

# Translation Control of *trpG* from Transcripts Originating from the Folate Operon Promoter of *Bacillus subtilis* Is Influenced by Translation-Mediated Displacement of Bound TRAP, While Translation Control of Transcripts Originating from a Newly Identified *trpG* Promoter Is Not<sup>∇†</sup>

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*Bacillus subtilis trpG* encodes a glutamine amidotransferase subunit that participates in the biosynthesis of both tryptophan and folic acid. TRAP inhibits translation of *trpG* in response to tryptophan by binding to a site that overlaps the *trpG* Shine-Dalgarno sequence, thereby blocking ribosome binding. Similar mechanisms regulate *trpP* and *ycbK* translation. The equilibrium binding constants of tryptophan-activated TRAP for the *trpG*, *ycbK*, and *trpP* transcripts were determined to be 8, 3, and 50 nM, respectively. Despite TRAP having a higher affinity for the *trpG* transcript, TRAP exhibited the least control of *trpG* expression. The *trpG* Shine-Dalgarno sequence overlaps the stop codon of the upstream *pabB* gene, while six of nine triplet repeats within the TRAP binding site are located upstream of the *pabB* stop codon. Thus, ribosomes translating the upstream *pabB* cistron could be capable of reducing TRAP-dependent control of TrpG synthesis by displacing bound TRAP. Expression studies using *pabB-trpG'-lacZ* fusions in the presence or absence of an engineered stop codon within *pabB* suggest that translation-mediated displacement of bound TRAP reduces TRAP-dependent inhibition of TrpG synthesis from transcripts originating from the folate operon promoter ( $P_{pabB}$ ). A new *trpG* promoter ( $P_{trpG}$ ) was identified in the *pabB* coding sequence that makes a larger contribution to *trpG* expression than does  $P_{pabB}$ . We found that TRAP-dependent regulation of *trpG* expression is more extensive for a transcript originating from  $P_{trpG}$  and that transcripts originating from  $P_{trpG}$  are not subject to translation-mediated displacement of bound TRAP.

The *trpEDCFBA* operon of *Bacillus subtilis* carries six of the seven tryptophan biosynthetic genes and is contained within a larger aromatic amino acid supraoperon (Fig. 1A). The seventh tryptophan biosynthetic gene, *trpG* (*pabA*), is present in a folic acid biosynthesis operon. Expression of the tryptophan biosynthetic genes is regulated in response to tryptophan by *trp* RNA-binding attenuation protein (TRAP) via transcription attenuation and translation control mechanisms (reviewed in references 3 and 13). TRAP also regulates translation of *trpP*, a gene that encodes an apparent tryptophan transporter (22, 29), and *ycbK*, a gene that shares sequence homology to known efflux protein genes (23, 28). TRAP consists of 11 identical subunits arranged in a single ring (2). When activated by tryptophan, 11 KKR motifs on the perimeter of TRAP interact with multiple NAG trinucleotide repeats in target transcripts, with a preference for N of  $G \approx U > A > C$ , thereby wrapping the RNA around TRAP's perimeter (1, 6, 32). Optimal spacing between triplet repeats is 2 nucleotides (nt) (5, 7), although 1-nt spacers

and spacers as large as 14 nt have been observed in natural TRAP targets (Fig. 1B).

The *trpEDCFBA* operon leader transcript is capable of folding into mutually exclusive antiterminator and terminator structures that participate in the transcription attenuation mechanism. When activated by tryptophan, TRAP can bind to 11 triplet repeats present in the nascent *trp* leader transcript (Fig. 1B). Bound TRAP prevents formation of the antiterminator structure because six of the triplet repeats are present within its stem. As a consequence, formation of the overlapping terminator hairpin causes RNA polymerase (RNAP) to terminate transcription in the leader region. In the absence of TRAP binding, formation of the antiterminator allows transcriptional readthrough into the *trp* operon structural genes (4, 6, 19). TRAP also regulates translation initiation of *trpE* (Fig. 1A). TRAP binding to *trp* operon readthrough transcripts promotes formation of a *trpE* Shine-Dalgarno (S-D) sequence-sequestering hairpin. Formation of this RNA structure inhibits translation initiation by preventing ribosome binding (11, 18).

A third TRAP-dependent regulatory mechanism is responsible for controlling translation initiation of *trpG*, *ycbK*, and *trpP*, with bound TRAP directly blocking ribosome binding (12, 18, 22, 23, 28, 29, 31). TrpG functions as a common glutamine amidotransferase subunit in the biosynthesis of both tryptophan and folic acid. TRAP binds to nine triplet repeats that overlap the *trpG* S-D sequence (Fig. 1B). The TRAP binding site in the *trpP* transcript also contains nine triplet repeats that overlap its S-D sequence (Fig. 1B). One distinc-

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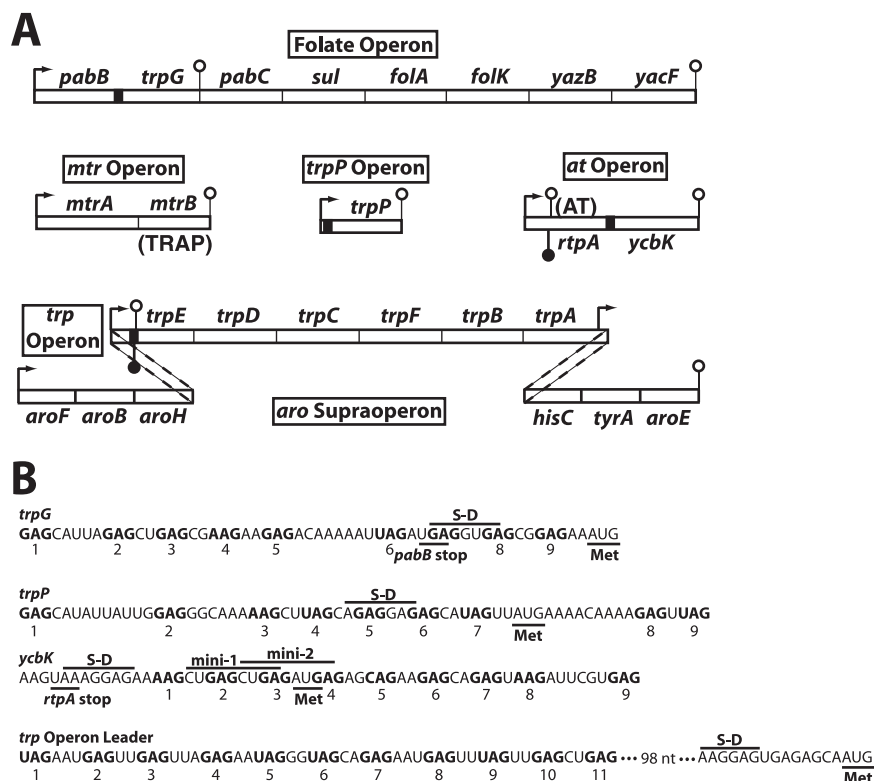


FIG. 1. Organization of *B. subtilis* tryptophan metabolism genes and sequence comparison of the four known TRAP binding sites. (A) The six genes of the *trp* operon, which is part of the aromatic amino acid supraoperon, and *trpG* encode the tryptophan biosynthetic enzymes. *trpP* encodes a putative tryptophan transporter, while the *ycbK* gene product is similar to known efflux proteins. *mtrA* and the folate operon encode proteins involved in folic acid metabolism. *mtrB* encodes TRAP. TRAP regulates the expression of tryptophan metabolism genes by transcription attenuation (*trpEDCFBA* operon) and translation control (*trpG*, *trpP*, *ycbK*, and *trpE*) mechanisms. *rtpA* encodes anti-TRAP (AT), a protein that binds to and inactivates tryptophan-activated TRAP (25). Filled and open lollipop structures represent antiterminator and terminator structures, respectively. The four known TRAP binding sites are represented by black boxes, and bent arrows represent promoters. (B) The triplet repeats of the four known TRAP binding sites are shown in bold and are numbered. The S-D sequences and translation start codons (Met) are shown for *trpG*, *trpP*, *ycbK*, and *trpE* (first gene in the *trp* operon). There is a 98-nt gap in the sequence between the last triplet repeat in the *trp* operon leader and the *trpE* S-D sequence. The positions of the two dipeptide-encoding minigenes in the *ycbK* translation initiation region are marked. Expression of minigene 1 has a small inhibitory effect on translation of *ycbK* (28). The *pabB* and *rtpA* stop codons overlap the S-D sequences of *trpG* and *ycbK*, respectively.

tion between *trpP* and *trpG* translation control is that the TRAP binding site in *trpP* mRNA extends into the *trpP* coding sequence, whereas the TRAP binding site in *trpG* ends just prior to the coding sequence. TRAP also inhibits translation initiation of *ycbK* by a similar mechanism; however, in this case, all nine triplet repeats are downstream from the S-D sequence and extend further into the *ycbK* coding sequence than is the case for *trpP* (Fig. 1B). In addition, expression of a dipeptide-encoding minigene that utilizes the *ycbK* S-D sequence has a small inhibitory effect on YcbK synthesis (Fig. 1B) (28).

In the present study, we compared the extents of TRAP-mediated translation inhibition of *trpG*, *ycbK*, and *trpP*. Our results led to the hypothesis that the *pabB* and *trpG* gene arrangement might contribute to the low level of TRAP-dependent regulation of *trpG*. Our data suggest that ribosomes that translate *pabB* are capable of displacing bound TRAP from the *trpG* S-D sequence. A new *trpG* promoter was also identified that is not subject to translation-mediated displacement of bound TRAP.

## MATERIALS AND METHODS

**Bacterial strains and plasmids.** The *B. subtilis* strains used in this study are listed in Table 1. The cloning vectors pTZ18U and pTZ19R (U.S. Biochemical) contain a T7 RNA polymerase promoter upstream from their polylinkers. Plasmids pPB77, containing the *B. subtilis* *trp* operon leader (4), pPB31, containing *trpG* (6), and pHZB6, containing *trpP* (29), and the *B. subtilis* integration vector ptrpBGI-PLK (18) were described previously.

Plasmid pYH53 contains a partial in-frame deletion of *pabB* cloned into pTZ18U. The resulting fragment (nt -92 to +138 and +1336 to +1498 relative to the *pabB* transcriptional start site) was subcloned into ptrpBGI-PLK, thereby producing a *trpG*'-*lacZ* translational fusion (pYH55) driven exclusively by *P*<sub>*pabB*</sub> (prime symbols indicate truncations of the genes) (Fig. 2, fusion 1). Plasmid pYH54 contains a TAA stop codon in place of the GGA glycine codon (nt +1336 to +1338 relative to the *pabB* transcriptional start site) that is present in pYH53. Plasmid pYH56 is identical to pYH55 except that it contains the mutation of the GGA glycine codon to a TAA stop codon. Plasmids pYH55 and pYH56 were linearized and integrated into the chromosomal *amyE* locus of *B. subtilis* strain PLBS338 (prototrophic), resulting in strains PLBS442 and PLBS443, respectively.

Plasmid pYH66 contains nt -92 to +1498 relative to the *pabB* transcriptional start site cloned into pTZ19R. Plasmid pYH67 is identical to pYH66 except that it contains the mutation of the GGA glycine codon to a TAA stop codon. The folate operon-specific DNAs from pYH66 and pYH67 were ligated into pTrpBGI-

TABLE 1. *B. subtilis* strains used in this study

Strain	Genotype <sup>a</sup>	Reference
BG4233	$\Delta mtrB$	14
PLBS338	Prototroph	29
PLBS339	$amyE::P_{trpB}trpP'-lacZ$ Cm <sup>r</sup>	29
PLBS341	$\Delta mtrB amyE::P_{trpB}trpP'-lacZ$ Cm <sup>r</sup>	29
PLBS343	$\rho::neo$ (Km <sup>r</sup> ) $amyE::P_{trpB}trpP'-lacZ$ Cm <sup>r</sup>	29
PLBS418	$amyE::P_{aycbK}'-lacZ$ Cm <sup>r</sup>	28
PLBS420	$\Delta mtrB amyE::P_{aycbK}'-lacZ$ Cm <sup>r</sup>	28
PLBS421	$\rho::neo$ (Km <sup>r</sup> ) $amyE::P_{aycbK}'-lacZ$ Cm <sup>r</sup>	28
PLBS442	$amyE::P_{pabB}(-92 \text{ to } +138 \text{ and } +1336 \text{ to } +1498) trpG'-lacZ^b$ Cm <sup>r</sup>	This study
PLBS443	$amyE::P_{pabB}(-92 \text{ to } +138 \text{ and } +1336 \text{ to } +1498) trpG'-lacZ(pabB \text{ stop codon})^{b,c}$ Cm <sup>r</sup>	This study
PLBS486	$amyE::P_{pabB}(-92 \text{ to } +1498) trpG'-lacZ^d$ Cm <sup>r</sup>	This study
PLBS487	$amyE::P_{pabB}(-92 \text{ to } +1498) trpG'-lacZ(pabB \text{ stop codon})^{c,d}$ Cm <sup>r</sup>	This study
PLBS496	$\rho::neo$ (Km <sup>r</sup> ) $amyE::P_{pabB}(-92 \text{ to } +1498) trpG'-lacZ^d$ Cm <sup>r</sup>	This study
PLBS497	$\rho::neo$ (Km <sup>r</sup> ) $amyE::P_{pabB}(-92 \text{ to } +1498) trpG'-lacZ(pabB \text{ stop codon})^{c,d}$ Cm <sup>r</sup>	This study
PLBS498	$\Delta mtrB amyE::P_{pab}(-92 \text{ to } +1498) trpG'-lacZ^d$ Cm <sup>r</sup>	This study
PLBS499	$\Delta mtrB amyE::P_{pabB}(-92 \text{ to } +1498) trpG'-lacZ(pabB \text{ stop codon})^{c,d}$ Cm <sup>r</sup>	This study
PLBS503	$amyE::P_{trpG}(+1040 \text{ to } +1498) trpG'-lacZ^e$ Cm <sup>r</sup>	This study
PLBS504	$amyE::P_{trpG}(+1040 \text{ to } +1498) trpG'-lacZ(pabB \text{ stop codon})^{c,e}$ Cm <sup>r</sup>	This study
PLBS508	$\Delta mtrB amyE::P_{trpG}(+1040 \text{ to } +1498) trpG'-lacZ^e$ Cm <sup>r</sup>	This study
PLBS509	$\Delta mtrB amyE::P_{trpG}(+1040 \text{ to } +1498) trpG'-lacZ(pabB \text{ stop codon})^{c,e}$ Cm <sup>r</sup>	This study
PLBS510	$\Delta mtrB amyE::P_{pabB}(-92 \text{ to } +138 \text{ and } +1336 \text{ to } +1498) trpG'-lacZ^b$ Cm <sup>r</sup>	This study
PLBS511	$\Delta mtrB amyE::P_{pabB}(-92 \text{ to } +138 \text{ and } +1336 \text{ to } +1498) trpG'-lacZ(pabB \text{ stop codon})^{b,c}$ Cm <sup>r</sup>	This study
PLBS512	$\rho::neo$ (Km <sup>r</sup> ) $amyE::P_{trpG}(+1040 \text{ to } +1498) trpG'-lacZ^e$ Cm <sup>r</sup>	This study
PLBS513	$\rho::neo$ (Km <sup>r</sup> ) $amyE::P_{trpG}(+1040 \text{ to } +1498) trpG'-lacZ(pabB \text{ stop codon})^{c,e}$ Cm <sup>r</sup>	This study
PLBS514	$\rho::neo$ (Km <sup>r</sup> ) $amyE::P_{pabB}(-92 \text{ to } +138 \text{ and } +1336 \text{ to } +1498) trpG'-lacZ^b$ Cm <sup>r</sup>	This study
PLBS515	$\rho::neo$ (Km <sup>r</sup> ) $amyE::P_{pabB}(-92 \text{ to } +138 \text{ and } +1336 \text{ to } +1498) trpG'-lacZ(pabB \text{ stop codon})^{b,c}$ Cm <sup>r</sup>	This study

<sup>a</sup>  $P_{pabB}$  and  $P_{trpG}$  denote the *pabB* and *trpG* promoters, respectively. A prime symbol indicates truncation of the gene.

<sup>b</sup> This *trpG'-lacZ* translational fusion is driven exclusively by the  $P_{pabB}$  promoter.

<sup>c</sup> This *trpG'-lacZ* translational fusion contains an engineered stop codon that replaces the GGA glycine codon at nt +1336 to +1338 relative to the  $P_{pabB}$  promoter transcription start site.

<sup>d</sup> This *trpG'-lacZ* translational fusion is driven by the  $P_{pabB}$  and  $P_{trpG}$  promoters.

<sup>e</sup> This *trpG'-lacZ* translational fusion is driven exclusively by the  $P_{trpG}$  promoter.

PLK. The resulting circular plasmids were linearized and subsequently integrated into the *amyE* locus by transforming strain PLBS338, thereby generating strains PLBS486 and PLBS487, respectively. The resulting *trpG'-lacZ* translational fusions were driven by both  $P_{pabB}$  and  $P_{trpG}$  (Fig. 2, fusion 2). Proper fusions were confirmed by sequencing PCR products from both strains.

Plasmids pYH68 and pYH69 were constructed by subcloning a DNA fragment containing nt +1040 to +1498 relative to the *pabB* transcription start site from pYH66 and pYH67 into pTRPBG1-PLK, respectively. In both cases, the resulting *trpG'-lacZ* translational fusions were driven exclusively by the  $P_{trpG}$  promoter (Fig. 2, fusion 3). Plasmids pYH68 and pYH69 were linearized and subsequently integrated into the chromosomal *amyE* locus of strain PLBS338 as described above, giving rise to strains PLBS503 and PLBS504, respectively.

Transformation of *B. subtilis* strains PLBS442, PLBS443, PLBS486, PLBS487, PLBS503, and PLBS504 with chromosomal DNA from strain BG4233 ( $\Delta mtrB$  TRAP<sup>-</sup>) (14) resulted in strains PLBS510, PLBS511, PLBS498, PLBS499,

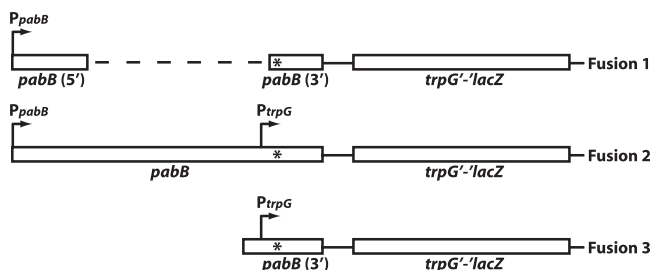


FIG. 2. Schematic representation of translational fusions used in this study. The  $P_{pabB}$  and  $P_{trpG}$  promoters are shown. The dashed line in fusion 1 represents a 1,197-nt partial in-frame deletion of the *pabB* coding sequence. This deletion removes  $P_{trpG}$ . Each fusion was engineered with and without a TAA stop codon in the *pabB* coding sequence (\*).

PLBS508, and PLBS509, respectively. The null  $\rho::neo$  allele from linearized plasmid pYH14 (30) was used to replace the wild-type (WT)  $\rho$  gene in strains PLBS442, PLBS443, PLBS486, PLBS487, PLBS503, and PLBS504 to yield strains PLBS514, PLBS515, PLBS496, PLBS497, PLBS512, and PLBS513, respectively.

**$\beta$ -Galactosidase assay.** *B. subtilis* cultures were grown at 37°C in minimal acid casein hydrolysate (0.2%) medium containing 5  $\mu$ g of chloramphenicol/ml in the absence or presence of 200  $\mu$ M tryptophan. Growth medium for  $\rho$  null strains also contained 10  $\mu$ g of kanamycin/ml. Cells were harvested in late exponential phase and assayed for  $\beta$ -galactosidase activity as described previously (11, 20).

**Gel mobility shift assay.** Quantitative gel mobility shift assays used to examine TRAP-RNA interactions were performed by following published procedures (27, 29). TRAP was purified as described previously (27). *trpG*, *trpP*, *ycbK*, and *trp* operon leader RNAs were synthesized in vitro and 5' end labeled with [ $\gamma$ -<sup>32</sup>P]ATP. Gel-purified transcripts were renatured by heating to 80°C for 1 min, followed by slow cooling. Binding reactions contained 40 mM Tris-HCl (pH 8.0), 4 mM MgCl<sub>2</sub>, 30 mM KCl, 1 mM dithiothreitol (DTT), 10% glycerol, 0.2 mg *Escherichia coli* tRNA/ml, 400 U of RNasin/ml, 5'-end-labeled RNA, 1.2 mM L-tryptophan, TRAP, and 0.1 mg of xylene cyanol/ml. The concentrations of labeled *trpG*, *trpP*, *ycbK*, and *trp* operon leader RNAs were 50 pM, 400 pM, 50 pM, and 10 pM, respectively. TRAP-RNA complexes were allowed to equilibrate at 37°C for 30 min. Samples were fractionated in native polyacrylamide gels. Radioactive bands were visualized and quantified, and  $K_d$  values were calculated as described previously (27).

**Filter binding assay.** Filter binding reactions were performed following a published procedure (24). Purified TRAP and labeled RNAs were identical to those described for the gel mobility shift assay. Binding reactions contained 40 mM Tris-HCl (pH 8.0), 4 mM MgCl<sub>2</sub>, 250 mM KCl, 1 mM DTT, 0.2 mg of *E. coli* tRNA/ml, 5'-end-labeled RNA, 1.2 mM L-tryptophan, and TRAP. TRAP-RNA complexes were allowed to equilibrate at 37°C for 30 min. Samples were filtered and washed twice with 0.5 ml of 40 mM Tris-HCl (pH 8.0) and 250 mM KCl. Radioactive spots were visualized and quantified as described for the gel mobility shift assay.

**Primer extension reaction.** Total RNA was isolated from *B. subtilis* strain PLBS338. Twenty micrograms of RNA was hybridized to a <sup>32</sup>P-end-labeled DNA

TABLE 2. Affinities of TRAP for *trpG*, *trpP*, *ycbK*, and *trpL* leader RNA

Transcript	$K_d$ (nM) <sup>a</sup>	
	Gel shift assay	Filter binding assay
<i>trpG</i>	8.4 ± 1.7	8.0 ± 3.0
<i>trpP</i>	34 ± 10	78 ± 5
<i>ycbK</i>	2.9 ± 0.9	3.4 ± 1.4
<i>trpL</i>	0.6 ± 0.3	0.7 ± 0.3

<sup>a</sup> Values are averages for at least three independent experiments ± standard deviations.

oligonucleotide complementary to nt +1325 to +1355 relative to the *pabB* transcriptional start site. Reaction mixtures (10 μl) containing 3 μl of hybridization mixture, a 375 μM concentration of each deoxynucleoside triphosphate, 10 mM DTT, 100 μg/ml of bovine serum albumin, 1× Moloney murine leukemia virus (MMLV) reaction buffer, and 20 U/μl MMLV reverse transcriptase were incubated for 15 min at 42°C. Reactions were terminated by the addition of stop solution (29), and samples were fractionated through 6% sequencing gels. Sequencing reactions were performed using pYH66 as the template and the same end-labeled DNA oligonucleotide as a primer.

**In vitro transcription.** Single-round in vitro transcription reactions and data analysis were performed as described previously (26). *B. subtilis* σ<sup>A</sup> RNAP was purified as described previously (21), with an additional HiTrap Heparin HP (Amersham) chromatography step. Stable transcription elongation complexes were formed in a reaction mix containing 10 μM ATP, 10 μM UTP, 2 μM GTP, 2 μCi [α-<sup>32</sup>P]UTP, and *trpG* DNA templates. Transcription elongation was halted after incorporation of the 21st nt due to the absence of CTP. Elongation of halted transcription complexes was resumed by the addition of all four nucleoside triphosphates together with heparin. The final concentrations were 150 μM CTP, 150 μM GTP, 150 μM UTP, 10 μM ATP, and 100 μg/ml of heparin. Elongation reactions were stopped after 10 min by the addition of 2× loading buffer. Samples were fractionated through 6% polyacrylamide sequencing gels. DNA templates used in this analysis included a 350-bp EcoRI-HindIII fragment of pYH66 (positions -200 to +150 relative to the start of *P<sub>trpG</sub>* transcription) and a 188-bp PCR product (positions -75 to +113 relative to the start of *P<sub>trpG</sub>* transcription). A DNA template giving rise to a 139-nt transcript was used as a size marker.

## RESULTS

**TRAP-mediated inhibition of *trpG* expression is modest despite high TRAP-*trpG* RNA affinity.** The affinities of tryptophan-activated TRAP for the four known TRAP binding sites in *B. subtilis* (Fig. 1B) were compared by gel mobility shift and filter binding assays (Table 2). TRAP exhibited the highest affinity for the *trp* operon leader RNA, followed by the *ycbK*, *trpG*, and *trpP* transcripts. With the exception of the twofold difference for *trpP*, the  $K_d$  values using the two methods were virtually identical. The affinities of TRAP for the four tran-

scripts partially reflect the number of repeats as well as the number of repeats separated by optimal 2-nt spacers (Fig. 1B). For example, TRAP exhibited the highest affinity for the *trp* leader transcript, which contains 11 triplet repeats and 7 optimal spacers, whereas TRAP had the lowest affinity for *trpP*, a transcript that contains 9 repeats and only 4 optimal spacers. TRAP had an intermediate affinity for the *trpG* and *ycbK* transcripts, both of which contain nine repeats and six optimal spacers.

TRAP is responsible for regulating expression of the tryptophan metabolism genes in response to tryptophan. The *trpEDCFBA* operon is regulated by TRAP-dependent transcription attenuation and translation control mechanisms, while *trpG*, *trpP*, and *ycbK* are regulated by TRAP at the level of translation only (Fig. 1). The extents of TRAP-dependent translation control of *trpG*, *trpP*, and *ycbK* were compared by examining the expression of translational fusions with *lacZ* (Table 3). The effect of exogenous tryptophan was assessed by the ratio of expression when cells were grown in the absence and presence of added tryptophan (-Trp/+Trp ratio). TRAP exhibited the tightest control of *trpP* expression (150-fold inhibition), whereas there was considerably less regulation of *ycbK* (9-fold) and *trpG* (5-fold) expression. Because TRAP is responsible for regulating the translation of *trpG*, *trpP*, and *ycbK* in response to tryptophan, expression of the three fusions did not respond to tryptophan in a  $\Delta mtrB$  (TRAP-deficient) genetic background. The observation that expression levels of all three fusions under Trp-negative conditions were lower in WT strains than in the corresponding *mtrB* mutant strains indicates that the level of endogenously synthesized tryptophan is sufficient to partially activate TRAP. The extent of TRAP-mediated regulation in vivo was determined by comparing expression in the  $\Delta mtrB$  strains with that in the WT strains grown in the presence of tryptophan ( $\Delta mtrB$ /WT ratio). TRAP-dependent inhibition was highest for *trpP* (>900-fold), followed by *ycbK* (21-fold) and *trpG* (12-fold).

**Translation-mediated displacement of bound TRAP reduces TRAP-dependent translation inhibition of *trpG*.** TRAP-dependent regulation is far more extensive for *trpP* than for *trpG* and *ycbK*, despite TRAP having 5- and 10-fold higher affinities for the *trpG* and *ycbK* transcripts, respectively (Tables 2 and 3). What could account for this apparent discrepancy? A possible explanation for the large difference in TRAP-dependent control of *trpP* and *trpG* translation could be related to the gene arrangement of the two operons. Interestingly, the *pabB* stop

TABLE 3. TRAP-mediated regulation of *trpG*, *trpP*, and *ycbK* expression

Relevant genotype	Fusion	$\beta$ -Galactosidase activity <sup>a</sup>			Inhibition ( $\Delta mtrB$ /WT) <sup>b</sup>
		-Trp	+Trp	-Trp/+Trp	
WT	<i>trpG'</i> - <i>lacZ</i>	110 ± 20	21 ± 4	5.2	12
$\Delta mtrB$	<i>trpG'</i> - <i>lacZ</i>	260 ± 35	260 ± 20	1.0	
WT	<i>trpP'</i> - <i>lacZ</i>	200 ± 30	1.3 ± 0.3	150	920
$\Delta mtrB$	<i>trpP'</i> - <i>lacZ</i>	1,200 ± 160	1,200 ± 220	1.0	
WT	<i>ycbK'</i> - <i>lacZ</i>	12 ± 2	1.3 ± 0.2	9.2	21
$\Delta mtrB$	<i>ycbK'</i> - <i>lacZ</i>	30 ± 6	27 ± 4	1.1	

<sup>a</sup>  $\beta$ -Galactosidase activities are given in Miller units (20). Values are averages for at least three independent experiments ± standard deviations.

<sup>b</sup> The extent of TRAP-dependent inhibition was determined by dividing the value for the *mtrB* mutant by the value for the corresponding WT strain in the presence of tryptophan.



TABLE 4. Ribosome-mediated displacement of bound TRAP influences *trpG*'-'*lacZ* expression

Row	Relevant genotype	Fusion <sup>a</sup>	Stop codon <sup>b</sup>	β-Galactosidase activity <sup>c</sup>			Inhibition ( $\Delta mtrB$ /WT) <sup>d</sup>
				-Trp	+Trp	-Trp/+Trp	
1	WT	1	No	8 ± 2	2.8 ± 0.2	2.9	3.6
2	$\Delta mtrB$	1	No	10 ± 1	10 ± 2	1.0	
3	$\Delta rho$	1	No	7 ± 2	3.8 ± 1.6	1.8	
4	WT	1	Yes	7.4 ± 0.6	0.5 ± 0.1	15	28
5	$\Delta mtrB$	1	Yes	14 ± 2	14 ± 1	1.0	
6	$\Delta rho$	1	Yes	5 ± 1	0.5 ± 0.1	10	
7	WT	2	No	110 ± 20	21 ± 2	5.2	12
8	$\Delta mtrB$	2	No	260 ± 40	260 ± 20	1.0	
9	$\Delta rho$	2	No	75 ± 15	16 ± 3	4.7	
10	WT	2	Yes	100 ± 20	11 ± 2	9.1	25
11	$\Delta mtrB$	2	Yes	290 ± 10	270 ± 15	1.1	
12	$\Delta rho$	2	Yes	70 ± 10	8 ± 1	8.8	
13	WT	3	No	60 ± 10	5 ± 1	12	50
14	$\Delta mtrB$	3	No	230 ± 40	250 ± 50	0.9	
15	$\Delta rho$	3	No	50 ± 5	7 ± 1	7.1	
16	WT	3	Yes	80 ± 5	6 ± 1	13	37
17	$\Delta mtrB$	3	Yes	270 ± 30	220 ± 30	1.2	
18	$\Delta rho$	3	Yes	55 ± 10	10 ± 2	5.5	

<sup>a</sup> The three fusions are (1)  $P_{pabB}$ - $pabB$ '- $\Delta$ '- $pabB$ - $trpG$ '-' $lacZ$ , (2)  $P_{pabB}$ - $pabB$ - $P_{trpG}$ - $trpG$ '-' $lacZ$ , and (3)  $P_{trpG}$ - $trpG$ '-' $lacZ$ , where  $P$  denotes a promoter and  $\Delta$  indicates a partial in-frame deletion of *pabB*.

<sup>b</sup> Presence (yes) or absence (no) of an engineered stop codon within the coding sequence of *pabB*.

<sup>c</sup> β-Galactosidase activities are given in Miller units (20). Values are averages for at least three independent experiments ± standard deviations.

<sup>d</sup> The extent of TRAP-dependent inhibition was determined by dividing the value for the *mtrB* mutant by the value for the corresponding WT strain in the presence of tryptophan.

codon lies within the *trpG* S-D sequence (Fig. 1B). Since seven of the triplet repeats in the *trpG* TRAP binding site lie upstream or within the *pabB* stop codon, we hypothesized that translation of *pabB* could result in translation-mediated displacement of bound TRAP, resulting in a *trpG* S-D sequence that was transiently free of bound TRAP. Thus, translation-mediated displacement of bound TRAP might allow translation initiation of *trpG*, thereby reducing the inhibitory influence of TRAP. Since *trpP* is a single-gene operon, translation-mediated displacement of bound TRAP would not be a factor.

To determine whether translation-mediated displacement of bound TRAP influences the expression of *trpG*, a stop codon was introduced into the *pabB* coding sequence upstream from the TRAP binding site that overlaps the *trpG* translation initiation region. Thus, translation of *pabB* would terminate 36 codons upstream from the natural *pabB* stop codon, thereby eliminating any possibility of translation-mediated displacement of bound TRAP. To simplify integration of the *trpG*'-'*lacZ* translational fusion into the chromosomal *amyE* locus, the stop codon was combined with a large in-frame deletion of the *pabB* coding sequence (Fig. 2, fusion 1). β-Galactosidase activity was determined for WT and TRAP-deficient ( $\Delta mtrB$ ) strains grown in the absence and presence of tryptophan. If translation-mediated displacement of bound TRAP participated in regulating the expression of *trpG*, then reduced expression of fusions containing the stop codon mutation would be expected for WT strains grown in the presence of tryptophan but not for  $\Delta mtrB$  strains. To test for the possible influence of transcriptional polarity caused by the engineered stop codon, experiments were also carried out in *rho::neo* (Rho<sup>-</sup>) strains. Expression from the *trpG*'-'*lacZ* translational fusion containing the partial in-frame deletion of *pabB* (fusion 1) was 8 Miller units in the absence of exogenously added tryptophan and was reduced threefold by the addition of tryptophan to the

growth medium (Table 4, row 1). Expression from this fusion was not regulated in response to tryptophan in the  $\Delta mtrB$  strain (Table 4, row 2), nor was expression influenced by the *rho* mutation (compare row 1 with row 3). The extent of TRAP-mediated regulation was determined by comparing expression in the  $\Delta mtrB$  and WT strains grown in the presence of tryptophan. TRAP-dependent inhibition of fusion 1 was about fourfold (10/2.8). Importantly, when cells were grown in the presence of tryptophan, introduction of the stop codon resulted in a five- to sevenfold reduction in expression of fusion 1 in the WT (Table 4, compare row 1 with row 4) and *rho* mutant (compare row 3 with row 6) strains but not in the  $\Delta mtrB$  strain (compare row 2 with row 5). These results are consistent with translation-mediated displacement of bound TRAP participating in the TRAP-dependent *trpG* translation control mechanism.

**Identification of a new *trpG* promoter.** As described above, expression from fusion 1 was only 8 and 3 Miller units in the absence and presence of added tryptophan, respectively (Table 4, row 1). For comparison, expression from a *trpG*'-'*lacZ* translational fusion containing the complete *pabB* coding sequence was previously reported to be 56 Miller units in the absence of tryptophan and 8 Miller units in its presence (31). Accordingly, we examined the expression of *trpG*'-'*lacZ* translational fusions containing the entire *pabB* coding sequence in the absence or presence of the engineered stop codon described above (Fig. 2, fusion 2). Expression from fusion 2 was about 10-fold higher than that from fusion 1 (Table 4, compare row 1 with row 7). Fusion 2 expression was regulated fivefold in response to tryptophan (Table 4, row 7). As previously observed for fusion 1, expression from fusion 2 was not regulated in response to tryptophan in the  $\Delta mtrB$  strain (Table 4, row 8), nor was expression influenced by the *rho* mutation (compare row 7 with row 9). TRAP-dependent inhibition of fusion 2 was

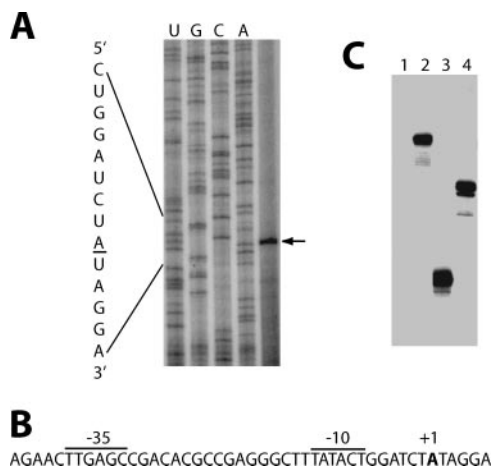


FIG. 3. Identification of a new *trpG* promoter. (A) Primer extension mapping of the 5' end of the *trpG* transcript. Total RNA was isolated from a WT *B. subtilis* strain, hybridized to an end-labeled DNA primer, and subsequently extended with MMLV reverse transcriptase. Sequencing reactions were performed using the same end-labeled DNA primer. An arrow marks the single reverse transcriptase product corresponding to the 5' end of the transcript originating from  $P_{trpG}$ . (B)  $\sigma^A$  *trpG* promoter sequence. The *trpG* promoter sequences (-35 and -10) and the transcription start site (+1) are marked. (C) In vitro transcription analysis of the *trpG* promoter, using  $\sigma^A$ -containing *B. subtilis* RNAP. Lane 1, no-template control; lane 2, DNA restriction fragment template giving rise to a 150-nt *trpG* transcript; lane 3, PCR-derived template giving rise to a 113-nt *trpG* transcript; lane 4, PCR-derived template giving rise to a 139-nt control transcript.

12-fold (260/21). As previously observed for fusion 1, the stop codon did not influence expression of the  $\Delta mtrB$  strain (Table 4, compare row 8 with row 11). Notably, fusion 2 expression was reduced only twofold by the engineered stop codon when cells were grown in the presence of excess tryptophan for the WT (Table 4, compare row 7 with row 10) and *rho* mutant (compare row 9 with row 12) strains. One likely explanation for these results is that the in-frame *pabB* deletion associated with fusion 1 removed a previously unidentified promoter that was not subject to translation-mediated displacement of bound TRAP.

Primer extension experiments were carried out using total cellular RNA to map the 5' ends of transcripts within the region delineated by the deletion endpoints from fusion 1. A single primer extension product was identified (Fig. 3A). A likely  $\sigma^A$ -dependent promoter ( $P_{trpG}$ ) was identified just upstream that matched the -35 and -10 consensus sequences in four of six and five of six positions, respectively. The spacing between the -35 and -10 sequences matched the 17-nt consensus (Fig. 3B). In vitro transcription reactions using purified *B. subtilis* RNAP confirmed that this was a  $\sigma^A$ -dependent promoter (Fig. 3C).

Expression studies similar to those performed with fusions 1 and 2 were carried out with *trpG'*-*lacZ* translational fusions driven exclusively by  $P_{trpG}$  (Fig. 2 and Table 4, fusion 3). Expression from  $P_{trpG}$  (fusion 3) was considerably higher than expression from the corresponding fusion containing only  $P_{pabB}$  (fusion 1) (Table 4, compare rows 1 and 13). Fusion 3 expression was regulated 12-fold in response to tryptophan (Table 4, row 13). As previously observed for fusions 1 and 2,

expression from fusion 3 was not regulated in response to tryptophan in the  $\Delta mtrB$  strain (Table 4, row 14), nor was expression influenced by the *rho* mutation (compare row 13 with row 15). In this case, TRAP-dependent inhibition was 50-fold (250/5). Expression from fusion 3 was not subject to translation-mediated displacement of bound TRAP; expression levels were not altered by the engineered stop codon in the WT or the  $\Delta mtrB$  or *rho* mutant strain (Table 4, compare row 13 with row 16, row 14 with row 17, and row 15 with row 18). Taken together, our results establish that *trpG* transcription initiates from two promoters ( $P_{pabB}$  and  $P_{trpG}$ ), that transcription levels are considerably higher from  $P_{trpG}$  than from  $P_{pabB}$ , and that only transcripts originating from  $P_{pabB}$  are subject to translation-mediated displacement of bound TRAP.

One would expect that the expression levels of fusion 1 ( $P_{pabB}$ ) and fusion 3 ( $P_{trpG}$ ) would add up to the expression level of fusion 2, containing both promoters. In the absence of added tryptophan (-Trp), the combined expression levels of fusions 1 and 3 were similar to the expression level of fusion 2 in all strains (Table 4). However, in the presence of added tryptophan (+Trp), the combined expression levels of fusions 1 and 3 in the WT strains (with or without the stop codon) were two- to threefold lower than those from fusion 2. Note that this discrepancy was not observed for the  $\Delta mtrB$  or *rho* mutant strain. While we do not have an explanation for this observation for the WT strains, we have ruled out mRNA stability as a source of this apparent discrepancy (data not shown).

## DISCUSSION

TRAP regulates translation initiation of *trpG*, *trpP*, and *ycbK* by directly blocking ribosome access to their cognate S-D sequences (12, 18, 22, 23, 28, 29, 31). The TRAP binding sites in *trpG*, *trpP*, and *ycbK* each contain nine triplet repeats (Fig. 1B). While *trpG* is present in an operon primarily concerned with folic acid biosynthesis (Fig. 1A), the TrpG polypeptide functions as the common glutamine amidotransferase subunit of anthranilate synthase (TrpE-TrpG) and *para*-aminobenzoate synthase (PabB-TrpG) in tryptophan and folic acid biosynthesis, respectively (13). The finding that *trpG* is regulated less tightly than *trpP*, despite having a higher affinity for TRAP (Tables 2 and 3), combined with the arrangement of the *pabB* and *trpG* coding sequences with respect to the TRAP binding site (Fig. 1B), led to the hypothesis that translation-mediated displacement of bound TRAP could play a role in the *trpG* translation control mechanism. Genetic results from this study suggest that ribosomes translating the *pabB* coding sequence are capable of displacing tryptophan-activated TRAP from its binding site in the *trpG* transcript (Table 4). Since seven of the nine triplet repeats in the *trpG* TRAP binding site lie upstream or within the *pabB* stop codon, and because the *pabB* stop codon overlaps the *trpG* S-D sequence (Fig. 1B), translation of *pabB* would displace bound TRAP, resulting in a *trpG* S-D sequence that is transiently free of TRAP. The same or a different ribosome can then bind and initiate translation before another tryptophan-activated TRAP molecule binds to the *trpG* transcript. Since *trpP* is a single-gene operon, translation-mediated displacement of bound TRAP cannot be a factor. It is also interesting that the stop codon for *trpA* overlaps the *ycbK* S-D sequence, although all nine of the triplet repeats are

downstream from the *trpA* stop codon (Fig. 1B). Like the case for *trpG*, we engineered a stop codon to test whether ribosome-mediated displacement of bound TRAP affected expression of *ycbK*. However, in this case, our results indicate that ribosome-mediated displacement of bound TRAP, if it occurs, does not influence the expression of *ycbK* (data not shown).

Two promoters control the transcription of *trpG*. The  $P_{pabB}$  promoter is located just upstream from *pabB* (Fig. 1A), while  $P_{trpG}$  is located ~250 bp upstream from the *trpG* initiation codon within the 3' end of the *pabB* coding sequence (Fig. 2 and 3). Expression from  $P_{trpG}$  is considerably higher than expression from  $P_{pabB}$  (Table 4). Furthermore, our results indicate that translation-mediated displacement of bound TRAP can only occur for transcripts originating from  $P_{pabB}$ . One physiological role for this translation displacement mechanism would be to ensure a sufficient level of TrpG synthesis to maintain folic acid biosynthesis in the presence of excess tryptophan; expression from fusion 1, which is only driven by  $P_{pabB}$ , is low when cells are grown in the presence of excess tryptophan (Table 4). However, the presence of  $P_{trpG}$  would appear to be sufficient to maintain folic acid biosynthesis under excess tryptophan growth conditions (Table 4, fusion 3). While our initial hypothesis was that translation-mediated displacement of bound TRAP would provide an answer for how translation of *trpG* can be regulated less tightly than that of *trpP*, despite TRAP having a higher affinity for the *trpG* message, it is apparent that this mechanism can only partially explain this dichotomy (Tables 2 and 3). Perhaps  $P_{trpG}$  evolved relatively recently and at one time  $P_{pabB}$  was the only available promoter for *trpG* expression. Thus, it is possible that the translation-mediated displacement mechanism was necessary for survival at some point during the evolution of *B. subtilis*. It is also possible that transcription initiation from these promoters is differentially regulated. While it was previously shown that folate operon transcript levels increased markedly when stationary-phase cells were diluted in fresh medium (9), it is unclear whether  $P_{pabB}$ ,  $P_{trpG}$ , or both were responsible for the increased expression.

Of the various *Bacillus* species for which sequence information is available for the *trp* and folate operons, *B. subtilis*, *Bacillus licheniformis*, *Bacillus halodurans*, and *Bacillus clausii* contain TRAP. In each of these organisms, a single *trpG* (*pabA*) gene is located immediately downstream from *pabB* in the folate operon. It appears that the internal  $P_{trpG}$  promoter is conserved in all four organisms (see Fig. S1 in the supplemental material). Examination of the *pabB-trpG* intercistronic region reveals the presence of appropriately spaced triplet repeats such that TRAP would be capable of inhibiting translation of *trpG*. Moreover, the potential of ribosome-mediated displacement of bound TRAP from transcripts originating from  $P_{pabB}$  is conserved as well; in each case, the majority of the triplet repeats that constitute the putative TRAP binding site lie upstream of or within the *pabB* stop codon (see Fig. S2 in the supplemental material). Interestingly, the *Bacillus* species that do not contain TRAP have separate *trpG* and *pabA* genes. In these instances, *trpG* is the second gene of the *trp* operon, while *pabA* is immediately downstream of *pabB* in the folate operon. It appears that the internal  $P_{trpG}$  ( $P_{pabA}$ ) promoter is conserved in these species as well (see Fig. S1 in the supplemental material). The *trp* operons in the *Bacillus* species

that do not contain TRAP are regulated by the T box antitermination mechanism, in which uncharged tryptophanyl tRNA binds to the untranslated leader and promotes transcription readthrough (15). Surprisingly, despite the absence of TRAP in these organisms, five appropriately spaced triplet repeats are present in the analogous *pabB-pabA* intercistronic region, like the case for the TRAP-regulated *trpG* (*pabA*) genes (see Fig. S2 in the supplemental material).

We are not aware of any previous studies demonstrating the role of translation-mediated displacement of a specific RNA binding protein in regulating gene expression. However, translation-mediated displacement of RNA binding proteins has been implicated in the nonsense-mediated decay mechanism in eukaryotes. Nonsense-mediated decay rids eukaryotic cells of aberrant mRNAs containing premature termination codons. Premature stop codons are distinguished from true termination codons by downstream exon-exon junctions (17). It was shown that hUpf3 remains bound at exon-exon junctions following splicing. It was proposed that during the first round of translation, the progressing ribosome displaces hUpf3 and associated hUpf2 when bound upstream from the termination codon. If these proteins are not displaced, subsequent binding of hUpf1 triggers mRNA degradation (17). Using an in vitro system, it was shown that ribosome-mediated displacement of Y14, an RNA binding protein that is part of the exon-exon junction complex, occurs from translationally active mRNAs (10). More recently, it was suggested that movement of the ribosome along the mRNA might displace AUF1, a protein that promotes the rapid decay of AU-rich element-containing RNAs. This last mechanism would be distinct from those described above because these AU-rich elements are present in the 3'-untranslated regions of certain eukaryotic messages (16).

The precise mechanism or energy required for protein displacement has not been addressed in any of the studies just described. However, footprinting data indicate that the 5'- and 3'-most triplet repeats were least protected by bound TRAP (6, 28). A weak interaction between TRAP and the 3' triplet plays a critical role in the degradation of terminated *trp* operon leader RNA so that TRAP can be recycled (8). Thus, it is conceivable that a relatively weak interaction between TRAP and the 5'-most triplet repeat in the *trpG* transcript is important for translation-mediated displacement of bound TRAP. Disruption of the TRAP interaction with the first repeat by the ribosome would generate a new 5'-most TRAP-bound triplet (i.e., the second repeat). Sequential disruption of TRAP interaction with each repeat by the translating ribosome may ultimately lead to TRAP dissociation and, hence, translation of *trpG*.

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