for the desired mutations. Following steps 1 and 2 (Fig. 1), the desired mutations were screened by determining the susceptibility of the replicative-form DNAs to cutting by the restriction endonuclease *Fsp*I; step 1 introduced an *Fsp*I site, and step 2 removed the site. All of the mutations were confirmed by direct sequencing of the single-stranded phage DNA templates.

TABLE 1. Bacterial strains used in this study[a]

Strain	Genotype	Source
AZ302	Δ*hisGe3419 trp*::Tn*5*/F′123 *finP301 trp*$^+$	Laboratory collection
AZ314	Φ(*hisD-lac*) Δ*hisGa1242 trp*::Tn*5*	Laboratory collection
AZ315	Same as AZ314 but *hisGa*$^+$	Laboratory collection
AZ316	*hisGp3367* Δ*hisGa1242* Φ(*hisD-lac*) *trp*::Tn*5*	Laboratory collection
AZ1460	Φ(*hisD-lac*) Δ*hisGa1242*	Reference 21
AZ7505	*hisGp3400 trp*::Tn*5*/F′123 *finP301 trp*$^+$	This work
AZ7506	*hisGp3424 trp*::Tn*5*/F′123 *finP301 trp*$^+$	This work
AZ7507	*hisGp3425 trp*::Tn*5*/F′123 *finP301 trp*$^+$	This work
AZ7516	*hisGp3400* Φ(*hisD-lac*) *trp*::Tn*5*	This work
AZ7517	Same as AZ7516 but Δ*hisGa1242*	This work
AZ7518	Same as AZ7516 but *hisGp3424*	This work
AZ7519	Same as AZ7518 but Δ*hisGa1242*	This work
AZ7520	Same as AZ7516 but *hisGp3425*	This work
AZ7521	Same as AZ7520 but Δ*hisGa1242*	This work

[a] The Φ(*hisD-lac*) transcriptional fusion carried by some of the strains resulted from an immobilized Mu d1 *c*ts(Ap^r *lac*) insertion that has already been described (2, 20, 21). The immobilization procedure (2) allowed the fusion to be moved by phage P22 generalized transduction for strain constructions and rendered the strains heat resistant so that growth experiments could be done at 37°C. The fusion previously was designated Φ(*hisGD-lac*) (20, 21) because, based only on a histidinol-negative growth phenotype, the location of the insertion was imprecisely known. Direct sequencing of *his* DNA PCR amplified from strain AZ1460 has since determined that the Mu d1 *c*ts(Ap^r *lac*) insertion lies within *hisD* with the left endpoint of the insertion 151 bp downstream of the *hisD* translation initiation codon (15).

Allele replacement. To efficiently recombine the promoter mutations into the *S. typhimurium* chromosome, the 3.1-kb *his* DNA fragments containing the different mutations were first subcloned into the M13mp11*cat* phage (3). The M13mp10*cat* and M13mp11*cat* phage vectors allow any gene that has been cloned into the phage multiple cloning site to be integrated into the homologous region of the bacterial chromosome by selecting for resistance to chloramphenicol. Segregants were selected by resistance to the bile salt sodium deoxycholate. Details of the events associated with the allele replacement have already been described (3).

The recipient used in the allele replacement, strain AZ302, contained a deletion in the *his* leader region extending from and including positions +20 to +89 with respect to the transcriptional start point (designated +1). This deletion, Δ*hisGe3419*, causes a tight His$^-$ phenotype and allowed easy screening of His$^+$ segregants in which the deletion had been replaced. Strain AZ302 was grown in LB broth for 5 h at 37°C to mid-log phase. Samples (0.1 ml) of the culture were dispensed into small, sterile glass test tubes, and 0.1-ml volumes of the recombinant M13mp11*cat* phage donors were added to the tubes to give a multiplicity of infection in the range of 10 to 100. The tubes were incubated for 1 h on a 37°C shaker, after which 0.1 ml of each mixture was spread onto 2×YT-chloramphenicol (20 μg/ml) plates. The plates were incubated overnight at 37°C, by which time integrant colonies appeared. The donors and recipient were spread separately on the same medium, and no colonies appeared on these control plates.

Several chloramphenicol-resistant colonies were picked at random from each cross and streaked directly onto nutrient agar plates containing 0.3% sodium deoxycholate to select segregants that had lost the M13mp11*cat* phage. The plates were incubated overnight at 37°C, and isolated colonies were picked and streaked onto LB plates for single-colony purification. The segregants were retested for loss of the M13mp11*cat* recombinant phage on the chloramphenicol-containing medium, as well as for the ability to grow on minimal medium lacking histidine. The ability of the segregants to grow on minimal medium was a good indication of successful allele replacement, since the recipient strain, AZ302, is a *his* auxotroph. Based on the results of experiments that measured relative expression of the different mutant *his* promoters in vitro (7), segregants that had retained either the *hisGp3400* or the *hisGp3424* mutant promoter were expected to grow well on minimal medium lacking histidine. An exception was the *hisGp3425* promoter mutant, which had low *his* expression in vitro and, in fact, grew poorly without histidine. To verify the presence of the promoter mutations, the *his* promoter regions in representative segregants were PCR amplified and the DNA was sequenced as described below. The strains carrying the three mutant promoters that resulted from the allele replacement procedure were designated AZ7505, AZ7506, and AZ7507 (Table 1).

Fusion of Mu d1 *c*ts(Ap^r *lac*) to *his* promoter mutations for measurement of *his-lac* expression. Donor P22 phage lysates were made on strains AZ316 and AZ1460. Both donor strains contain the Δ*hisGa1242* mutation and an immobilized Mu d1 *c*ts(Ap^r *lac*) insertion in the *hisD* gene [Φ(*hisD-lac*); Table 1].

MUTANT -10 AND WILD-TYPE DISCRIMNATOR

-10 +1
5'-GTGGTTTATAATAAAAGGTATCAAATGAATAA-3' ***hisGp3400***

3'-CCAAATATTA**CGCG**TCCATAG-5' primer

↓ STEP 1

MUTANT -10 AND MUTANT DISCRIMINATOR

*Fsp*I
-10 ↓ +1
5'-GTGGTTTATAATGCGCAGGTATCAAATGAATAA-3' ***hisGp3423***

3'-CAAATATTACGCG**GGG**TAGTTTAC-5' primer

↓ STEP 2

MUTANT - 10 AND MUTANT DISCRIMINATOR

-10 +1
5'-GTGGTTTATAATGCGCCCCATCAAATGAATAA-3' ***hisGp3424***

3'-CAAAT**CCA**ACGCGGGGTAGTTTAC-5' primer

↓ STEP 3

WILD-TYPE -10 AND MUTANT DISCRIMINATOR

-10 +1
5'-GTGGTTTAGGTTGCGCCCCATCAAATGAATAA-3' ***hisGp3425***

FIG. 1. Construction of mutations *hisGp3423*, *hisGp3424*, and *hisGp3425* by oligonucleotide-directed mutagenesis. The top strand in each step represents the uracil-incorporated template strand, and the bottom strand represents the oligonucleotide primer, containing the desired base changes (in boldface). Each mutant, in turn, served as the template for the subsequent mutant (progressing from step 1 to step 3; *hisGp3400-hisGp3423-hisGp3424-hisGp3425*). The arrow points to the cleavage site in the *Fsp*I recognition sequence (5′-TGCGCA-3′) that served to identify the *hisGp3423* mutation after step 1. The −10 region and the transcriptional start point (+1) are underlined.

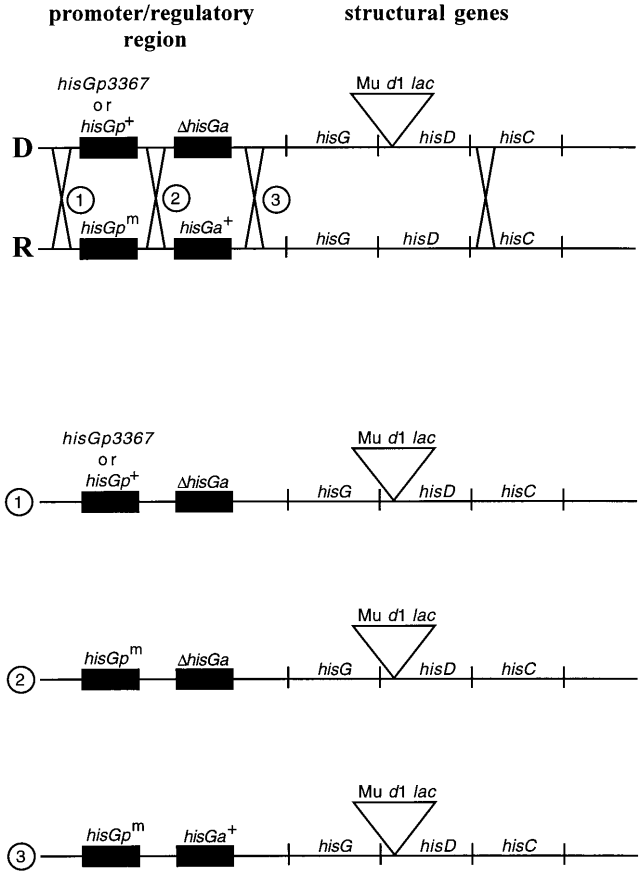

FIG. 2. Recombination events associated with construction of *his-lac* fusions. Donor P22 phage lysates (D) were made on strains AZ1460 and AZ316 and were either wild type for the *his* promoter or carried the *hisGp3367* down promoter mutation, respectively. Both strains AZ1460 and AZ316 contained the *hisGa1242* attenuator deletion and an Mu d1 *c*ts(Ap^r *lac*) insertion in the *hisD* gene. Recipient strains (R) AZ7505, AZ7506, and AZ7507 each contained one of the three promoter mutations (*hisGp*^{m} represents *hisGp3400*, *hisGp3424*, or *hisGp3425*) described in Materials and Methods. In every case, the rightmost crossover was selected but there were three possible locations for the second crossover event (shown as encircled numbers 1, 2, and 3). The recombinants resulting from the different crossover events are shown at the bottom. Crossovers 2 and 3 produced the desired recombinants. In these cases, the recipient promoters (*hisGp*^{m}) were retained and the *hisGa1242* deletion was inherited together with the Mu d1 *c*ts(Ap^r *lac*) insertion in crossover 2 but not in crossover 3, which resulted in inheritance of the wild-type attenuator site.

Whereas strain AZ1460 is wild type for the *his* promoter, strain AZ316 has a strong promoter down mutation (*hisGp3367*) which is a substitution at the sixth position of the −10 hexamer (20). Consequently, strain AZ316 forms white colonies when plated on MacConkey lactose agar while strain AZ1460 forms dark red colonies. The rationale for using these two donor lysates was based on the results of in vitro experiments which indicated that the *hisGp3400* and *hisGp3424* mutations gave high levels of *his* expression while the *hisGp3425* mutation resulted in low levels of *his* expression (7). Crossing the AZ7505 (*hisGp3400*) and AZ7506 (*hisGp3424*) recipients with the P22/AZ316 lysate and the AZ7507 (*hisGp3425*) recipient with the P22/AZ1460 lysate allowed the color phenotypes of the desired recombinants to be distinguished from the respective donor color phenotype on MacConkey plates. The recipient strains AZ7505, AZ7506, and AZ7507 were grown for 3 h at 37°C in LB broth, and 0.1 ml of each culture was applied to a MacConkey-lactose-ampicillin (50 μg/ml) plate. A sample (5 to 100 μl) of the appropriate phage P22 donor lysate (about 10^{10} PFU/ml) was added to the cells, and the mixture was spread. After the plates had dried, they were incubated overnight at 37°C, by which time transductants appeared. Figure 2 shows the various recombinants that can arise from the transductional crosses. Since the selection was for ampicillin resistance, all of the transductants that arose from the cross must have received the Mu d1 *c*ts(Ap^r *lac*) genes. However, depending on the location of the second crossover, *his* promoter mutations may segregate with or without the Δ*hisGa1242* mutation.

In the AZ7505 and AZ7506 (recipient) × P22/AZ316 (donor) transduction, crossovers 2 and 3 resulted in dark red and light red recombinant colonies, respectively. For both of these crossovers, the mutant promoter of the recipient was retained and the attenuator deletion was inherited from the donor in crossover 2 but not in crossover 3; both recombinant classes were distinguishable from the donor class (crossover 1), which gave white colonies due to inheritance of the *hisGp3367* mutation from the donor. In the AZ7507 (recipient) × P22/AZ1460 (donor) transduction, crossovers 2 and 3 gave rise to pink and white colonies, respectively. Pink colonies were due to inheritance of the Δ*hisGa1242* mutation, whereas white recombinants retained the wild-type attenuator. Both classes were distinguishable from the donor class (crossover 1), which gave dark red colonies.

Fourteen colonies representing each of the six recombinant classes of interest (*hisGp3400*, *hisGp3424*, and *hisGp3425*, each combined with either the wild-type attenuator or the Δ*hisGa1242* mutation) were chosen, and genomic DNA was isolated and PCR amplified (see below). The PCR-amplified DNAs were sequenced to verify the mutations present. All six recombinant classes were obtained, and 11 of the 14 templates sequenced were of the predicted genotype; the remaining three recombinants apparently resulted from quadruple crossover events. Strains AZ7516 through AZ7521 (Table 1) are the representatives of the six recombinant classes that were used to study *his* expression as a function of

β-galactosidase synthesis in the *lac* fusion derivatives. F′123 was segregated from these strains following growth in media nonselective for retention of the episome.

β-Galactosidase assays with samples obtained during steady-state growth and following serine hydroxamate addition. Strains were grown in 2 ml of nutrient-rich medium or minimal medium with required supplements at 37°C overnight and then subcultured into 25 ml of the same medium to give an initial optical density at 650 nm (OD_{650}) of about 0.01. β-Galactosidase specific activities were determined from the slopes of differential rate plots of five samples taken in approximately equivalent incremental steps between OD_{650}s of about 0.1 and 0.5 for cultures in steady-state growth. A mild downshift condition was induced by addition of DL-serine hydroxamate (Sigma) to a concentration of 2 mM (21). Serine hydroxamate was prepared as a 0.5 M stock solution immediately prior to use and kept on ice. The analog was added to cultures in exponential growth in nutrient-rich medium at an OD_{650} of about 0.15. Samples were taken every 2 min for 0.5 h and every 10 min thereafter for the next 0.5 h following addition of serine hydroxamate. β-Galactosidase assays were performed and specific activities were calculated as described previously (21).

Preparation of DNA templates, PCR amplifications, and DNA sequencing. Genomic DNA was isolated by the following method (15). Cultures were grown in 2 ml of LB broth supplemented with 1.2% (wt/vol) glucose and 0.7× Vogel and Bonner salts and incubated overnight at 37°C. A sample (1.5 ml) of the culture was then centrifuged for 5 min in a microcentrifuge, and the supernatant was discarded. The cell pellet was resuspended in 567 μl of TE buffer (14), 30 μl of 10% sodium dodecyl sulfate plus 3 μl of 20 mg/ml proteinase K (Sigma) was added, and the tubes were incubated for 1 h at 37°C. Proteinase K was denatured by incubating the tubes for 5 min at 80°C, and the lysate was treated with 5 μl of 10-mg/ml DNase-free RNase A (Sigma) for a further 15 min at 37°C. The DNA was purified of protein by phenol-chloroform extraction (1:1, vol/vol) and precipitated with isopropanol. DNA pellets were washed with 70% ethanol, dried, and resuspended in 0.5 ml of TE buffer.

Two primers were designed to amplify the *his* region by PCR. The primers were spaced about 1,500 bp apart. Primer XAV1, a 20-mer, has the sequence 5′-CCTGCATCGCCTGCAGATAA-3′ and was designed to anneal to the template strand approximately 400 bp upstream of the *his* transcriptional start point. The sequence contains a restriction site for the enzyme *Pst*I (5′-CTGCAG-3′). Primer XAV2, a 26-mer, has the sequence 5′-GGTCTAGACAGTACCAGAA TCGAGCT-3′ and was designed to anneal to the nontemplate strand approximately 1,100 bp downstream of the transcriptional start site. A restriction site for the enzyme *Xba*I (5′-TCTAGA-3′) was designed into the primer, near its 5′ end. PCRs were performed under standard conditions by using components of the Perkin-Elmer GeneAmp DNA Amplification Reagent Kit. Samples of the amplified reactions were electrophoresed to verify the product size. The PCR-amplified DNAs were purified by using Amicon 100 Microconcentrators (W. R. Grace & Co.) and sequenced by using Sequenase Version 2.0 enzyme (United States Biochemical). The template DNAs were sequenced by using a primer-template molar ratio of about 20:1. The primers used to sequence the *his* promoters have already been reported (17). A region of DNA encompassing approximately 200 nucleotides downstream and 100 nucleotides upstream was sequenced for each mutant construct to ensure that genetic and biochemical manipulations did not introduce extraneous mutations that might affect *his* expression. In particular, the presence of the wild-type attenuator site or the Δ*hisGa1242* mutation was determined for each strain.

RESULTS

Expression of wild-type and mutant *his* promoters under steady-state conditions in minimal and nutrient-rich media. Figure 3 and Table 2 show the effects on *his* expression of growing strains containing either the wild-type *his* promoter or *his* promoter mutations in a minimal medium versus a nutrient-rich, complex medium. The data in Table 2 are summarized as specific activities which were taken from the slopes of differential rate plots of β-galactosidase accumulation obtained during balanced, steady-state growth (21). Representative examples of the differential rate plots for cultures grown in minimal and nutrient-rich media are shown in Fig. 3, which gives results for strain AZ314 carrying the wild-type *his* promoter and strain AZ7521 carrying one of the promoter mutations (*hisGp3425*). Linearity of the data and extrapolation of the straight lines approximately to the origin of the graph indicate that the cultures, in fact, approached balanced growth and that β-galactosidase accumulated as a constant proportion of cell mass during the experiment. All of the strains grew with very similar generation times in the nutrient-rich medium (28 min) and in the minimal medium (55 min).

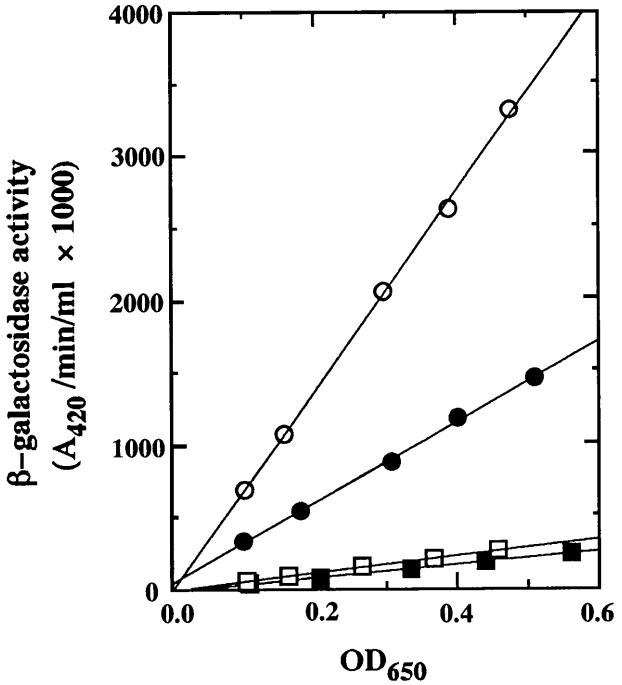

FIG. 3. Differential rates of *his* expression obtained with strains AZ314 (wild-type promoter [○, ●]) and AZ7521 (*hisGp3425* mutant promoter [□, ■]). The strains were grown in minimal medium (open symbols) or nutrient-rich medium (solid symbols).

To simplify interpretation of the data, experiments reported in Fig. 3 and Table 2 were done with strains completely missing the operon-specific attenuation mechanism owing to deletion of the attenuator site by the Δ*hisGa1242* mutation. Thus, these experiments measured only attenuator-independent regulatory events. Results obtained with the attenuator present will be addressed subsequently.

Expression of the *his* operon by strain AZ314 (*hisGp*$^+$) was about 2.5-fold lower in nutrient-rich medium than in minimal medium (Fig. 3 and Table 2). In replicate experiments, the repression ratio varied from about 2.5-fold to 3.5-fold. This moderate fluctuation was observed to result primarily from error in the measurement of the differential rate of β-galactosidase synthesis in rich medium, apparently because of the constant depletion of nutrients in this medium, especially at higher ODs. The magnitude of the regulatory effect correlates well with the change in steady-state ppGpp levels found in strains grown under similar conditions (5). In contrast, regulation of *his* expression in strains AZ7517, AZ7521, and AZ7519 was relatively unaffected by the nutrient richness of the growth medium. Strain AZ7517 carries a mutation (*hisGp3400*) that converts the wild-type *his* promoter −10 hexamer to the consensus sequence for interaction with Eσ^{70} RNA polymerase (i.e., 5′-TAGGTT → 5′-TATAAT). This mutation was previously shown to render the *his* operon relatively insensitive to ppGpp stimulation in the DNA-dependent, coupled transcription-translation (S-30) system in vitro (17). In vivo, the *hisGp3400* mutant gave somewhat elevated *his* expression in minimal medium compared to the *hisGp*$^+$ strain and was highly resistant to repression in nutrient-rich medium (decrease of about 23%; Table 2). Strain AZ7521 carries a mutation (*hisGp3425*) that converts the wild-type, AT-rich *his* discriminator sequence to a GC-rich sequence (i.e., 5′-AAAAGGT → 5′-GCGCCCC). This mutation resulted in a

TABLE 2. Effects of promoter mutations on differential rates of *his* expression in attenuator-deleted strains in minimal medium and nutrient-rich medium

Strain	Relevant DNA sequence[a]		β-Galactosidase sp act[b]	
	−10 Hexamer	Discriminator	Minimal medium	Nutrient-rich medium
AZ314	TAGGTT (wt)	AAAAGGT (wt)	6,895	2,757
AZ7517	TATAAT	AAAAGGT (wt)	8,052	6,221
AZ7521	TAGGTT (wt)	GCGCCCC	556	427
AZ7519	TATAAT	GCGCCCC	10,865	8,502

[a] DNA sequences of the wild-type (wt) promoter elements are indicated.
[b] β-Galactosidase activities, reported as specific activities with units of A_{420} per minute per unit of $OD_{650} \times 1{,}000$, were obtained as the slopes of differential rate plots (see Materials and Methods).

strong down promoter effect. The *hisGp3425* mutant was also highly resistant to repression in nutrient-rich medium, showing about 23% lower *his* operon expression in the latter medium than in minimal medium (Fig. 3 and Table 2). The *hisGp3425* mutation has recently been shown to render *his* expression relatively insensitive to ppGpp stimulation in the coupled system in vitro (7). Strain AZ7519 carries the *hisGp3424* mutation, which combines the sequence changes of the *hisGp3400* and *hisGp3425* mutations. The *hisGp3424* mutant promoter was resistant to nutrient-rich medium repression, similar to the single mutants, and *his* expression was at about the *hisGp3400* level found in strain AZ7517 (Table 2).

Table 3 shows the regulatory effects on *his* expression of growth in minimal medium versus nutrient-rich medium with strains isogenic to those in Table 2, except for the presence of the wild-type attenuator site. Expression of the *his* operon in Table 3, therefore, is much lower than that observed in Table 2 because of repression caused by the attenuation mechanism. Repression by attenuation in minimal medium was about 10-fold for strains with the wild-type promoter (compare strain AZ314 [6,895 U/OD_{650}; Table 2] to strain AZ315 [676 U/U of OD_{650}; Table 3]).

Nutrient-rich medium repression of *his* expression in strain AZ315 (Table 3) is a combined effect of attenuation and promoter regulation. Expression of the *his* operon by strain AZ315 (wild-type promoter, wild-type attenuator) was about 13-fold lower in nutrient-rich medium than in minimal medium. Strains AZ7516, AZ7520, and AZ7518, which carry promoter/regulatory mutations in the −10 and/or discriminator regions, showed reduced nutrient-rich medium regulation (3-fold to 4.5-fold) because only the attenuation mechanism was working in these strains. The results of Tables 2 and 3, taken together, are consistent with the interpretation that the steady-state, 13-fold nutrient-rich medium repression in strain AZ315 results from additivity of two regulatory phenomena: ppGpp-dependent promoter regulation (about 2.5- to 3.5-fold) and attenuation (about 3- to 4.5-fold).

We assume that repression via the attenuation mechanism in nutrient-rich medium is due primarily to increased tRNA charging and growth rate-dependent changes in the relative rates of transcription and translation (5) with consequential effects on formation of the alternative RNA secondary structures that govern the mechanism (10). It is unlikely that changes in ppGpp levels directly influence *his* attenuation in vivo, since we have shown that the presence or absence of the attenuator does not significantly alter regulation by ppGpp in the DNA-dependent transcription-translation system in vitro (17).

Effects of promoter mutations on differential rates of *his* expression following addition of serine hydroxamate to elicit the stringent response. Serine hydroxamate is a competitive inhibitor of seryl-tRNA synthetase and elicits the stringent response by mimicking an amino acid downshift and provoking *relA*-dependent synthesis of ppGpp (6, 21). We previously described in detail the close correlation between attenuator-independent *his* expression and ppGpp levels during downshift caused by serine hydroxamate addition to cultures of *S. typhimurium* strains containing the wild-type *his* operon promoter (21). In those experiments, as well as the ones reported here, a moderate concentration of serine hydroxamate was used to allow growth to continue so that differential rates of enzyme synthesis could be monitored accurately during the downshift (21).

Table 4 shows the effects on *his* operon expression of adding serine hydroxamate to strains containing wild-type or mutant *his* operon promoters. All strains contained the Δ*hisGa1242* attenuator deletion mutation. At the time of serine hydroxamate addition, the cultures were growing exponentially in nutrient-rich, complex medium. Samples for β-galactosidase assays were taken periodically before and after serine hydroxamate addition to establish preshift and postshift differential rates of *his* expression. The preshift differential rates in Table 4 are close to those shown in Table 2 for the same strains grown under steady-state conditions in nutrient-rich medium.

Following serine hydroxamate addition, the differential rate in strain AZ314 (wild-type promoter) increased fourfold, from a preshift level of 2,434 U/OD_{650} to a postshift level of 10,285 U/OD_{650} (Table 4). This increase in *his* operon expression

TABLE 3. Effects of promoter mutations on differential rates of *his* expression with attenuation in minimal medium and nutrient-rich medium[a]

Strain	Relevant DNA sequence		β-Galactosidase sp act	
	−10 Hexamer	Discriminator	Minimal medium	Nutrient-rich medium
AZ315	TAGGTT (wt)	AAAAGGT (wt)	676	52
AZ7516	TATAAT	AAAAGGT (wt)	2,105	625
AZ7520	TAGGTT (wt)	GCGCCCC	42	9
AZ7518	TATAAT	GCGCCCC	2,502	827

[a] See the footnotes to Table 2.

TABLE 4. Effects of promoter mutations on differential rates of *his* expression in attenuator-deleted strains following addition of serine hydroxamate[a]

Strain	Relevant DNA sequence		β-Galactosidase sp act	
	−10 Hexamer	Discriminator	Preshift	Postshift
AZ314	TAGGTT (wt)	AAAAGGT (wt)	2,434	10,285
AZ7517	TATAAT	AAAAGGT (wt)	6,569	10,188
AZ7521	TAGGTT (wt)	GCGCCCC	447	506
AZ7519	TATAAT	GCGCCCC	6,538	9,363

[a] Strains were grown in nutrient-rich medium at 37°C, and 2 mM DL-serine hydroxamate was added at an OD_{650} of about 0.15. Sampling for β-galactosidase assays was done as described in Materials and Methods. For other information, see the footnotes to Table 2.

correlates closely to that observed previously for a strain containing the wild-type *his* promoter (21). In contrast, the differential rates of *his* expression increased much less in the *his* promoter mutants following serine hydroxamate addition: strain AZ7517 gave about a 55% increase, strain AZ7521 gave about a 14% increase, and strain AZ7519 gave a 43% increase. These results show that *his* expression in the −10 hexamer and discriminator *his* promoter mutants is quite insensitive to shift conditions that increase levels of ppGpp.

DISCUSSION

Previous evidence established a direct correlation between the level of *his* operon expression and the concentration of ppGpp both in vivo (18, 21, 22, 27, 28) and in vitro (17, 22). The correlation persisted in the absence of the *cis*-acting attenuator site, indicating that some regulatory mechanism other than the attenuation mechanism is involved. In addition, evidence obtained in vitro suggested the *his* promoter as the site at which the regulatory response to ppGpp occurs (17). The latter evidence was based on the finding that certain mutations that altered a domain of the *his* promoter, including the −10 hexamer and the adjacent discriminator sequence, greatly diminished the stimulation of *his* expression by ppGpp in a DNA-dependent, coupled transcription-translation (S-30) system (17). Whereas expression of the *his* operon containing the wild-type promoter was stimulated 10- to 20-fold by optimal ppGpp concentrations in the coupled system (17, 22), that of the promoter mutants were stimulated less than 2-fold (17).

Results reported here demonstrate for the first time that well-defined *his* promoter mutations disrupt the correlation between the level of *his* operon expression and the concentration of ppGpp in vivo. The results, therefore, provide important physiological evidence to supplement the evidence of the effects of *his* promoter mutations on ppGpp regulation in vitro. The results also lend added support to the conclusion that ppGpp regulates RNA polymerase interaction with the *his* promoter and point specifically to the region of the promoter containing the −10 hexamer and discriminator sequences as the target of ppGpp stimulation (17).

Analysis of the properties of the *hisGp3425* mutation alleviates a reasonable objection to the interpretation that ppGpp regulates *his* expression at the level of RNA polymerase interaction with the promoter (6). The objection was based on the observation that the mutant promoters found to be ppGpp-insensitive in vitro resulted from up promoter mutations (17). It could be argued, therefore, that the coupled system expressed the mutant promoters at the maximum capacity of the system, thus masking any further stimulation of *his* expression by ppGpp. If this were the case, then stimulation by ppGpp could occur at some step of protein synthesis other than RNA polymerase interaction with the promoter. The *hisGp3425* mutation resulted in a strong down promoter effect and rendered attenuator-independent *his* expression insensitive to growth conditions that change ppGpp levels (Fig. 3 and Tables 2 and 4). In agreement with these in vivo results, the *hisGp3425* mutant promoter has been found to be ppGpp insensitive in the coupled system in vitro despite being expressed at a low level compared to the wild-type *his* promoter (7). The behavior of the *hisGp3425* mutant promoter, therefore, can most readily be explained by a defect in ppGpp regulation at or near the promoter.

Despite the validity of the conclusion described above, the data in Tables 2 to 4 are, interestingly, consistent with the interpretation that the combined effects of the Δ*hisGa1242* attenuator mutation and the *hisGp3400* up promoter mutation cause the *his* operon to be expressed in vivo at a level approaching a maximum. This maximum apparently corresponds to a level of β-galactosidase specific activity in the range of about 8,000 to 10,000 U/OD_{650} under the conditions analyzed. The up promoter effect of the *hisGp3400* mutation, by itself, was about threefold in minimal medium (compare strain AZ315 [676 U/OD_{650}] to strain AZ7516 [2105 U/OD_{650}] in Table 3). When the effect of the *hisGp3400* mutation was measured in the attenuator deletion background, however, the up promoter effect was reduced to less than 20% (compare strain AZ314 [6,895 U/OD_{650}) to strain AZ7517 [8,052 U/OD_{650}] in Table 3). This lack of additivity suggests that some step in β-galactosidase synthesis, other than initiations by RNA polymerase, became limiting when the *hisGp3400* mutation was combined with the attenuator deletion. The limiting step could be, for example, transcription elongation or a step in translation. In addition, the specific activity of β-galactosidase achieved during nutritional downshift was about 10,000 U/OD_{650} (Table 4), which is consistent with the interpretation that this level is near the maximum level possible. (For comparison, the corresponding minimum level of *his* expression in the absence of ppGpp [measured in a double-null *relA* and *spoT* mutant] is about 1,000 U/OD_{650} [31]. The overall regulatory range of the ppGpp stimulatory effect at the *his* promoter in vivo is therefore about 10-fold.)

There is an interesting relationship between the *hisGp3400* and *hisGp3425* mutations. The data in Tables 2 to 4 and Fig. 3 all indicate that *hisGp3400* (in strains AZ7516 and AZ7517) is an up promoter mutation, while *hisGp3425* (in strains AZ7520 and AZ7521) is a strong down promoter mutation. However, when the two mutations were combined to make a new mutation, *hisGp3424* (in strains AZ7518 and AZ7519), the effect on *his* expression mimicked that of the *hisGp3400* mutation. That is, *hisGp3400* is epistatic to *hisGp3425*. Assuming that the *his* transcription start site remains unaltered by the promoter mutations introduced, this epistatic relationship is consistent with the classical bacterial genetic interpretation (4) that *hisGp3400* and *hisGp3425* alter different steps in a common pathway and that the step altered by *hisGp3400* precedes that altered by *hisGp3425*. In biochemical terms, the epistatic relationship between the two mutations may be explained by the idea that melting of *his* promoter DNA by RNA polymerase proceeds in at least two steps, with the first step being initiated within the −10 hexamer and the second step progressing into the discriminator region. It will be interesting to analyze this relationship in more detail with in vitro techniques.

Finally, it is interesting that the *hisGp3425* mutation converted the wild-type *his* discriminator sequence (5′-AAAAG GT) to the consensus sequence (5′-GCGCCNC) normally as-

sociated with promoters that are ppGpp inhibitable (11, 23, 24, 32). Although the *hisGp3425* mutation caused a down promoter effect and rendered *his* expression insensitive to ppGpp stimulation (similar to a previously studied up promoter, *his* discriminator mutation [17]), the mutation did not introduce negative control. Thus, our results are consistent with other evidence indicating that the GC-rich consensus discriminator is necessary but not sufficient for negative control by ppGpp (30). How the discriminator sequences of promoters that are susceptible to stimulation or inhibition by ppGpp contribute to this important regulation remains to be determined.

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REFERENCES

1. **Artz, S. W., and D. Holzschu.** 1983. Histidine biosynthesis and its regulation, p. 379–404. *In* K. M. Herrmann and R. L. Somerville (ed.), Amino acids: biosynthesis and genetic regulation. Addison-Wesley Publishing Co., New York, N.Y.
2. **Blum, P., L. Blaha, and S. Artz.** 1986. Reversion and immobilization of phage Mu *d*1 *cts* (Ap^r *lac*) insertion mutations in *Salmonella typhimurium*. Mol. Gen. Genet. **202:**327–330.
3. **Blum, P., D. Holzschu, H.-S. Kwan, D. Riggs, and S. Artz.** 1989. Gene replacement and retrieval using recombinant M13mp bacteriophages. J. Bacteriol. **171:**538–546.
4. **Botstein, D., and R. Maurer.** 1982. Genetic approaches to the analysis of microbial development. Annu. Rev. Genet. **16:**61–83.
5. **Bremer, H., and P. P. Dennis.** 1987. Modulation of chemical composition and other parameters of the cell by growth rate, p. 1527–1542. *In* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology. American Society for Microbiology, Washington, D.C.
6. **Cashel, M., and K. Rudd.** 1987. The stringent response, p. 1410–1438. *In* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology. American Society for Microbiology, Washington, D.C.
7. **Da Costa, X. J., D. Riggs, and S. Artz.** Unpublished results.
8. **Gaal, T., and R. L. Gourse.** 1990. Guanosine 3′-diphosphate 5′-diphosphate is not required for growth rate-dependent control of rRNA synthesis in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **87:**5533–5537.
9. **Gentry, D. R., and M. Cashel.** 1995. Cellular localization of the *Escherichia coli* SpoT protein. J. Bacteriol. **177:**3890–3893.
10. **Johnston, H. M., W. M. Barnes, F. G. Chumley, L. Bossi, and J. R. Roth.** 1980. Model for regulation of the histidine operon of *Salmonella*. Proc. Natl. Acad. Sci. USA **77:**508–512.
11. **Josaitis, C. A., T. Gaal, and R. L. Gourse.** 1995. Stringent control and growth-rate dependent control have nonidentical promoter sequence requirements. Proc. Natl. Acad. Sci. USA **92:**1117–1121.
12. **Kunkel, T. A., J. D. Roberts, and R. A. Zakour.** 1987. Rapid and efficient site-specific mutagenesis without phenotypic selection. Methods Enzymol. **154:**367–382.
13. **Lagosky, P. A., and F. N. Chang.** 1980. Influence of amino acid starvation on guanosine 5′-diphosphate 3′-diphosphate basal-level synthesis in *Escherichia coli*. J. Bacteriol. **144:**499–508.
14. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
15. **Ness, J., and S. Artz.** Unpublished results.
16. **Primakoff, P., and S. W. Artz.** 1979. Positive control of *lac* operon expression *in vitro* by guanosine 5′-diphosphate 3′-diphosphate. Proc. Natl. Acad. Sci. USA **76:**1726–1730.
17. **Riggs, D. L., R. D. Mueller, H.-S. Kwan, and S. W. Artz.** 1986. Promoter domain mediates guanosine tetraphosphate activation of the histidine operon. Proc. Natl. Acad. Sci. USA **83:**9333–9337.
18. **Rudd, K. E., B. R. Bochner, M. Cashel, and J. R. Roth.** 1985. Mutations in the *spoT* gene of *Salmonella typhimurium*: effects on *his* operon expression. J. Bacteriol. **163:**534–542.
19. **Seyfzadeh, M., J. Keener, and M. Nomura.** 1993. *spoT* dependent accumulation of guanosine tetraphosphate in response to fatty acid starvation in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **90:**11004–11008.
20. **Shand, R. F., P. H. Blum, D. L. Holzschu, M. S. Urdea, and S. W. Artz.** 1989. Mutational analysis of the histidine operon promoter of *Salmonella typhimurium*. J. Bacteriol. **171:**6330–6337.
21. **Shand, R. F., P. H. Blum, R. D. Mueller, D. L. Riggs, and S. W. Artz.** 1989. Correlation between histidine operon expression and guanosine 5′ diphosphate–3′-diphosphate levels during amino acid downshift in stringent and relaxed strains of *Salmonella typhimurium*. J. Bacteriol. **171:**737–743.
22. **Stephens, J. C., S. W. Artz, and B. N. Ames.** 1975. Guanosine 5′-diphosphate 3′-diphosphate (ppGpp): positive effector for histidine operon transcription and general signal for amino-acid deficiency. Proc. Natl. Acad. Sci. USA **72:**4389–4393.
23. **Travers, A. A.** 1980. Promoter sequence for stringent control of bacterial ribonucleic acid synthesis. J. Bacteriol. **141:**973–976.
24. **Travers, A. A.** 1984. Conserved features of coordinately regulated *E. coli* promoters. Nucleic Acids Res. **12:**2605–2618.
25. **Vogel, H., and D. Bonner.** 1956. Acetylornithinase of *Escherichia coli*: partial purification and some properties. J. Biol. Chem. **218:**97–106.
26. **Winkler, M. E.** 1987. Biosynthesis of histidine, p. 395–411. *In* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology. American Society for Microbiology, Washington, D.C.
27. **Winkler, M. E., D. J. Roth, and P. E. Hartman.** 1978. Promoter- and attenuator-related metabolic regulation of the *Salmonella typhimurium* histidine operon. J. Bacteriol. **133:**830–843.
28. **Winkler, M. E., R. V. Zawodny, and P. E. Hartman.** 1979. Mutation *spoT* of *Escherichia coli* increases expression of the histidine operon deleted for the attenuator. J. Bacteriol. **139:**993–1000.
29. **Xiao, H., M. Kalman, K. Ikehara, S. Zemel, G. Glaser, and M. Cashel.** 1991. Residual guanosine 3′,5′-bispyrophosphate synthetic activity of *relA* null mutants can be eliminated by *spoT* null mutations. J. Biol. Chem. **266:**5980–5990.
30. **Zacharias, M., G. Theissen, C. Bradaczek, and R. Wagner.** 1991. Analysis of sequence elements important for the synthesis and control of ribosomal RNA in *E. coli*. Biochimie **73:**699–712.
31. **Zhang, W., J. Ness, R. Provost, and S. Artz.** Unpublished results.
32. **Zhang, X., and H. Bremer.** 1995. Control of the *Escherichia coli rrnB* P1 promoter strength by ppGpp. J. Biol. Chem. **270:**11181–11189.