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Differential Responses of *Bacillus subtilis* rRNA Promoters to Nutritional Stress[∇]†

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The *in vivo* expression levels of four rRNA promoter pairs (*rrnp*$_1$*p*$_2$) of *Bacillus subtilis* were determined by employing single-copy *lacZ* fusions integrated at the *amyE* locus. The *rrnO*, *rrnJ*, *rrnD*, and *rrnB* promoters displayed unique growth rate regulation and stringent responses. Both *lacZ* activity and mRNA levels were highest for *rrnO* under all growth conditions tested, while *rrnJ*, *rrnB*, *and rrnD* showed decreasing levels of activity. During amino acid starvation induced by serine hydroxamate (SHX), only the strong *rrnO* and *rrnJ* promoters demonstrated stringent responses. Under the growth conditions used, the *rrn* promoters showed responses similar to the responses to carbon source limitation induced by α-methyl glucoside (α-MG). The ratio of P2 to P1 transcripts, determined by primer extension analysis, was high for the strong *rrnO* and *rrnJ* promoters, while only P2 transcripts were detected for the weak *rrnD* and *rrnB* promoters. Cloned P1 or P2 promoter fragments of *rrnO* or *rrnJ* were differentially regulated. In wild-type (*relA*$^+$) and suppressor [*relA*(S)] strains under the conditions tested, only P2 responded to carbon source limitation by a decrease in RNA synthesis, correlating with an increase in (p)ppGpp levels and a decrease in the GTP concentration. The weak P1 promoter elements remain relaxed in the three genetic backgrounds [*relA*$^+$, *relA*, *relA*(S)] in the presence of α-MG. During amino acid starvation, P2 was stringently regulated in *relA*$^+$ and *relA*(S) cells, while only *rrnJp*$_1$ was also regulated, but to a lesser extent. Both the *relA*$^+$ and *relA*(S) strains showed (p)ppGpp accumulation after α-MG treatment but not after SHX treatment. These data reveal the complex nature of *B. subtilis rrn* promoter regulation in response to stress, and they suggest that the P2 promoters may play a more prominent role in the stringent response.

The major products of all cellular transcription in *Escherichia coli* and *Bacillus subtilis* are rRNA and tRNA, which constitute more than 95% of the total RNA (19, 37). There are 7 *rrn* operons in *E. coli* and 10 in *B. subtilis*, each controlled by tandem (P1 and P2) promoters that are tightly regulated in response to changes in nutritional status and other stress conditions (6, 7, 8, 49). A significant number of studies on the regulation of *rrn* synthesis in *E. coli* have been published, while only a few reports focusing on the spore-forming Gram-positive bacterium *B. subtilis* exist.

In *E. coli*, the core (−10/−35) region in *rrnp*$_1$ promoters is preceded by an UP element that increases promoter activity 20- to 50-fold because it is strongly bound by the C-terminal domains of the two α subunits of RNA polymerase (6, 7). Binding sites for the transcription factor FIS, centered at positions −71, −102, and −143 upstream of *rrnBp*$_1$, increase overall activity by an additional 3- to 8-fold (21). The downstream P2 promoters have been reported to be less active during rapid steady-state growth but are more active at lower growth rates (1a, 12, 21, 30, 31). In addition, it has been suggested that when RNA polymerase molecules initiate at the upstream promoter (P1), the enzyme directly interferes with the ability of additional molecules to initiate from the downstream promoter (P2) as suggested by Adhya and Gottesman (1) in their promoter occlusion model. Several studies indicate that most of the regulation of *E. coli rrnp*$_1$ and *rrnp*$_2$ activity is attributable to the changes in the concentrations of the initiating nucleoside triphosphate and the highly phosphorylated guanosine nucleotide (p)ppGpp (4, 11, 14, 30). Amino acid starvation and other nutritional stress conditions lead to the "stringent response," accompanied by increasing levels of (p)ppGpp via a *relA* gene-dependent mechanism (6). Changes in the levels of newly synthesized rRNA are influenced by the ATP/GTP concentrations (11, 13, 30). The P2 promoters are less affected by the changes in the concentrations of these signaling molecules than are the P1 promoters (31).

The spore-forming Gram-positive bacterium *B. subtilis* has 10 *rrn* operons; 9 are located 1° to 90° on one side of *oriC*, and 1 (*rrnB*) is located on the opposite side at 271° on the chromosome (19, 22). The operons are transcribed from the H strand in the same direction as the chromosome replicates (28, 35). Five of the *rrn* operons (*rrnO*, *rrnA*, *rrnE*, *rrnD*, and *rrnB*) have approximately the same organization: tandem promoters P1 and P2–16S gene–spacer–23S gene–5S gene–terminator re-

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† This paper is dedicated to the memory of Inga R. Richter and Steffen R. Buchholz, who were members of our research group and died in an automobile accident (8 June 1988).

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TABLE 1. *Bacillus subtilis* strains and plasmids used in this study

Strain or plasmid	Relevant genotype and/or promoter fragment (length)	Source
Strains		
IS58	*trpC2 lys-3 relA*$^+$	I. Smith
IS56	*trpC2 lys-3 relA*	I. Smith
L3	*trpC2 lys-3 relA*(S)	R. Rudner
Plasmids		
pDH32	Amp^r Cm^r *amyE spoVG-lacZ*	D. Henner
pDG268	Amp^r Cm^r *amyE spoVG-lacZ*	P. Stragier
pPW810	pDH32-(P1)-*veg* (0.48 kb)	C. Stewart
pPW4	pDH32-(P1-P2)-*rrnO* (1.9 kb)	C. Stewart
pAWR118	pDG268-(P1-P2)-*rrnJ* (1.5 kb)	A.-M. White
pAWR116	pDG268-(P1-P2)-*rrnD* (2.2 kb)	A.-M. White
pAWR123	pDG268-(P1-P2)-*rrnB* (1.8 kb)	A.-M. White
pLR501	pDG268-(P2)-*rrnO* (339 bp)	D. Liu
pLR512	pDG268-(P1)-*rrnO* (241 bp)	D. Liu
pLR105	pDG268-(P2)-*rrnJ* (222 bp)	D. Liu
pLR210	pDG268-(P1)-*rrnJ* (192 bp)	D. Liu
pLR203	pDG268-(P1-P2)-*rrnJ* (438 bp)	D. Liu

gion. In the closely spaced operons *rrnJ-rrnW* and *rrnI-rrnH-rrnG*, there are single promoters (P2) upstream of *rrnW* and *rrnH-rrnG* (16, 19, 22). The upstream promoter regions (−36 to −80) are enriched in short A and T tracts, typical for upstream (UP) elements (see Table 2).

Several differences have been noted in the *rrn* promoter strengths of these operons in both *E. coli* and *B. subtilis* (7, 16, 35a). In this paper, we report the differential expression of the tandem promoters P1-P2 of *rrnO*, *rrnJ*, *rrnD*, and *rrnB* in *B. subtilis* by employing single-copy *lacZ* fusions integrated at the *amyE* locus. We asked whether the operons closest to *ori*, at map positions 1° (*rrnO*) and 8° (*rrnJ*), exhibit higher transcription activity than operons located farther away, at 81° (*rrnD*) and 271° (*rrnB*) (2, 19, 35a, 49). We also wanted to determine whether *B. subtilis* $rrnp_2$ promoters are more active and more highly regulated than $rrnp_1$ promoters, as was shown by others in early studies for *rrnO* and *rrnB* (9, 35a, 49). Finally, we inquired whether both $rrnp_1$ and $rrnp_2$ promoters are similarly regulated during amino acid starvation or during carbon source limitation in cell strains differing in their *relA* backgrounds. Our studies also aimed to evaluate the expression of four *rrn* operons with respect to their chromosomal positions relative to *ori* and to establish whether position effects exist in *B. subtilis.*

MATERIALS AND METHODS

Strains, plasmids, and PCR constructions. The *B. subtilis* strains used in this study are listed in Table 1. Strains IS58 (*relA*$^+$), IS56 (*relA*), and L3 [*relA*(S)] are isogenic except for the *relA* gene (33). L3 has a stringent response to glucose but not to amino acid starvation and is resistant to 3-amino-1,2,4-triazole (AT) (6, 20). In addition, the *relA*(S) and *relA* mutant forms cause the cells to require certain amino acids when grown in minimal medium, as reported for *E. coli* and *Salmonella enterica* serovar Typhimurium (6, 17, 39, 41, 50). The transcriptional plasmids pDH32 and pDG268 (9,894 and 9,327 bp, respectively), which contain a promoterless *spoVG-lacZ* fusion gene, a *B. subtilis* ribosomal binding site from the *spoVG* gene, two large regions of the *amyE* gene, and the *cat* gene determinants like *erm* from pC194 (3, 21b, 45), were used. Another plasmid, pDG793, also known as pDG1663 (8,396 bp), was examined; it is similar to pDH32 and pDG268 but instead contains two large regions of *thrC* and the pE12 *erm* gene (3, 21a). The *lacZ* gene in all constructs is transcribed in the opposite direction from the *amyE* or *thrC* gene (3, 45). Table 1 lists the constructs derived by cloning *rrn* promoter fragments into the plasmids described above, and Table 2 lists their respective promoter sequences. The intact cloned promoters from *rrnO* and *veg* (plasmids pPW4 and pPW810, respectively) have been described previously (10, 36) and were kindly provided by C. Stewart. Plasmid pAWR118 was constructed by inserting a 1.5-kb PstI *rrnJ* fragment from *B. subtilis* strain 168 into pDG268 (Table 1) (51, 52). Plasmid pAWR116 contains a 2.2-kb HindIII *rrnD* fragment from *B. subtilis* strain SB25 (51, 52) inserted into pDG268. Plasmid pAWR123 contains a 1.8-kb EcoRI *rrnB* fragment from pGS227 (15) cloned into plasmid pDG268. The fragments derived from *rrnO* and *rrnB* extend to the EcoRI site 0.8 kb into the 16S gene; the *rrnJ* fragment extends to the PstI site at 0.9 kb of the 16S gene; and the *rrnD* fragment terminates 0.2 kb into the 23S gene. These *rrn-lacZ* fusions were transformed into the competent IS58, IS56, and L3 strains (Table 1) using standard techniques, and Cm^r *amyE* (or Erm^r *thrC*) cells were selected for these studies.

The individual promoters of *rrnO* and *rrnJ*, as well as $rrnJp_1p_2$, without the upstream activation sequences (UAS) were created by PCRs using standard techniques. The primers used, along with their restriction enzyme termini, are listed in Table 3 (27). The *rrn* promoter fragments generated by PCR were cloned into pDG268 and are listed in Table 1. The $rrnOp_1$ fragment is a 241-bp HindIII-BamHI fragment extending from −186 to +40 with respect to the +1 transcription start site; $rrnOp_2$ is a 339-bp BamHI fragment extending from −84 to +243; $rrnJp_1$ is a 192-bp EcoRI-HindIII fragment extending from −162 to +20; $rrnJp_2$ is a 222-bp HindIII-BamHI fragment extending from −82 to +133 (27); and $rrnJp_1p_2$ is an EcoRI-BamHI fragment extending from −162 of P1 to +133 of P2. The DNA sequences of the *rrnJ* PCR products were confirmed.

Bacterial growth conditions and *in vivo* labeling. Liquid cultures of *B. subtilis* with or without integrated *rrn-lacZ* fusions were grown in a fast complex medium composed of 2.5% veal infusion broth and 0.5% yeast extract (VY; Difco) (25). The basal medium (MM) used for growth rate experiments was Spizizen minimal salts (2a) supplemented with 50 μg/ml of L-tryptophan and 100 μg/ml of L-lysine. To achieve different growth rates, the basal medium was supplemented with the following: either 0.5% glucose and 1% sodium glutamate (MM1), 1% sodium succinate, 0.05% yeast extract, and 0.02% vitamin-free Casamino Acids (MM2), or 1% sodium acetate, 0.05% yeast extract, and 0.02% vitamin-free Casamino Acids (MM3). Overnight cultures of the various strains containing integrated *rrn-lacZ* fusions were centrifuged, washed, and resuspended 1:25 in the same medium or dilution salts, followed by inoculation of the cells into a 250-ml side-arm flask with an additional dilution of 1:10; hence, the cells were diluted

TABLE 2. Sequences of *B. subtilis rrnO*, *rrnJ*, *rrnD*, *rrnB*, and *veg* P1 and P2 promoters used in this study

Promoter	Map location (kb)	Sequence (UP element, −35, −10, +1)
$rrnOp_1$	10	GCGCTTTTTTGTGTCATAACCCTTTACAGTCATAAAAATTATGGTATAATCATTTCTG TTGTCT
$rrnOp_2$	10	TAAAAACTTTTTCAAAAAAGTATTGACCTAGTTAACTAAAAATGTTACTATTAAGTAG TCGCTT
$rrnJp_1$	90	TAGTATTTCTTCAAAAAAACTATTGCACTATTATTTACTAGGTGGTATATTATTATTG TTGCCG
$rrnJp_2$	90	AAAAGAACTTCAAAAAAAGTTATTGACTTCACTGAGTCAAGGAGTTATAATAATAAAG ACGTAC
$rrnDp_1$	945	GGATATTCTTTTAAAAAAGGTGTTGACTCTGATTCTTGACCGTGTTATATTATTAACG TCCTGA
$rrnDp_2$	945	GGAAAATAAATCAAAAAAACATTTGACAAAAGAAAGTCAAAATGTTATATTAATAAAG TCGCGT
$rrnBp_1$	3175	ATAGATTTTTTTTAAAAAACTATTGCAATAAATAAATACAGGTGTTATATTATTAAACG TCGCTG
$rrnBp_2$	3175	CAAAACAACTTGAAAAAAGTTGTTGACAAAAAAGAAGCTGAATGTTATATTAGTAAAG CTGCTT
$vegp_1$	53	GTTGATATAATTTAAATTTTATTTGACAAAAATGGGCTCGTGTTGTACAATAAATGTA GTGAGG

TABLE 3. Primers used for PCRs

Primer name	Sequence[a]	Restriction site(s)	Location[b]	Promoter fragment(s) generated[c]
RR15-71	GGG**GGATCC**GCTCGACTTGCATGTAT	BamHI	Downstream	*rrnJp*$_2$, *rrnJp*$_1$*p*$_2$, *rrnOp*$_2$
RR15-72	GGG**AAGCTT**GCCGCTAAACAAGGCG	HindIII	Upstream	*rrnJp*$_2$
RR15-73	GGGG**AAGCTT**CCCCTTCTATTCGCGAT	EcoRI	Upstream	*rrnJp*$_1$, *rrnJp*$_1$*p*$_2$
RR15-74	GGG**AAGCTT**CGCCTTGTTTAGCGGC	HindIII	Downstream	*rrnJp*$_1$
RR15-77	CC**GGATCCTGCAGA**CACAAGCATGACC	BamHI, PstI	Upstream	*rrnOp*$_2$
RR15-78	CC**GGATCC**TAGTCATAATGGTCATGC	BamHI	Downstream	*rrnOp*$_1$
RR15-79	GGG**AAGCTTCTGCAG**GTGCGTCTCAT	HindIII, PstI	Upstream	*rrnOp*$_1$

[a] Regions of primers complementary to target template DNA are underlined; restriction enzyme sites are in boldface.
[b] The priming site of the oligonucleotide with respect to the indicated promoter(s). Upstream primers are sense sequences, and downstream primers are antisense sequences.
[c] Some primers were used to generate several different promoter fragments.

1:250 with the same fresh, prewarmed medium or one of the other types of growth medium and were then shaken at 37°C. During their logarithmic growth, the cells were monitored on a Klett-Summerson spectrophotometer equipped with a red filter. Samples for β-galactosidase assays and for RNA extraction were withdrawn, inactivated with 0.05 M sodium azide, rapidly cooled to 0°C, and then processed. For stringent-response measurements, cultures were grown in MM1 and samples were withdrawn at varying times following the addition of serine hydroxamate (SHX) at 2 mg/ml (47) or α-methyl glucoside (α-MG) at 1% (23). For message decay assays, samples were withdrawn at different times after the addition of rifampin (150 μg/ml) at Klett readings of 100 to 120.

(p)ppGpp measurements. A low-phosphate Tris-glucose medium described previously (17, 33, 46) was used to label the pool of nucleotides. The medium was supplemented with 100 μg/ml of the L-amino acids lysine, proline, glycine, alanine, glutamic acid, aspartic acid, and arginine and with 40 μg/ml of cysteine, methionine, tyrosine, tryptophan, and phenylalanine to relieve the cells from the low-phosphate condition. Cultures were labeled with [^{32}P]phosphoric acid (50 to 100 μCi/ml) for one generation (1 h), followed by treatment with either SHX, α-MG, or rifampin as described above. Samples (100 μl) were withdrawn at 0, 5, 10, and 20 min and were mixed with 20 μl 13 M formic acid before freezing. The samples were centrifuged for 5 min, and 10 to 20 μl of the supernatants was applied to polyethyleneimine (PEI)-cellulose plates (Brinkmann Instruments) for separation by thin-layer chromatography (TLC) of the phosphorylated guanosine nucleotides in 1.5 M KH_2PO_4 as described previously (17, 33, 46). Radioactively labeled nucleotides were visualized by autoradiography. The relative concentrations of pppGpp, ppGpp, and GTP were determined by densitometry using a Zeineh soft scanning densitometer (model SL-DNA; Biomed Instruments). Actual nucleotide concentrations were determined by cutting out the appropriate areas of the chromatogram and measuring the radioactivity by liquid scintillation counting. Background radioactivity was corrected for by counting appropriate blank regions of the chromatogram.

Measurements of β-galactosidase activity. Frozen cell pellets were resuspended in 0.9 ml Z-buffer, solubilized with four drops of toluene, and assayed for β-galactosidase according to published procedures (29). In order to calculate Miller units, Klett readings of the sampled cell suspensions were converted to optical densities at 600 nm.

RNA isolation and quantification. RNA was isolated using a modified procedure described for *E. coli* (32). Samples (2.0 to 4.0 ml) were removed from cultures grown in MM1, MM2, or MM3 medium. These samples were diluted with an equal volume of ice-cold TMA buffer (50 mM Tris-HCl [pH 7.4], 1 mM $MgCl_2$, 10 mM $NaCN_3$) and were then centrifuged, and the pellets were immediately resuspended in 2.0 ml of freshly made lysis buffer (2.0 mg/ml lysozyme, 2 U/ml of RNase-free DNase [RQ1; Promega], 10 mM Tris-HCl [pH 8.0], 1 mM EDTA) and were placed on ice for 10 min. This was followed by the addition of 0.5 ml of 1 M NaCl, 50 mM EDTA (pH 8.0), and 2.5% sodium dodecyl sulfate (SDS). Samples were then placed in boiling water for 30 s. After phenol extraction and ethanol precipitation, the RNA was resuspended in sterile water, and the intactness of the preparations was judged by visualization of rRNAs in ethidium bromide-stained agarose gels. The RNA concentration was determined colorimetrically by the orcinol method (43).

Northern slot blot hybridization and primer extension analysis. To measure *lacZ* mRNA levels, 5 to 10 μg of total RNA was loaded per slot of a Minifold II slot blotter (Schleicher & Schuell) fitted with Nytran nylon membranes. The membranes were baked, prehybridized, hybridized, and washed as described previously for Northern blot analysis (48). A *lacZ* probe was generated from pDEB1 (44) by labeling with [α-^{32}P]dCTP using a random primer extension kit as directed by the supplier (USB, Cleveland, OH). Specific activity was consistently 1×10^8 to 3×10^8 cpm/μg of DNA. Membranes were exposed for 17 h to X-Omat AR film (Eastman Kodak Co., Rochester, NY) with intensifying screens at −70°C. Hybridization intensity was determined by scanning autoradiograms using the Zeineth soft scanning densitometer. Primer extension reactions were performed by modification (48) of a procedure previously described for *E. coli* (12, 32). Briefly, 75 μg of total RNA was mixed with 8 to 10 ng of a 5′-end-labeled primer in 0.1 M KCl–0.05 M Tris-HCl (pH 8.3). This mixture was denatured by heating for 1 min at 90°C, followed by 2 min at 60°C, and was then placed on ice for 15 min to anneal. Then 5XRT buffer (0.25 M Tris-HCl [pH 7.9], 0.2 M KCl, 0.036 M magnesium acetate, 0.01 M dithiothreitol [DTT]), 1 mM deoxynucleoside triphosphates (dNTPs), and 2 U/μl RNasin (Promega) were added to a final concentration of 1×. The primer extension reaction was initiated by the addition of 20 U of avian myeloblastosis virus (AMV) reverse transcriptase (Molecular Genetic Resources); the reaction mixture was incubated for 1 h at 45°C; and the reaction was stopped by the addition of an equal volume of dye mixture (0.1% bromophenol, 0.1% xylene cyanol in deionized formamide). The mixture was heated for 3 min at 100°C, and the primer extension products were separated by electrophoresis on a 6% acrylamide-urea sequencing gel and were visualized by autoradiography. The primer extension products were quantitated by densitometry using the Zeineth soft scanning densitometer. The two primers used were (i) 5′ TGC AGG CCC TAG TTT GAC TGA CTA C 3′, complementary to the unique sequence of *rrnO* at −256 to −221 (34), and (ii) 5′ TCA GTA ACT TCC ACA GTA GTT CAC CAC CTT 3′, complementary to the *spoVG-lac*Z junction between the SalI and BamHI sites at pDH32 positions 3771 to 3741 (45). The primers were purified by gel filtration using a 10-ml Sephadex G25X Fine column, and the peak fractions were concentrated by evaporation. The purified primers (100 ng) were then end labeled with 40 μCi of [γ-^{32}P]ATP in hybridization solution (5× kinase buffer, 0.5 M Tris-HCl [pH 9.5], 50 mM $MgCl_2$), 50 mM DTT, and 5 to 10 U of T4 polynucleotide kinase (Boehringer Mannheim). The reaction mixture was incubated at 37°C for 30 min, followed by purification through a Quick Spin G-25 Sephadex column, and was stored at a concentration of 70 to 100 μg/ml.

mRNA half-life determinations. RNA turnover was determined by the decay of hybridizable *lacZ* mRNA following inhibition of transcription by rifampin as described above. mRNA half-lives ($t_{1/2}$) were quantitated by analyzing autoradiograms of Northern slot blots using a densitometer as described above (5, 48).

RESULTS

Do the four intact *rrn* promoters inserted into the *amyE* gene display unique levels of expression as a function of growth rate? To evaluate the intrinsic promoter strength of a representative sample of rRNA gene sets, four promoter-bearing fragments were inserted upstream of the *spoVG-lacZ* fusion of either pDH32 or pDG268. Fragments from the *rrnO*, *rrnJ*, and *rrnB* operons contained 16S sequences of similar lengths (0.8, 0.9, and 0.8 kb, respectively), while the *rrnD* fragment included a longer segment of 1.67 kb. The control promoter *vegp* (0.48 kb), which is constitutively expressed during vegetative growth, was also included (10, 36). The five plasmids bearing the *rrn- or veg-lacZ* fusions were linearized, and each

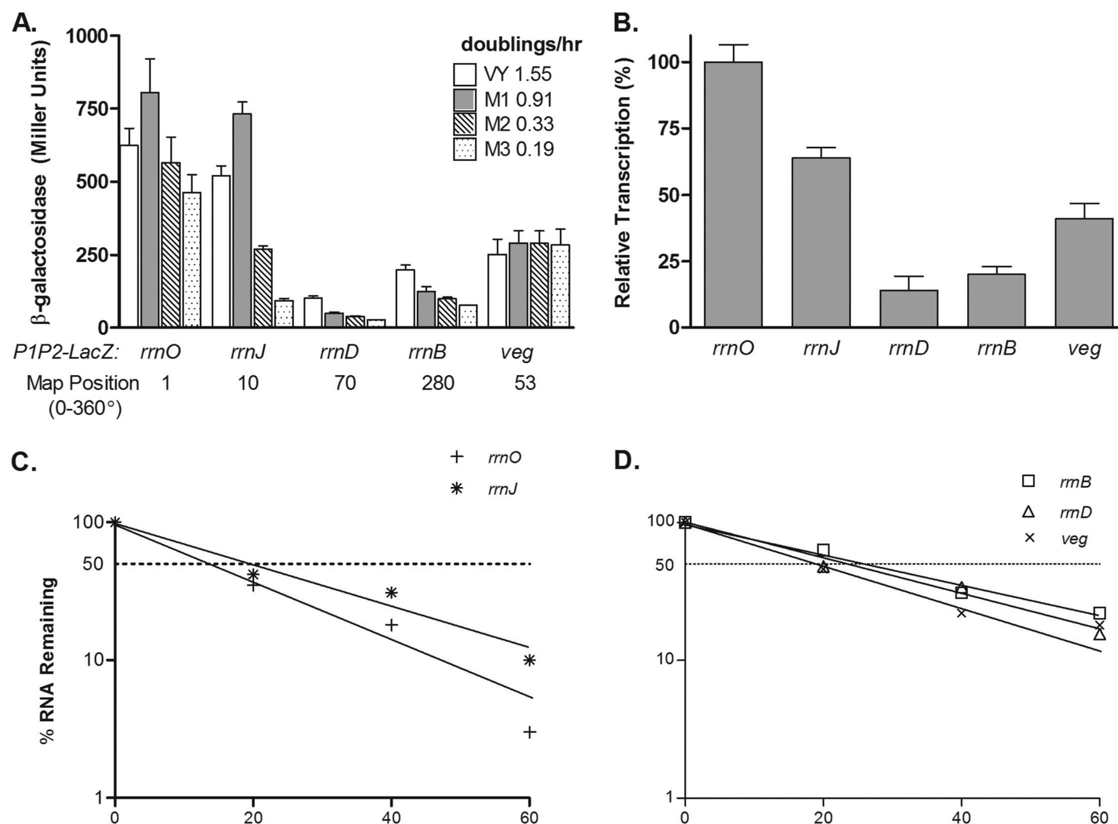

FIG. 1. Relative promoter strengths of four *rrn* operons and the *veg* gene integrated into the *amyE* locus. (A) β-Galactosidase activity measured in strains with *lacZ-rrn* promoter fusions with *rrnO*, *rrnJ*, *rrnB*, *rrnD*, and *veg* integrated at the *amyE* locus cultured in the indicated media to produce different growth rates. One-milliliter samples from the growing cultures were removed at a reading of 100 Klett units and were assayed for β-galactosidase activity by the method of Miller (29) as described in Materials and Methods. (B) The steady-state transcript levels driven by the indicated promoters were determined by Northern slot blotting. Five- and 10-μg portions of total RNA prepared from *B. subtilis relA*$^+$ strains grown in MM1-glucose were loaded into individual slots and hybridized with an excess of a ^{32}P-labeled *lacZ* probe. Autoradiograms were analyzed by densitometry, and the values shown are expressed relative to the signal from *rrnO*. (C and D) The stabilities of *rrn-lacZ* fusion transcripts initiating at the indicated promoters were determined by Northern slot blot hybridization of a *lacZ* probe to total RNA prepared at the indicated times (minutes) after rifampin treatment. The half-life was calculated by plotting the relative transcript levels obtained from the densitometric measurements of the autoradiograms versus time on semilog graph paper.

was transformed into strain IS58 (*relA*$^+$). The bacteria that harbored integration events of the single-copy intact *rrn*- or *veg-lacZ* fusions into the *amyE* gene were isolated as chloramphenicol-resistant *amyE* colonies that were blue on minimal agar plates supplemented with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal). Colonies that arose by a Campbell-like insertion and yielded the *amyE*$^+$ phenotype (3, 45) were not chosen for these studies. The cloned *rrn-spoVG-lacZ* fragments were oriented in the opposite direction from the other functional parts of the plasmids and their drug resistance genes; hence, readthrough of these sequences was unlikely, and only the cloned promoters directed *lacZ* expression.

The *B. subtilis* transformants carrying the various *rrn-spoVG-lacZ* fusions were grown in four different media to achieve growth rates from 0.2 to 1.6 doublings per h. These cultures were then assayed for β-galactosidase activities. As shown in Fig. 1A, each promoter exhibited a distinct expression level, with *rrnO* showing the highest expression, as in our earlier studies (51). In VY medium, the relative expression levels of the promoters were 100, 84, 16, 33, and 40% for *rrnO*, *rrnJ*, *rrnD*, *rrnB*, and *veg*, respectively. The levels of β-galactosidase expression driven by *rrnO* were the highest in all media tested, while those for *rrnJ* were high in complex medium (VY) and in MM1 (glucose) and showed a reduction in promoter strength at lower growth rates. The *rrnD* and *rrnB* promoters were weak at all growth rates, while *veg* displayed a constant activity level under all growth conditions (Fig. 1A). We have repeatedly noticed that cultures growing in VY medium tend to exhibit premature lysis, which occurs as the cells are centrifuged; hence the lower levels of β-galactosidase seen for *rrnO* and *rrnJ* in VY than in MM1 medium (Fig. 1A). At the *thrC* locus at 284° on the map (2), the same *rrnJ* and *rrnD* promoter fragments exhibited further decreases from their levels at the *amyE* locus (data not shown).

Do the *lacZ*-mRNA levels correlate with the enzymatic activities of β-galactosidase? Northern slot blotting was used to determine the transcript levels of *lacZ* mRNA from cultures grown in MM1, and the results are presented in Fig. 1B. The relative transcript levels (100, 64, 14, 20, and 41% for *rrnO*, *rrnJ*, *rrnD*, *rrnB*, and *veg*, respectively) correlated well with the β-galactosidase activity levels (100, 90, 7, 15, and 36%) (Fig. 1A), verifying the heterogeneity in promoter strength among the *rrn* operons of *B. subtilis* (42).

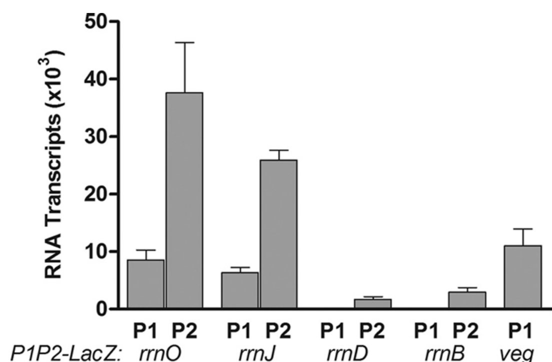

FIG. 2. Relative strengths of individual promoter elements as determined by primer extension analysis. Total RNA was isolated from strains containing the indicated promoter-*lacZ* fusion constructs and was used for primer extension analysis with a ^{32}P-labeled primer that hybridizes to the *spoVG-lacZ* junction present in the constructs. Primer extension products were separated by gel electrophoresis, and arbitrary values were calculated from densitometric scans of autoradiograms. Transcripts initiating at P1 or P2 were quantitated separately. Each experiment was repeated at least 3 times. Error bars represent the standard errors of the means.

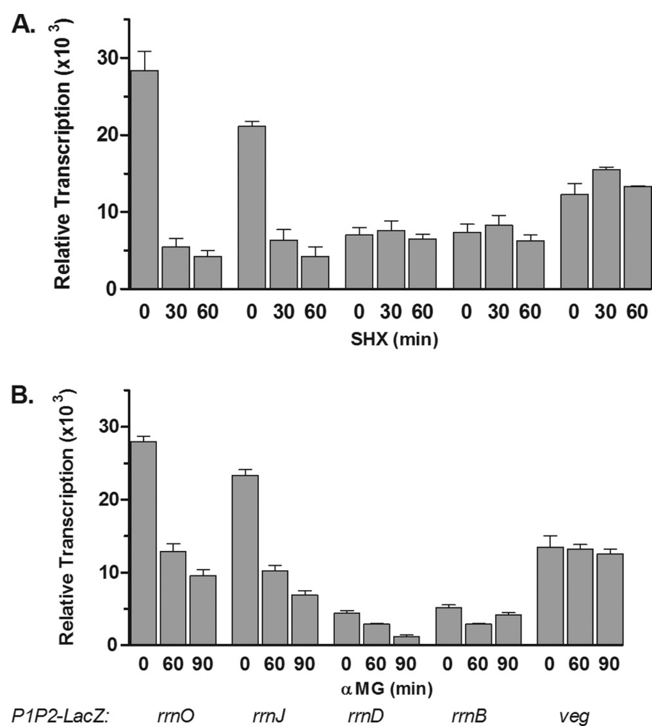

FIG. 3. Effects of nutritional stress on intact *rrn* promoters. Cultures of *B. subtilis* containing the indicated promoter-*lacZ* fusion constructs were grown in MM1-glucose medium and were treated either with serine hydroxamate (A) or with α-methyl glucoside (B) for the indicated times. Total RNA extracted from these cultures was analyzed by Northern slot blotting using a *lacZ* probe. The resulting filters were subjected to autoradiography and were then scanned. The values shown are average arbitrary densitometric units determined from at least 3 experiments. Error bars represent the standard errors of the means.

Are the half-lives of *lacZ* mRNAs transcribed from the different *rrn* promoters similar? To ensure that changes in *lacZ* mRNA levels and β-galactosidase activities were not due to differential message stability, the half-lives of the various mRNAs transcribed from intact *rrnO*, *rrnJ*, *rrnD*, *rrnB*, and *veg* promoters after standard rifampin treatment were determined by Northern slot blot hybridization using the *lacZ* probe. As shown in Fig. 1C and D, the half-lives of the reporter mRNAs differed over a small range. *lacZ* mRNA driven by *rrnO*, *rrnJ*, *rrnB*, *rrnD*, or *veg* had a $t_{1/2}$ of 14.4, 19.1, 26.4, 22.4, or 19.5 min, respectively. Thus, the large differences in mRNA transcript levels and β-galactosidase activities controlled by these *rrn* promoters must be due to intrinsic promoter activity (42). Our results are consistent with the 2003 report of Hambraeus et al. (18), which showed clearly that more than 30 mRNAs, including both mono- and polycistronic transcripts, were extremely stable, with half-lives of ≥15 min. In addition, total-RNA concentrations, determined colorimetrically by the orcinol method (43) during the rifampin treatments, did not increase; hence, the long $t_{1/2}$ reported above are reliable and were not due to residual RNA synthesis (data not shown).

Is there a differential response of the individual promoter elements expressed under nutritional stress from the native tandem (P1-P2)? Both of the core *rrn* promoters (P1 and P2) stimulate the production of the same rRNA; thus, it was of interest to determine their relative contributions to the observed activity of the promoter tandems in integrant strains. These strains were grown in MM1 medium, and promoter activities were compared by quantitative primer extension analysis using a primer that recognizes the *spoVG-lacZ* fusion transcript. As shown in Fig. 2, transcripts initiating from P2 were more abundant than those starting from P1. In fact, no P1 transcripts from *rrnD* or *rrnB* were detectable in this assay. For *rrnO* and *rrnJ*, approximately 4-fold fewer transcripts initiated at the P1 promoter than at the P2 promoter. These data suggest that during growth in MM1 with 0.6 doubling per hour, most of the *lacZ* expression seen in integrant strains is the result of P2 activity. Hence, these data imply that the P2 promoter is dominant over P1 in *rrn* promoter tandems under these growth conditions.

Is there a unique stringent response of solitary PCR-constructed P1 and P2 elements of *rrnO* and *rrnJ* during amino acid starvation and carbon limitation? In order to examine stringent regulation of *rrn* promoter tandems, *relA*$^+$ strains containing the *rrnp*$_1$*p*$_2$-*lacZ* fusions were stressed with either serine hydroxamate (SHX), a competitive inhibitor of aminoacylation of serine tRNA (47), or α-MG, a competitive inhibitor of glucose uptake (23). The stressed cultures were monitored for their abilities to transcribe *lacZ* mRNA from the *rrn* promoter tandems, and the results are shown in Fig. 3A and B. Within 30 min of SHX challenge, levels of *lacZ* mRNA transcripts controlled by the strong *rrnO* and *rrnJ* promoters decreased dramatically, by 80 to 85%, respectively (Fig. 3A). For SHX (or *O*-methyl threonine [*O*-MT]) to act completely effectively, incubation periods of 30, 60, and 90 min have been used for both *E. coli* and *B. subtilis* cultures (9, 17, 38, 47). In contrast, the two weak promoters of *rrnD* and *rrnB*, as well as the *veg* promoter, failed to respond to SHX treatment; levels of *lacZ* mRNA that were not insignificant remained essentially unchanged (Fig. 3A).

Similar measurements with the *rrn-lacZ* fusions in the same strains after exposure to α-MG are reported in Fig. 3B. As

shown, transcription by the strong *rrnO* and *rrnJ* promoters decreased, while the *rrnB* promoter showed a small decline of only 60 to 70% in *lacZ* mRNA transcription after 90 min of α-MG treatment. As with SHX treatment, the *veg* promoter failed to respond to carbon source limitation. These data, taken together, demonstrate that whereas all *rrn* promoters are downregulated during carbon source limitation, only the stronger *rrnO* and *rrnJ* promoters can be modulated by amino acid starvation. It is also clear that these two types of stress elicit different cellular responses.

Do the individual *rrn* promoter elements P1 and P2 respond to nutritional stress? The roles of the individual *rrn* promoters within the P1-P2 tandem during the stringent response were investigated using multiple approaches. First, quantitative primer extension analysis was performed on *B. subtilis* strains differing only in their *relA* phenotypes [i.e., *relA*$^+$, *relA*, *relA*(S)]. The results, shown in Fig. 4A, suggest that under these conditions, most of the transcripts initiated at the endogenous *rrnOp*$_2$ promoter, while fewer were from the *rrnOp*$_1$ promoter, and were stringently regulated in response to both SHX and α-MG treatments in a *relA*$^+$ background. As expected, neither *rrnO* promoter element was inhibited by the same treatments in a *relA* background. Interestingly, in the *relA*(S) strain, α-MG regulated *rrnOp*$_2$, but SHX did not. This response is most likely related to the uneven cellular production of highly phosphorylated guanosine nucleotides between the two treatments (see below).

The stringent responses of the native P1 and P2 promoter elements of *rrnO* were then compared to those of the same elements found in the *rrnO-lacZ* fusion integrated at the *amyE* locus. Transcript levels were determined by primer extension using the *lacZ*-specific probe, and the results are shown in Fig. 4B and C. SHX treatment of the *relA*$^+$-*rrnO* integrant strain led to similar reductions in *rrnO-lacZ* transcripts initiating from either *rrnOp*$_1$ or *rrnOp*$_2$ (Fig. 4B). SHX had no effect on the levels of these transcripts in either *relA*- or *relA*(S)-*rrnO* integrant strains. In contrast, glucose exhaustion induced by α-MG revealed strong stringent responses of *rrnOp*$_2$ in the *relA*$^+$ and *relA*(S) backgrounds (Fig. 4C). Very low levels of transcripts from the P1 promoters were detected. These did not appear to be regulated by α-MG in any of the integrant strains tested.

Are the stringent responses of solitary *rrn* promoters also asymmetric when they are expressed from the *amyE* gene? Individual P1 or P2 elements from *rrnO* and *rrnJ* were amplified by PCR in order to investigate stringent control imposed by the isolated *rrn* promoters (27). Additionally, an *rrnJ* fragment containing the promoter tandem and immediate upstream elements (UP), but lacking the upstream activation sequences (UAS), was also isolated. These fragments were cloned into the *spoVG-lacZ* fusion plasmid, and the resulting constructs were integrated into the *amyE* locus in both the *relA*$^+$ and *relA* backgrounds. Expression of the integrated PCR constructs was compared by RNA dot blots, and the results are shown in Fig. 5A to D. Following the induction of the stringent response by SHX treatment in *relA*$^+$ integrants, transcript levels controlled by the isolated P2 promoters of *rrnO* and *rrnJ* showed dramatic decreases of 84 and 81%, respectively (Fig. 5A and B, shaded bars). The decreases in the levels of *lacZ* transcripts regulated by the P2 elements were similar to those seen when both the P1 and P2 elements were present (Fig. 5A and B, filled bars). The levels of *lacZ* transcripts initiating at the isolated P1 promoters of *rrnO* and *rrnJ* decreased by only 28 and 59%, respectively (Fig. 5A and B, open bars) As expected, no downregulation of these constructs was observed when they were integrated into a *relA* strain (Fig. 5A and B).

Glucose exhaustion induced by the addition of α-MG produced a greater differential effect on the expression of *lacZ* mRNA controlled by the solitary promoters. The P2 promoters responded to glucose starvation in the *relA*$^+$ strains, demonstrating 89% and 76% inhibition of *lacZ* transcription from *rrnOp*$_2$ and *rrnJp*$_2$, respectively (Fig. 5C and D, shaded bars). As in the case of SHX treatment, the decreases in the levels of *lacZ* transcripts regulated by the P2 elements were similar to those seen when both the P1 and P2 elements were present (Fig. 5C and D, filled bars). In contrast, the solitary P1 promoters were totally insensitive to carbon deprivation. As expected, no constructs were downregulated in a *relA* background.

lacZ mRNA levels were measured after rifampin treatment in order to establish whether transcripts initiating at the isolated P1 and P2 promoter elements had different half-lives. The results, shown in Fig. 5E and F, actually indicate that transcripts directed by the strong P2 promoters of *rrnO* and *rrnJ* have shorter half-lives than those initiating at P1. Transcripts starting at the isolated *rrnOp*$_2$ and *rrnJp*$_2$ had half-lives of 9 and 13 min, respectively. The respective P1 promoters gave rise to transcripts with half-lives of 25 and 27 min. These data suggest that, due to differing half-lives, the increases in the levels of transcripts driven by isolated P2 versus P1 are underestimated, and thus, it is possible that the differential stringent regulation is even more pronounced than that observed. Similarly, the constructed individual promoter elements (P1 versus P2) of *rrnO* and *rrnJ* had different decay rates, where the weak P1 promoters had considerably longer $t_{1/2}$ than the active P2 promoters. In MM1 medium, the *lacZ* mRNAs transcribed from *rrnOp*$_1$ and *rrnOp*$_2$ had $t_{1/2}$ of 24.7 and 8.8 min, respectively, and the $t_{1/2}$ of *rrnJp*$_1$ and *rrnJp*$_2$ were 27.1 and 12.9 min, respectively (Fig. 5E and F).

Does the stringent control of the P2 promoter correlate with the formation of highly phosphorylated guanosine nucleotides? The accumulation of highly phosphorylated guanosine nucleotides correlates well with the induction of the stringent response. Since the *rrnp*$_1$ and *rrnp*$_2$ promoters demonstrated differential responses to SHX versus α-MG, it was of interest to assess the status of phosphorylated guanosine nucleotides after exposure to these two agents. As shown in Fig. 6 and Table 4, carbon limitation induced by α-MG resulted in the accumulation of (p)ppGpp in the *B. subtilis relA*$^+$ strain [results for the *relA*(S) strain are not shown here but were reported previously (17)] but not in the *relA* mutant. This considerable increase in ppGpp and pppGpp accumulation was accompanied by a simultaneous decline in the level of GTP to 22 to 25% of the starting levels of GTP found in untreated cultures (Fig. 6 and Table 4). The use of SHX to induce amino acid starvation resulted in larger accumulations of (p)ppGpp both in *E. coli* and in *B. subtilis* (6, 17). In the *relA*$^+$ strain, the levels of pppGpp and ppGpp increased dramatically following 15 min of SHX exposure, while levels of radiolabeled GTP decreased to 20% of the starting amount. In the *relA* and the *relA*(S) strains, the levels of GTP did not decrease, and no accumulations of guanosine polyphosphates were detectable (Table 4) (17, 41).

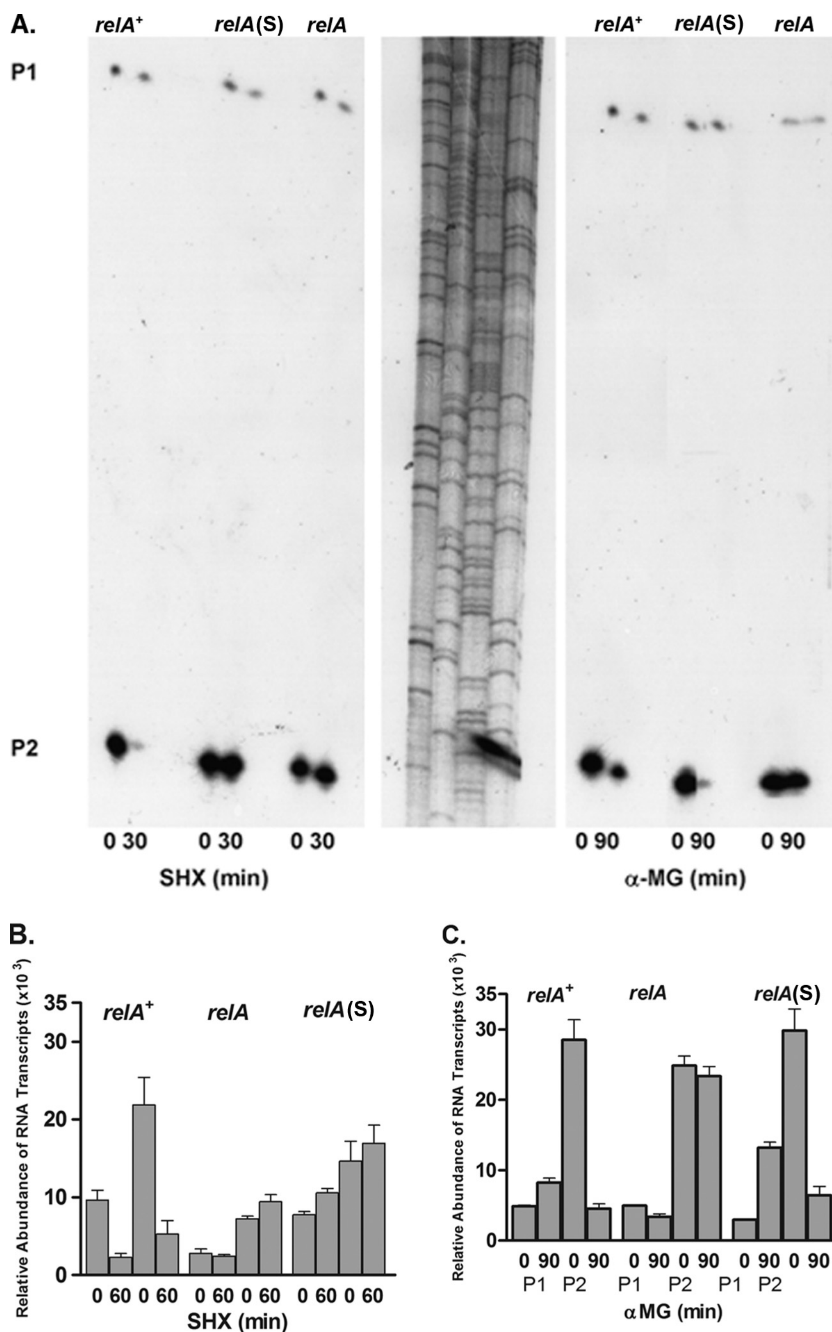

FIG. 4. Effects of nutritional stress on individual *rrn* promoter elements of *rrnO*. (A) Autoradiogram of a representative primer extension experiment performed using RNA isolated from *B. subtilis relA*$^+$, *relA*, and *relA*(S) strains and an *rrnO*-specific ^{32}P-labeled primer. Cells were treated with SHX or α-MG for the indicated times prior to RNA extraction. P1 and P2 primer extension products were separated by gel electrophoresis. A sequence ladder of ^{32}P-labeled lambda DNA was included as a size marker for the primer extension products. (B and C) Primer extension analysis using RNA from the different *relA* strains with the integrated *rrnO-lacZ* fusion and the *spoVG-lacZ* primer. Cells were grown in MM1-glucose and were treated with either SHX (B) or α-MG (C) for the indicated times. The relative levels of P1 and P2 transcripts were determined as described for Fig. 2.

DISCUSSION

Although there is little variation in the primary sequences of the 10 rRNA genes in *B. subtilis* or the 7 in *E. coli*, they respond differently to a variety of physiological conditions. To date, we have examined the activities of four specific rRNA promoters as single-copy integrants into the *amyE* locus and of two as integrants into the *thrC* locus. Previously, we constructed 6 of the 10 *rrn* genes as single-copy insertional plasmids that integrated at their native loci (52). These studies were carried out with fusions of plasmid pDEB1 that originated from pCED6, containing a promoterless *E. coli lacZ* gene with translational

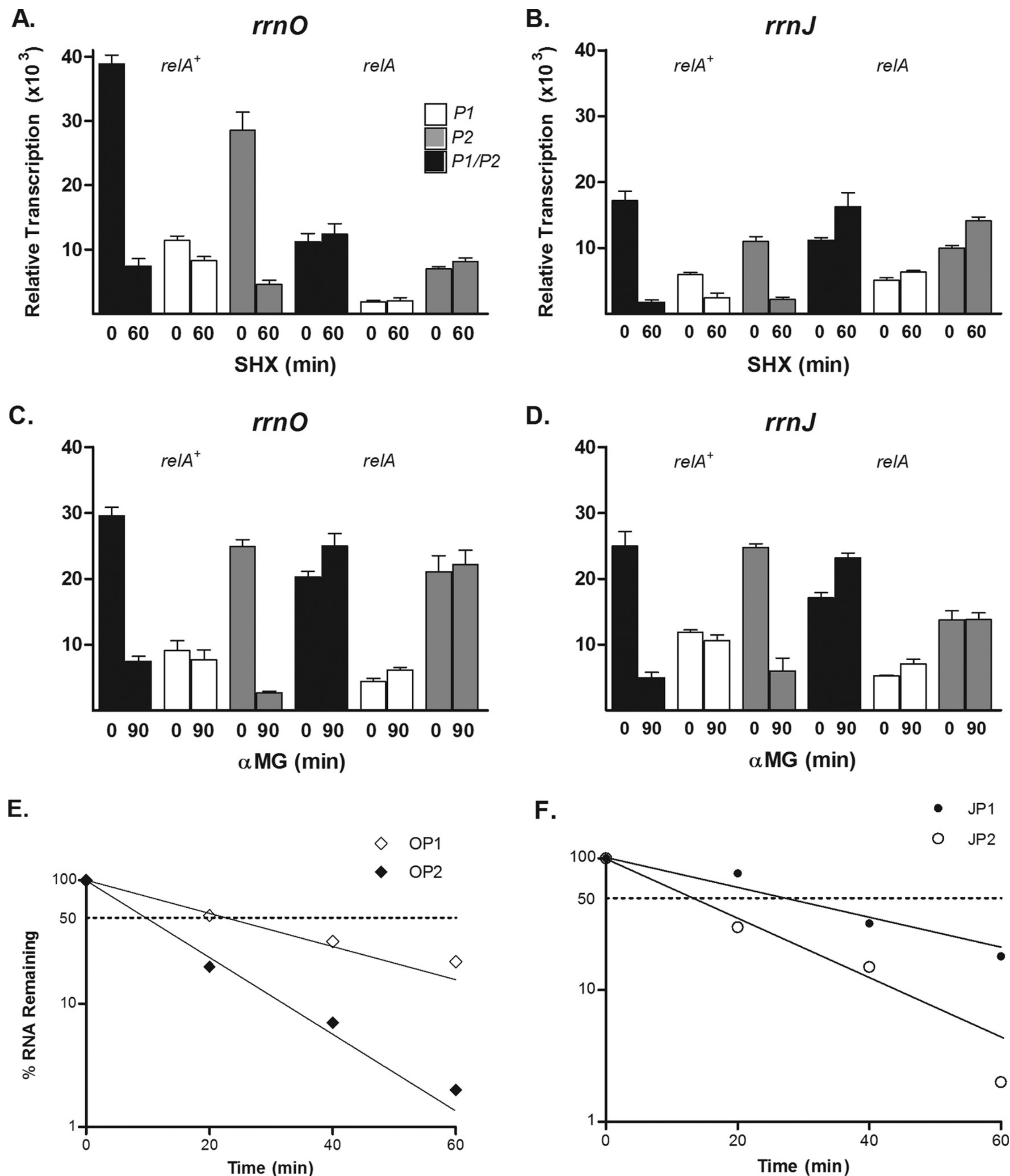

FIG. 5. Responses of separated promoter elements to nutritional stress. Wild-type *relA*$^+$ cells containing the indicated *lacZ* promoter fusion constructs were grown in MM1 and were treated with SHX (A and B) or with α-MG (C and D) for the indicated times. The relative activities of the promoter fragments of *rrnO* and *rrnJ* were measured by densitometric scans of autoradiograms from RNA slot blots before and after treatments. The values were calculated as described in the legend for Fig. 3. (E and F) The chemical half-lives of *lacZ* mRNAs transcribed from the isolated P1 or P2 elements of *rrnO* and *rrnJ* were determined and plotted as described in the legend for Fig. 1C and D.

trp sequences of *E. coli* and a selectable Cm^r marker (44). The presence of the *E. coli* translational sequences in the plasmids used resulted in lower levels of β-galactosidase (20 to 120 Miller units), yet the same gradient of promoter strength (i.e., *rrnO*, *rrnA*, *rrnW*, *rrnE*, *rrnD*, and *rrnB*) was observed (51). The use of another fusion system (*rrn-bgaB*) from the thermostable organism *Bacillus stearothermophilus* (20a), integrated at the *amyE* locus, resulted in a similar gradient of promoter strength (i.e., *rrnO*, *rrnE*, and *rrnD*) (8). In *B. subtilis* and *E. coli*, the expression hierarchy of β-galactosidase or chloramphenicol acetyltransferase (CAT) activities from fusions in various media correlates with their original genomic locations relative to *oriC* (**7**, 8, 19, 51). Even at the *amyE* locus, located at 25° on the *B. subtilis* map (2), the promoters of *rrnO* and *rrnJ*, located at 1° and 10°, respectively, clearly retained their high activities, while the promoters of *rrnD* and *rrnB*, located at 70° and 280°,

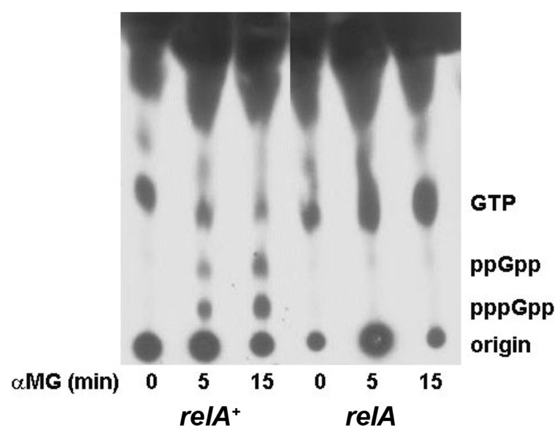

FIG. 6. Accumulation of (p)ppGpp in *B. subtilis relA*$^+$, *relA*, and *relA*(S) strains during nutritional stress. The indicated strains were grown in low-phosphate medium, followed by a 1-h labeling with [^{32}P]phosphoric acid. Labeled cells were stressed with SHX or α-MG, and samples were removed at the indicated times and were processed for thin-layer chromatography on PEI plates as described in Materials and Methods. The autoradiogram shows results for a representative carbon source starvation experiment.

respectively, remained weak (2) (Table 2; Fig. 1A and B). Moreover, at their native locations, *rrnD* and *rrnB* are linked to the two largest tRNA gene clusters, *trnD* (16 tRNA genes) and *trnB* (21 tRNA genes) (19). These clusters are symmetrically situated downstream of these low-expressing operons and are located farthest away from the *oriC* region (2, 16, 40). Since these tRNA genes are actually part of the rRNA transcriptional unit (16), it has been suggested that this arrangement may offer an explanation for the differences in strength and could define a temporal mechanism of regulation for the 10 ribosomal operons in the endospore-forming bacterium *B. subtilis* (35a, 40). Our results do not agree with those of another report dealing with similar *lacZ* rRNA promoter fusions integrated at the *amyE* locus, which showed that the two elements (P1 and P2) have similar expression levels and that $rrnp_1$ promoters from *rrnO* or *rrnB* are essentially as regulated as $rrnp_2$ promoters (see Fig. 1E in reference 24). Krásný and Gourse (24) did not detect the asymmetry in expression between these two promoters reported here and by Okamoto and Vold (35a). The differences in the findings could be related to the lengths of the promoter-bearing fragments. Specifically, our fragments (Table 1) and those used by Okamoto and Vold (35a) were considerably longer (and included additional "UP" sequences) than those used by Krásný and Gourse, which were 58 to 59 bases long (24). In 2006, Koga et al. (23a) reported similar results showing differential transcription of *rrn* during spore development in *B. subtilis*, where the P1 and P2 promoter activity levels of *rrnO*, *rrnE*, and *rrnD* were greater in rich medium than in poor medium.

In *B. subtilis* and *E. coli*, nutritional limitations trigger the stringent response, which results in the cessation of stable RNA synthesis (6, 19). We have compared the effects of SHX and α-MG on the expression of five promoter fusions in *B. subtilis*: *rrnO*-, *rrnJ*-, *rrnD*-, *rrnB*-, and *veg-lacZ*. Among the promoter fusions tested, only two (*rrnO* and *rrnJ*) were stringently regulated, exhibiting 8- to 15-fold decreases in expression upon amino acid or carbon source starvation (Fig. 3A and B and 4A and B). The weak promoters (*rrnD* and *rrnB*) and the nonregulated *veg* promoter did not respond to starvation. In *E. coli*, all seven operons are stringently regulated, exhibiting 2- to 3-fold decreases following SHX treatment (7). The extent of response in *E. coli* also varied, with *rrnD* and *rrnE* operons having the strongest effect (a 3-fold decrease) and *rrnB*, *rrnC*, and *rrnH* showing a lower decrease of 1.9-fold (7). While transcript stability may differ somewhat by the addition of SHX or α-MG, the relative contributions of the *lacZ* or CAT fusions in *B. subtilis* and *E. coli* should be equal, since they all contain the same portion of the 16S gene. Thus, the differences noted in both bacterial systems are valid measurements of the stringent control of each of the *rrn* promoters and are not likely to reflect differential effects of mRNA stability (8), as determined by their half-lives ($t_{1/2}$).

Starvation treatment of *B. subtilis relA*$^+$ strains led to significant accumulation of (p)ppGpp, while in *relA*(S) strains, α-MG, but not SHX, caused the appearance of highly phosphorylated guanosine nucleotides (Fig. 6A and Table 4). These findings are contrary to those of Krásný and Gourse (24), who reported no increase in ppGpp accumulation upon α-MG treatment in cells grown in minimal medium containing 0.5 mM KH_2PO_4, glucose, and 20 amino acids. Our accumulation studies of (p)ppGpp were performed under low-phosphate conditions (0.1 mM KH_2PO_4) and in the presence of 12 amino acids (see Materials and Methods) (33, 41), and this may account for the different observations.

Despite the relatively significant accumulations of (p)ppGpp induced during α-MG challenge in the *relA*$^+$ and *relA*(S) strains (41), transcription from the isolated P1 elements of *rrnO* and *rrnJ* persisted, while that from P2 and P1-P2 declined dramatically (Fig. 5C and D). The P1 promoter elements showed small reductions in activity after SHX treatment compared to those of the strong P2 elements (Fig. 5A and B). Since SHX treatment leads to higher levels of (p)ppGpp than does α-MG treatment (Table 4), the behavior of the P1 elements

TABLE 4. Relative GTP levels before and after treatments and accumulation of (p)ppGpp in three genetic backgrounds[a]

Treatment and strain	Treatment time (min)	GTP level (%)	Accumulation ($nmol/A_{690}$)	
			ppGpp	pppGpp
SHX				
relA$^+$	0	100	0	0
	15	20	0.58	0.75
relA	0	100	0	0
	15	170	0	0
relA(S)	0	100	0	0
	15	83	0	0
αMG				
relA$^+$	0	100	0	0
	15	22	0.42	0.43
relA	0	100	0	0
	15	86	0	0
relA(S)	0	100	0	0
	15	25	0.11	0.16

[a] The levels of GTP were calculated by densitometric scans of autoradiograms, The absolute levels of (p)ppGpp were determined by excision of the appropriate spots from TLC plates, followed by scintillation counting, performed as in a previous study (17).

suggests that they can mount a stringent response, but it requires a stronger stimulus, and the P2 element is still the major site of *rrn* promoter regulation.

The data presented in this study make it clear that *B. subtilis rrn* operons are heterogeneous with respect to both promoter strength and the stringent response. It also appears that, within the tandem promoters, the P2 element is responsible for most of the regulation seen in the intact promoter. Thus, in contrast to the situation in *E. coli*, the *rrn* operons in *B. subtilis* may have unique, dedicated functions based on the strong differences in stringent responses that we observed.

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