

TRAP binding to the *Bacillus subtilis* *trp* leader region RNA causes efficient transcription termination at a weak intrinsic terminator

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ABSTRACT

The *Bacillus subtilis* *trpEDCFBA* operon is regulated by a transcription attenuation mechanism controlled by the *trp* RNA-binding attenuation protein (TRAP). TRAP binds to 11 (G/U)AG repeats in the *trp* leader transcript and prevents formation of an antiterminator, which allows formation of an intrinsic terminator (attenuator). Previously, formation of the attenuator RNA structure was believed to be solely responsible for signaling RNA polymerase (RNAP) to halt transcription. However, base substitutions that prevent formation of the antiterminator, and thus allow the attenuator structure to form constitutively, do not result in efficient transcription termination. The observation that the attenuator requires the presence of TRAP bound to the nascent RNA to cause efficient transcription termination suggests TRAP has an additional role in causing termination at the attenuator. We show that the *trp* attenuator is a weak intrinsic terminator due to low GC content of the hairpin stem and interruptions in the U-stretch following the hairpin. We also provide evidence that termination at the *trp* attenuator requires forward translocation of RNA polymerase and that TRAP binding to the nascent transcript can induce this activity.

INTRODUCTION

In *Bacillus subtilis* the *trp* RNA-binding Attenuation Protein, TRAP, regulates expression of genes responsible for synthesis and transport of tryptophan (1). Six of the seven tryptophan biosynthetic genes are located within the *trpEDCFBA* operon (2). Transcription of the *trp* operon is regulated by an attenuation mechanism based on

formation of two alternative secondary structures in the 5' leader region RNA upstream of *trpE*. These structures consist of an intrinsic transcription terminator/attenuator comprised of segments A and B in Figure 1 and an upstream antiterminator comprised of segments C and D in Figure 1. As these structures share three residues, they are mutually exclusive (3).

TRAP is composed of 11 identical subunits, each encoded by the *mtrB* gene (4), arranged in a ring (5). When the intracellular concentration of tryptophan is high, it binds to TRAP and activates the protein to bind RNA (6). The TRAP-binding site in the *trp* leader segment is composed of 11 (G/U)AG repeats (7). Because this binding site overlaps the antiterminator region, TRAP binding prevents formation of the antiterminator, allowing the attenuator to form and halt transcription in the leader region (8). When tryptophan levels are low, TRAP does not bind RNA and the antiterminator forms allowing transcription of the *trp* genes.

In the current model for attenuation control of the *trp* operon, the only role of TRAP is to alter the secondary structure of the leader region RNA (Figure 1). To explore whether TRAP has any additional role in modulating attenuation, we examined the ability of the *trp* attenuator to induce transcription termination in the absence of the competing antiterminator. The efficiency of termination was examined with several constructs that contain substitutions designed to disrupt formation of the antiterminator structure and thus allow formation of the attenuator in the absence of TRAP. If the only function of TRAP is to promote formation of the attenuator, then transcription of these *trp* leader mutants should result in constitutive termination in the absence of TRAP. All of the mutant templates showed only slightly increased termination levels at the attenuator in the absence of TRAP as compared to the WT leader region, whereas transcription terminated efficiently in the presence of TRAP. These studies show that the *trp* attenuator is a weak intrinsic

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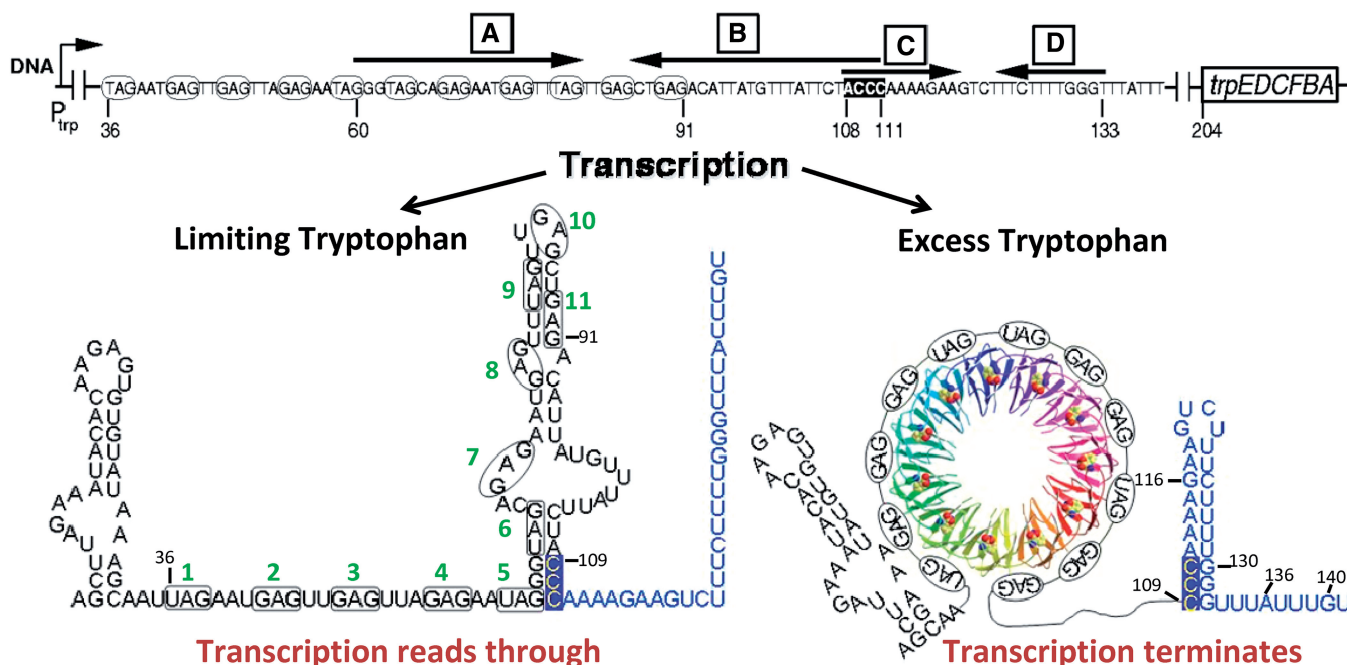


Figure 1. Model of transcription attenuation for the *B. subtilis trp* operon. Bold black letters designate the complementary strands of the terminator (highlighted in blue) and antiterminator stem-loops. TRAP is shown as a ribbon diagram with each subunit as a different color. The 11 (G/U)AG repeats of the TRAP-binding site are circled and numbered in green. Small black numbers indicate residues relative to the start of transcription.

terminator and suggest that TRAP has a role in the attenuation mechanism beyond influencing the structure of the leader region RNA. We show that the low GC content in the hairpin stem combined with two interruptions in the U-tract generates the weakness of the *trp* attenuator. One model for intrinsic termination suggests that formation of the hairpin in the nascent transcript causes hypertranslocation of RNAP without chain elongation (9). We found that impeding the forward movement of RNAP at the *trp* attenuator inhibits transcript release. Moreover, TRAP binding to the nascent *trp* transcript can induce forward translocation of RNAP. Together our results suggest that the *trp* attenuator represents a new type of bacterial transcription termination mechanism that is neither truly intrinsic nor dependent on Rho protein.

MATERIALS AND METHODS

Materials

All plasmids were propagated in *Escherichia coli* K802. Plasmid pUC119*trpL*, which contains a 730-bp EcoRI–HindIII fragment including the *B. subtilis trp* promoter and leader sequence (–411 to +318 relative to the start of transcription), was used to create templates for *in vitro* transcription by polymerase chain reaction (PCR) (10). Bead-bound DNA templates were created with 5' biotinylated DNA primers from IDT (10). PCR products were purified using QIAGEN MinElute, and were coupled to streptavidin-coated magnetic beads (Dynabeads M-280) according to the manufacturer's instructions.

Modifications to the antiterminator region of the leader sequence were created using the QuikChange kit (Stratagene) (AntiAB1: G61A, G62A, T63G and C87A) or by cloning overlapping oligonucleotide inserts between XbaI and PstI sites introduced at positions +29 and +139 (relative to the start of transcription) in pUC119*trpL* (AntiAB2: A67C, T77C, C87A, C93G, A94T, T95G, T96G, C106G, T107A, AntiAB3: A67C, T77C, C87A, C93G, A94T, T95A, T96A, C106G and T107A, AntiAB-GAGAU₁₁, AntiAB-GAGUU₁₁, No Binding Site and CCC/GGG Switch: C109G, C110G, C111G, G130C, G131C, G132C). The differences between AntiAB2 and 3 are highlighted in bold-type font. The sequence of the No Binding Site template from +36 to +91 replaced as: TTGACTGCTATTACTGACTACTT GATTACGTTAATCATGGATACGTCTCGAG. The restriction sites were then replaced with WT *trp* sequence by site-directed mutagenesis. Substitutions in the attenuator region were created by site-directed mutagenesis. The sequence of the complementary oligos: Oligo A; complementary to bases 70–84, Oligo B; complementary to bases 55–69.

Bacillus subtilis BG2087 (*argC4*) and BG4233 (*argC4* Δ *mtrB*) were used for analysis of *lacZ* gene fusions. BG4233 contains a deletion of *mtrB*, which encodes TRAP (11). *Bacillus subtilis* was transformed by natural competence (12) and blue colonies were selected on plates containing Vogel and Bonner minimal salts (13), 0.2% acid-hydrolyzed casein (ACH), 0.2% (w/v) glucose, 50 μ g/m X-gal, 10 μ g/ml Arg and 5 μ g/ml chloramphenicol. PCR was used to introduce EcoRI and BamHI sites at the ends of DNA fragments containing the *trp* promoter, leader region, and the first 40 codons of *trpE* followed by a

TAA stop codon. Transcriptional fusions with *lacZ* were created by ligating the PCR products into similarly digested pDH32 (14). The resulting plasmids were linearized with *PstI* and transformed into *B. subtilis*. Successful cloning and mutagenesis of plasmids were confirmed by DNA sequencing. Fusions were integrated into the *amyE* locus by homologous recombination, which was confirmed on starch plates (15). To determine the amount of β -galactosidase that corresponds to 100% read through of the *trp* attenuator, we created a transcriptional fusion in which the *trp* promoter is immediately upstream of *lacZ* with no regulatory sequences from the leader region present. TRAP was expressed in *E. coli* BL21(DE3) and was purified as described previously (16).

A plasmid with the gene for the cleavage deficient EcoRI E111Q (EcoRI*) protein was a kind gift from Paul Modrich (Duke) (17). The coding sequence for both EcoRI* and the associated methylase were amplified by PCR and ligated into *NdeI* and *BamHI* cut pET17b (Novogen). Six histidine (His) codons were added to the amino terminus of the EcoRI* gene using the QuikChange mutagenesis kit from Stratagene. In addition, the methylase gene was amplified by PCR separately and ligated into the tetracycline resistance gene of pACYC184 using *BamHI* and *SalI* such that the methylase gene is expressed from the *tet* gene promoter. Both plasmids were transformed into *E. coli* BL21(DE3). The resulting strain was grown in LB with ampicillin (100 μ g/ml) and chloramphenicol (30 μ g/ml) at 37°C until the A600 reached 0.5 at which point IPTG was added and growth was continued for 4 h. The cells were harvested by centrifugation broken with a French pressure cell and EcoRI* was purified using nickel agarose as per manufacturer's instructions (Qiagen).

***In vitro* transcription attenuation assay**

Transcription reactions contained 40 mM Tris-HCl (pH 8.0), 4 mM MgCl₂, 0.1 mM EDTA, 4 mM spermidine, 5 mM DTT, 1 mM L-tryptophan, 20 nM DNA template, 500 μ M NTPs, 1 μ Ci [α -³²P]UTP 3000 Ci/mmol and 50 μ g/ml *B. subtilis* σ^A RNA polymerase (RNAP). TRAP was added as indicated in figures and legends. Reaction mixtures were incubated 15 min at 37°C, then stopped by addition of an equal volume of 95% formamide/20 mM EDTA, pH 8.0/0.3 mg/ml bromophenol blue. Samples were denatured at 95°C for 2 min and products fractionated on 6% denaturing gels, which were exposed to a PhosphorImager screen. Quantification of band intensities was performed using IMAGEQUANT software (Molecular Dynamics). The number of U residues in the read through and terminated RNA transcripts was used to calculate the percentage of transcription termination.

EcoRI* blocked *in vitro* transcription

Prior to initiating transcription, EcoRI* (130 nM) was allowed to bind to DNA template (20 nM) for 5 min at 37°C in Transcription Buffer (20 mM Tris-HCl pH 8, 6 mM MgCl₂, 2 mM DTT and 100 mM KCl). Transcription was initiated with 50 μ g/ml RNAP and 8 μ M ATP, GTP, 2 μ M UTP and 1 μ Ci [α -³²P] UTP

(3000 Ci/mmol) at 37°C for 10 min. Heparin (0.1 mg/ml) was then added to prevent re-initiation, and transcription was continued for 10 min at 37°C in the presence of 20 μ M NTPs and 1 mM L-tryptophan in the absence or presence of TRAP. Transcripts that remained associated with the bead-bound DNA template were collected with a magnet, whereas released transcripts remained in the supernatant. Reactions were stopped and the products were fractionated as described above. For each reaction, the amount of RNA in the both the supernatant and remaining associated with the bead-bound DNA template was quantified using IMAGEQUANT as described above. The percentage of transcripts released was calculated by dividing the amount of RNA released by the total amount of RNA produced in each reaction.

β -Galactosidase assays

Bacillus subtilis strains were grown in, in MOPS minimal medium (400 mM MOPS pH 7, 500 mM KCl, 5 mM MgSO₄, 40 mM tricine and 100 mM NH₄Cl) with 1.32 mM K₂HPO₄, 10 μ M FeCl₃, 0.2% ACH, 5 μ g/ml Arg and 5 μ g/ml chloramphenicol in the presence or absence of 50 μ g/ml L-tryptophan at 37°C to an absorbance at 600 nm of 0.4–0.6; 1.5 ml of cells were pelleted, washed with TE buffer pH 8, and resuspended in 1.5 ml of Z-buffer (60 mM Na₂HPO₄, 40 mM NaH₂PO₄, 10 mM KCl, 1 mM MgSO₄ and 50 mM β -mercaptoethanol). Lysozyme was added to 0.1 mg/ml to 0.5 ml of cells and incubated at 37°C for 5 min followed by the addition of 0.1% triton X-100; 0.1 ml of cell lysate was added to 0.9 ml of Z buffer and assayed for β -galactosidase activity (18). Each value is the average of two or three independent experiments, each performed in triplicate.

RESULTS

The *trp* attenuator is a weak intrinsic terminator that requires TRAP bound to the nascent *trp* transcript to cause transcription termination

We created several DNA templates that contain mutant *trp* leaders with substitutions designed to disrupt base pairing in the A/B antiterminator (19) without altering the (G/U)AG repeats of the TRAP binding site. If the sole function of TRAP binding is to prevent formation of the antiterminator structure, then transcription of these mutant templates should terminate constitutively at the C/D attenuator in the absence of TRAP. The AntiABI template contains four substitutions designed to prevent the shared CCC sequence from participating in the antiterminator and thereby promote formation of the attenuator (Figure 2B). We tested the effects of these substitutions on the efficiency of termination at the *trp* attenuator using an *in vitro* transcription attenuation assay (8). For comparison, the wild-type (WT) *trp* leader region was also examined. In the absence of TRAP, transcription of the WT template resulted in virtually complete read through of the attenuator yielding >99% run-off transcripts (Figure 2A and C). As seen previously (6,8), adding increasing amounts of tryptophan-activated TRAP increased the fraction of transcripts that terminated at the

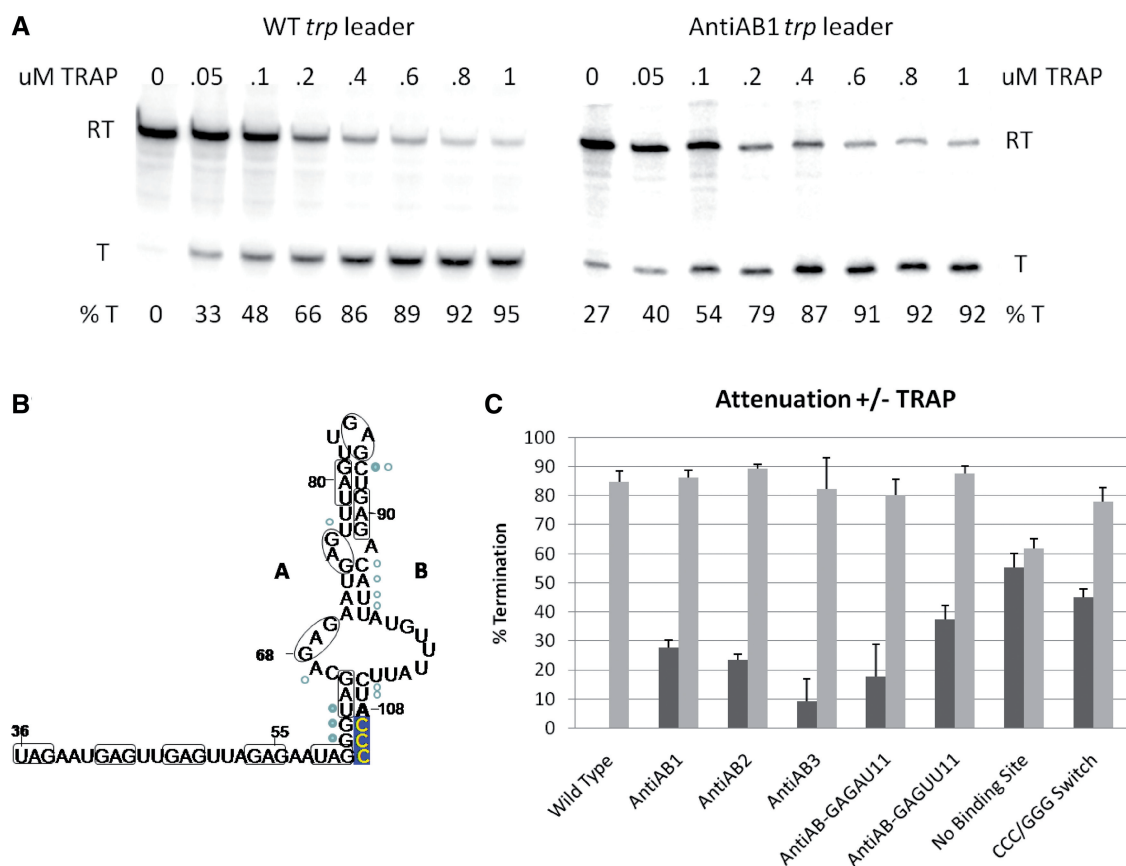


Figure 2. Effect of substitutions in the antiterminator on TRAP-mediated transcription termination *in vitro*. (A) PAGE analysis of the products of *in vitro* transcription of the WT and AntiAB1 mutant *trp* leader regions in the absence and presence of increasing amounts of tryptophan-activated TRAP. Positions of read through (RT = 320 nt) and terminated (T = 140 nt) transcripts are indicated. The percentage of termination for each reaction is at the bottom of each lane. Although a faint band is visible for the terminated transcript with the WT leader in the absence of TRAP, IMAGEQUANT analysis did not detect this as above background. (B) Diagram of the A/B antiterminator with the positions of residues that were altered in *trp* leader mutants indicated. (G/U)AG triplets of the TRAP binding site are circled. Small closed circles represent positions of substituted nucleotides in AntiAB1; open circles represent substituted residues in AntiAB2 and 3. AntiAB-GAGAU₁₁ and AntiAB-GAGUU₁₁ replace the WT sequence from +36 to +91 with 11 repeats of the respective five residue sequences. No Binding Site replaces +36-91 with an unstructured RNA sequence with no TRAP binding. (C) Bar graph of the percentage of termination for antiterminator constructs in the absence (dark gray) and presence (light gray) of 0.4 uM TRAP.

attenuator, indicative of TRAP mediated transcription attenuation (Figure 2A). Transcription of the AntiAB1 template in the absence of TRAP showed only slightly elevated termination at the attenuator (27%), while the presence of TRAP yielded termination levels similar to those observed with the WT template (Figure 2A). With both templates, transcription in the presence of 0.4 μ M TRAP resulted in nearly complete (>85%) termination at the attenuator (Figure 2A). Although higher levels of TRAP yielded slightly more termination, we also observed slight inhibition of transcription. Consequently, although TRAP was titrated with each template, results with 0 and 0.4 μ M TRAP are presented to represent transcription in the absence and presence of TRAP (Figures 2C and 3).

A potential explanation for observing only a slight increase in termination with the AntiAB1 template in the absence of TRAP could be that an antiterminator RNA structure formed despite the substitutions to allow the shared CCC sequence to participate in the attenuator. To address this possibility, we created five additional

templates with more extensive substitutions designed to prevent base-pairing in the antiterminator region. Two of these (AntiAB2 and AntiAB3) contain nine substitutions in the antiterminator region (Figure 2B), and two have the entire TRAP binding site from +36 to +91 (Figure 1) replaced with an optimized binding site composed of 11 repeats of either GAGUU or GAGAU. RNA composed of either of these repeated sequences has been shown to be unstructured (20) and mfold computer predictions support that this should also be true in the context of the *trp* leader. For the GAGAU containing RNA no structure was predicted by mfold. In the case of the GAGUU containing leader region several structures are predicted with an overall ΔG values of approximately -25 kcal/mol however, none of these structures include the GAG repeat portion of the transcript nor did they overlap the terminator. In the fifth template (CCC/GGG) the three GC pairs at base of the attenuator are changed to CG pairs, thus allowing formation of an equivalent terminator hairpin containing no sequence

the top of the stem during transcription (22), these base-pairs may be particularly important in the intrinsic termination mechanism. To examine the importance of this region of the attenuator, A:U to G:C substitutions were introduced in the context of the AntiAB1 construct, which allowed the effects to be examined without the influence of a competing RNA structure. Results presented below (Figure 3D) confirm that the AntiAB1 substitutions promote constitutive formation of the C/D attenuator in the absence of TRAP. Increasing the number of GC pairs in the top portion of the attenuator stem by changing two AU pairs at 117/124 and 118/123 to GC increased termination in the absence of TRAP from 27% (WT) to 45%, however, TRAP was still required to achieve $\geq 50\%$ termination (Figure 3B: GC Top). Replacing all of the AU pairs in the stem with GCs yielded virtually complete termination in the absence of TRAP in the context of the antiterminator deficient template (Figure 3B: All GC). Hence, the low GC content contributes to the weakness of the *trp* attenuator as an intrinsic terminator, and TRAP binding to the nascent *trp* transcript can overcome this deficiency and induce efficient transcription termination.

In the context of the wild-type antiterminator segment, increasing the GC content of the top of the stem (GC Top) only slightly enhanced transcription termination (15%) in the absence of TRAP while replacing all of the AU pairs in the stem with GCs (All GC) increased termination at the attenuator to 58% (Figure 3B). As increasing the GC content of the attenuator stem had a greater effect on termination in the AntiAB1 context than with the WT antiterminator sequence present, these results confirm that the antiterminator RNA structure contributes to regulating transcription (Figure 3B and C; GC Top and All GC).

A CC mismatch in the stem at positions 116 and 125 eliminated termination even in the presence of TRAP (Figure 3B: G116C), whereas a compensatory CG pair (as opposed to GC) restored termination to nearly WT levels (Figure 3B: G116C/C125G). These observations show that while TRAP binding can compensate for low GC content in the *trp* attenuator stem, TRAP cannot induce termination if there are unpaired residues in the stem.

Interruptions in the U-stretch of the attenuator contribute to the requirement for TRAP

A sequence of 7–9 U residues following the hairpin (21) has been implicated in the intrinsic termination mechanism both to pause RNAP (23) and make the RNA:DNA hybrid susceptible to shearing (22). The U-stretch of the *trp* attenuator is interrupted twice, at A136 and G140 (Figures 1 and 3A). Replacing either A136 or G140 with a U significantly increased termination efficiency in the absence of TRAP, especially in the AntiAB1 context (Figure 3D and E). When both interruptions were replaced with Us, virtually complete termination occurred in the absence of TRAP in the AntiAB1 context (Figure 3D and 3E A136U/G140U). Thus, interruptions to the U-tract also contribute to the weakness of the attenuator and its requirement for TRAP.

Substitutions in the U-stretch do not involve residues that overlap between the antiterminator and terminator, nor should they affect the stability of the terminator hairpin. Consequently, these changes should not influence which structure forms in the RNA during transcription. Hence, the observation that substitutions in the U-tract of the attenuator showed increased termination efficiency, up to 40% for A136U/G140U in the presence of the WT antiterminator (Figure 3E) suggests that the WT A/B antiterminator structure is not completely effective at preventing formation of the attenuator hairpin. Moreover, the dramatic increases in termination seen with the U-stretch substitutions in the context of AntiAB1 (up to 90%, Figure 3D) confirms that these substitutions effectively prevent formation of the antiterminator.

In vivo analysis of *trp* attenuator function

The properties of the *trp* attenuator were also investigated *in vivo* using *trpE'-lacZ* transcriptional fusions (Table 1). Each of the fusions tested was under control of the *trp* promoter followed by either the WT or AntiAB1 antiterminator coupled with the WT, All GC or A136U attenuator. β -Galactosidase expression from each construct was examined in BG2087 (*mtrB*⁺), which expresses WT TRAP, and BG4233 (Δ *mtrB*) which lacks TRAP (Table 1). None of the fusions were regulated in response to tryptophan in BG4233 (Table 1). As seen previously (24,25), the WT fusion was regulated in response to tryptophan in BG2087 (*mtrB*⁺) such that growth in the presence of excess tryptophan yielded <1% of that in the strain lacking TRAP, indicative of highly efficient (>99%) termination at the attenuator (Table 1). The level of β -galactosidase from all the fusions in BG4233 grown in either the absence or presence of tryptophan was greater than those seen for the same fusion grown in BG2087 in the absence of tryptophan. This difference reflects endogenously produced tryptophan in BG2087, which is *trp*⁺.

The AntiAB1 substitutions in the antiterminator region increased termination at the attenuator as evidenced by reduced β -galactosidase expression in the absence of TRAP. This effect can be seen in BG4233 by comparing the levels obtained with each of the three attenuators in the context of the WT antiterminator (Table 1; rows 1–3) to those obtained for the same attenuator in the context of the AntiAB1 substitutions (Table 1; rows 4–6). In all cases, these observations are consistent with our *in vitro* studies and suggest that the AntiAB1 substitutions prevent formation of the antiterminator. However, complete (>99%) termination at the wild-type attenuator is not achieved unless TRAP and tryptophan are present. This requirement for TRAP is seen by comparing 90% termination at the WT attenuator in the context of the AntiAB1 substitutions in the absence of TRAP (Table 1: row 4, BG4233) with >99% termination seen in the presence of TRAP and tryptophan (Table 1: row 4, BG2087). These observations again suggest that TRAP has an additional function that allows the *trp* attenuator to efficiently halt transcription beyond altering the RNA secondary structure.

Table 1. *In vivo* Analysis of antiterminator and attenuator substitutions on attenuation control of transcription from the *trp* promoter

| <i>Trp</i> leader RNA | | <i>In vivo</i> β -galactosidase activity (U) ^a | | | | | | <i>In vitro</i> transcription %Termination ^c | |
|-----------------------|-------------|---|--------------|------------------------|-------------------------|---------------|------------------------|---|-------|
| | | BG4233 ($\Delta mtrB$) | | | BG2087 (<i>mtrB</i> +) | | | -TRAP | +TRAP |
| Antiterminator | Attenuator | -Trp | +Trp | %T (+Trp) ^b | -Trp | +Trp | %T (+Trp) ^b | -TRAP | +TRAP |
| Wild-type | | | | | | | | | |
| 1 | Wild-type | 355 \pm 70 | 355 \pm 73 | 32.9 | 106 \pm 20 | 4.4 \pm 1.6 | 99.2 | 0 | 85 |
| 2 | All GC Stem | 60 \pm 22 | 58 \pm 25 | 89 | 21 \pm 5 | 1.9 \pm 0.9 | 99.6 | 60 | 90 |
| 3 | A136U | 318 \pm 36 | 319 \pm 28 | 39.7 | 111 \pm 11 | 3.4 \pm 1.2 | 99.4 | 25 | 95 |
| AntiA/B1 | | | | | | | | | |
| 4 | Wild-type | 55 \pm 9 | 52 \pm 10 | 90.2 | 14 \pm 6 | 3.6 \pm 4.4 | 99.3 | 28 | 88 |
| 5 | All GC Stem | 17 \pm 2 | 17 \pm 2 | 96.8 | 3.5 \pm 0.8 | 0.7 \pm 0.4 | 99.9 | 89 | 96 |
| 6 | A136U | 31 \pm 10 | 28 \pm 5 | 94.7 | 3.6 \pm 1.2 | 1.3 \pm 2 | 99.8 | 79 | 98 |

^a Values are the average of two or three independent experiments, each performed in triplicate, \pm the standard deviation.

^b %T is the estimate of the percentage of transcripts that terminate at the *trp* attenuator; in each case %read through is calculated by dividing the units of β -galactosidase obtained by 529U which maximum units from the *P_{trp}/lacZ* construct in BG4233 grown in the presence of tryptophan; %T = 100 - %read through.

^c These data are reproduced from Figure 3B and C and are included for comparison.

We also used *lac* fusions to examine two modified attenuators that showed increased intrinsic termination *in vitro* (Figure 3). The All GC stem increased termination efficiency at the attenuator in the absence of TRAP (BG4233) even in the context of the WT antiterminator (Table 1: row 2). This increased termination is likely due to two effects. First, the All GC attenuator is a better intrinsic terminator, which can be seen by the increase in termination from 90% for the WT attenuator to 97% for All GC in the AntiAB1 context (Table 1 rows 4 and 5: BG4233). Second, the increased stability of the All GC stem favors formation of the attenuator structure in the presence of the WT antiterminator. This effect is illustrated by the increase in termination from 32% for the WT attenuator to 89% for the All GC stem attenuator in the context of the WT antiterminator (Table 1, rows 1 and 2: BG4233). The presence of tryptophan-activated TRAP increased termination efficiency of the All GC Stem attenuator to over 99% regardless of the antiterminator context (Table 1: rows 2 and 4 in BG2087 +Trp).

Lengthening the U-stretch of the attenuator (A136U) increased termination *in vivo* in the absence of TRAP (Table 1; BG4233). The U-tract replacement had a smaller effect than the All GC stem substitutions, presumably because these stem substitutions favor formation of the attenuator structure over the antiterminator, whereas changes in the U-tract do not. In all cases the presence of tryptophan-activated TRAP increased termination to >99% (BG2087 +Trp). Thus, the effects observed *in vivo* of extending the U-tract (Table 1) are similar to those seen *in vitro* (Figure 3C and E).

Preventing formation of the antiterminator with complementary oligonucleotides

Prior studies showed that the presence of a DNA oligonucleotide complementary to residues 55–69 at the base of the A/B antiterminator induced termination at the *trp* attenuator in an *in vitro* transcription attenuation assay (8,26). These results suggested that preventing formation

of the antiterminator by competition for pairing with the oligonucleotide is sufficient to induce termination. When we compared oligo-mediated and TRAP-mediated termination we found that with the WT *trp* leader region, TRAP-mediated termination was more efficient (92%) than that produced by up to 1000-fold excess of competitor oligo (65%) (Figure 4; lane 2 versus lane 6). By contrast, when the DNA template encoded either the A136U or the All GC stem mutant *trp* attenuators (Figure 3A), the presence of the competitor oligo was as effective as TRAP (Figure 4; lane 8 versus 12 and lane 14 versus 18). These observations are again consistent with the suggestion that TRAP binding has additional functions beyond just altering the RNA secondary structure of the leader region.

Blocking forward movement of RNAP inhibits termination at the *trp* attenuator

To determine whether hypertranslocation is required for termination at the *trp* attenuator, we examined the effects of blocking the forward movement of RNAP downstream from the site of termination (position 140) (26). The cleavage defective E111Q mutant EcoRI (EcoRI*) protein binds to DNA and blocks elongation of RNAP \sim 14 bp upstream of the first G of its GAATTC recognition site (9,27,28). We created templates that position EcoRI* between 0 and 4 bp downstream of the front edge of RNAP when it reaches G140 and examined the effect of the presence of this roadblock on transcript release (Figure 5A).

Transcription of these templates in the presence of tryptophan-activated TRAP resulted in \geq 90% transcript release at the attenuator in the absence of EcoRI* (Figure 5A, lanes 1, 2 and 5–14). The presence of EcoRI* protein positioned either immediately adjacent to, or 1 bp downstream of the front edge of RNAP inhibited transcript release by 60–70% (Figure 5A, lanes 15–18). This inhibitory effect progressively diminished as the distance between RNAP and the roadblock increased to two or

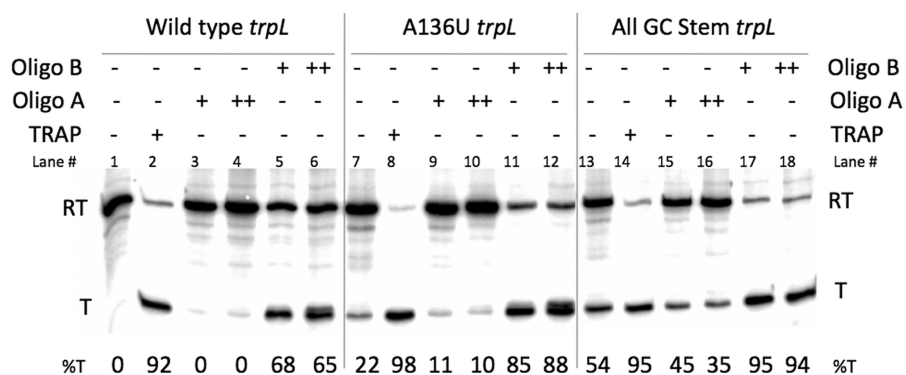


Figure 4. Effects of complementary DNA oligonucleotide (oligo) addition on an *in vitro* transcription attenuation in the *trp* leader region. A 6% polyacrylamide –8M Urea gel of the products of *in vitro* transcription of the wild-type, A136U and All GC Stem *trp* leader regions with *B. subtilis* RNAP in the absence or presence of 1 μM tryptophan-activated *B. subtilis* TRAP or oligos as indicated. Oligos were added to the reaction mixture before transcription and were complementary to either the top region (Oligo A, residues 70–84) or the base of the antiterminator (Oligo B, residues 55–69). Oligos were added either at 100 (+) or 4000 (++) fold molar excess over template DNA. Positions of read through (RT = 320 nt) and terminated (T = 140 nt) transcripts are indicated. The molar percentage of termination for each reaction is presented at the bottom of each lane.

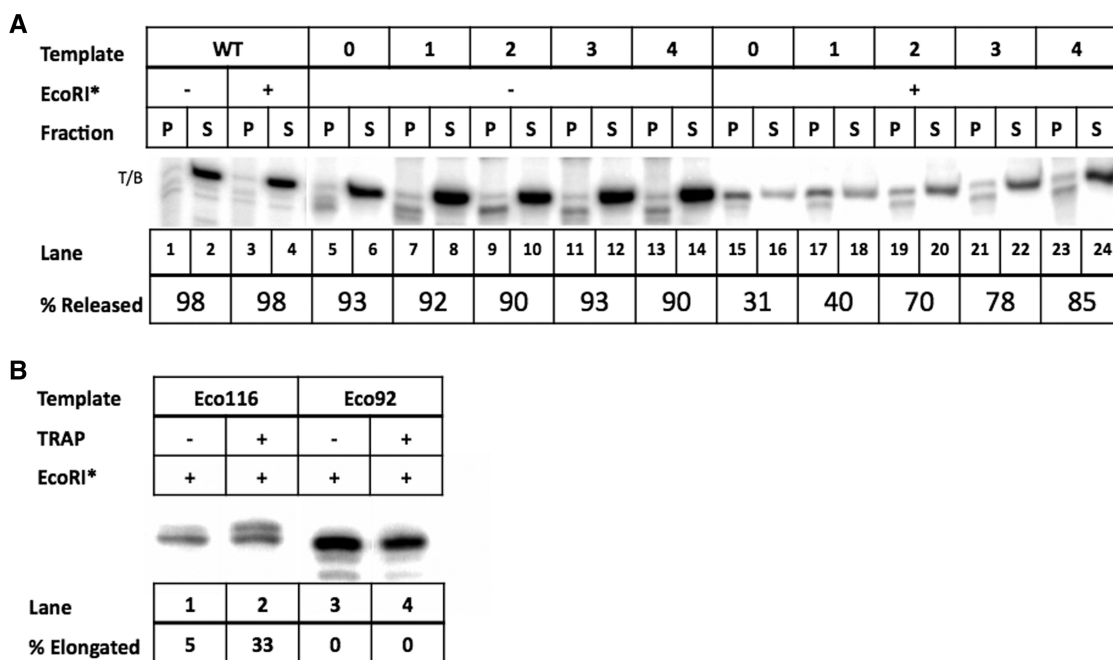


Figure 5. Blocking forward movement of RNAP at the *trp* attenuator. (A) 6% PAGE analysis of the products of *in vitro* transcription in the absence and presence of EcoRI* bound to the DNA template. All reactions contain 0.5 μM TRAP and 1 mM tryptophan. RNAs retained (P) on the TEC were separated from those that were released (S) using bead bound DNA templates (‘Materials and Methods’ section). Templates 0–4 include an EcoRI recognition site starting at positions 154, 155, 156, 157 and 158, respectively, which position EcoRI* 0, 1, 2, 3 and 4 bp downstream of the front edge of RNAP when it transcribes G140 (the site of termination at the *trp* attenuator). The percentage of transcripts released from the TEC are shown below each pair of lanes. (B) PAGE analysis of the products of *in vitro* transcription of the Eco116 and Eco92 *trp* leader template in the presence of EcoRI*. Note: the RNAs from Eco116 and Eco92 are not the same length but are presented in the figure side by side for space considerations. The percentage of transcripts that are elongated by one residue are presented at the bottom of each lane.

more base pairs (lanes 19–24). EcoRI* had no effect on transcript release from the WT template (Figure 5A, lanes 3 and 4). In the absence of TRAP none of the transcripts were released in the presence of EcoRI*. Together these results are similar to previous findings with the t500 terminator (9) and indicate that forward translocation of ~2 bp is required for transcript release at the *trp* attenuator.

The observations that the *trp* attenuator functions weakly in the absence of TRAP and that forward translocation is required for termination at the attenuator suggest that TRAP may function by inducing forward movement of RNAP. Hence, we examined whether TRAP binding to the nascent transcript can induce forward translocation of RNAP. To do so, we blocked the transcription elongation complex (TEC) with

EcoRI* such that either 6 or 10 of the (G/U)AG repeats of the TRAP-binding sites are exposed on the nascent transcript. EcoRI* binding to the DNA blocks the TEC such that the nascent RNA is exposed from RNAP ~26–27 residues upstream of the G in the restriction site (9,27,28). Hence, placing a GAATTC recognition site starting at either +92 (Eco92) or at +116 (Eco116) positions EcoRI* to block the TEC such that the nascent transcripts are exposed up to approximately residues +66 or +90, respectively. The exposed regions on these two nascent transcripts contain either 6 (Eco92) or 10 (Eco116) of the (G/U)AG repeats of the TRAP binding site (Figure 1).

The transcripts produced from these two templates with EcoRI* bound and transcription performed in the absence or presence of TRAP were then examined. Transcription of either template in the absence of TRAP produced a single transcript of the expected length. Adding TRAP to the transcription reaction resulted in incorporation of one additional residue on ~33% of the transcripts from the Eco116 template, but had no effect on transcription from Eco92 (Figure 5B). These observations are similar to those seen with Mfd or Rho (29), and suggest that TRAP binding to >6 (G/U)AG repeats in the nascent transcript can induce forward translocation of RNAP against an elastic force of the EcoRI* roadblock.

DISCUSSION

Until now, the only identified functions of TRAP in the attenuation mechanism that controls transcription of the *B. subtilis trp* operon were to sense the intracellular level of tryptophan and to influence the secondary structure of the RNA in the leader region (Figure 1). While the results presented here support this model, our evidence shows that TRAP plays an additional active role in promoting termination at the attenuator beyond simply preventing formation of the antiterminator. This additional function is necessary because the *trp* attenuator is an inefficient intrinsic terminator.

Substitutions in the leader region to prevent formation of the A/B antiterminator do not result in constitutive termination of transcription at the attenuator (Figure 2). A simple explanation for these observations would be that despite these changes, alternative RNA structures form in the nascent transcripts from all the mutant templates and these structures inhibit formation of the attenuator. Several observations indicate that this alternative explanation is incorrect. First, we made a total of six different substitutions in the antiterminator region (Figure 2) and none are predicted to form secondary structures that overlap the attenuator (Mfold). Second, in addition to these six templates, we also created a template in which the three C:G base pairs in the base of the terminator are replaced with G:C pairs. This template alters the three Cs (109–111) that overlap between the A/B and C/D structures (Figure 1) and thus allows formation of an alternative terminator with GC content similar to the WT attenuator that does not overlap with the antiterminator. Moreover, if all of the other AntiAB substitutions form

RNA secondary structure involving C₁₀₉–C₁₁₁, this construct should not do so. Similar to our other substituted *trp* leader regions, transcription of this template *in vitro* yielded only 42% termination at the modified attenuator in the absence of TRAP, which increased to 92% in its presence. Finally, in the context of the AntiAB1 substitutions in the antiterminator segment, replacing the interruptions in the U-tract at A136 and G140 with Us results in virtually complete termination, whereas only 40% termination is observed when the WT A/B segment is present (Figure 3). Because changes in the U-tract should not influence which RNA secondary structure forms in the leader RNA, these observations strongly indicate that the AntiAB1 substitutions efficiently allow formation of the attenuator.

Our results suggest that the combination of the low GC content (36%) of the base-paired stem together with interruptions of the U-stretch generates the requirement for TRAP to cause termination at the *trp* attenuator (Figure 3). The average GC content of the hairpin stems from over 400 intrinsic *B. subtilis* terminators is 64% (30). Similarly, the average length of uninterrupted Us following the stem of these intrinsic terminators is 4.8, with only 24% having three or fewer Us before an interruption, as seen with the *trp* attenuator (30). The low GC content of the stem as well as early interruptions of the U-stretch are conserved in TRAP-regulated *trp* attenuators from other bacteria. Inspection of such *trp* attenuators from 12 additional species shows that the GC content of the stem ranges from 36% to 75%, and with one exception, those above 45% are from thermophiles. Similarly, all but one of the U-tracts of these *trp* attenuators are interrupted within the first three to four residues following the stem. These similarities suggest that the need for the additional function of TRAP in the attenuation mechanism is conserved in these regulatory systems.

Attenuation and antitermination mechanisms control transcription of numerous bacterial genes (31,32). As with the *B. subtilis trp* operon (Figure 1), in many cases an antiterminator structure competes with formation of an intrinsic terminator because the two RNA structures share residues. In the *B. subtilis trp* operon, three residues (CCC) are shared between the antiterminator and the attenuator (Figure 1) (3). This overlap is more extensive in other attenuation/antitermination systems. For example, in *E. coli* and *S. typhimurium*, transcription of numerous amino acid biosynthetic operons is regulated by attenuation based on the ability of a ribosome to translate a leader peptide coding segment (33). In these systems, the regulatory structures overlap by 6–11 residues with most being nine or more. Similarly, in the leader region of genes regulated by the T-box antitermination mechanism these structures generally share seven residues (32). The short overlap of 0–5 residues between antiterminator and attenuator is also conserved in TRAP-regulated *trp* operons from other species.

These studies indicate that the *B. subtilis trp* operon antiterminator does not completely prevent formation of the attenuator. This is evident from the observation that in the context of the WT antiterminator segment, improving the U-tract of the attenuator (A136U) significantly

increased termination in the absence of TRAP (Figure 3E and Table 1). Hence, although the attenuator forms frequently in *trp* transcripts in the absence of TRAP, this does not compromise regulation of the operon because in the absence of TRAP the attenuator does not effectively prevent transcription of the *trp* genes. In other attenuation/antitermination systems with strong intrinsic terminators, their formation must be controlled more tightly and thus these systems employ greater overlap and more stable antiterminators.

Our results suggest that an attenuation system could function without the presence of an antiterminator RNA structure if termination at the attenuator was entirely dependent on a *trans*-acting factor. This appears to be true for the *Bacillus halodurans trp* operon (34). In this system, TRAP regulates transcription at an attenuator in response to tryptophan; however, there is no apparent overlapping antiterminator. Moreover, the *B. halodurans trp* attenuator bears only slight similarity to a conical intrinsic terminator. Most notably, it contains several mismatches within the stem. Hence, it appears that in this system regulation is based solely on the ability of TRAP to promote termination at a very weak intrinsic terminator.

One model for intrinsic termination suggests that formation of the hairpin induces forward translocation of RNAP at the U-tract without chain elongation (9). Recent studies of the energetics of transcription termination support this forward translocation model for intrinsic terminators containing two interruptions in the U-tract (35), such as the *trp* attenuator (Figure 3A). Terminators with uninterrupted U-tracts do not appear to require forward translocation of RNAP (35). We found forward translocation is required for termination at the *trp* attenuator (Figure 5A). Its weak intrinsic termination activity may result from ineffective pushing of RNAP by the AU-rich hairpin. The role of TRAP in causing termination at the attenuator may therefore be to assist forward movement of the TEC. We also show that, like the termination factor Mfd and Rho (29), TRAP can induce forward movement of RNAP on the DNA template (Figure 5B). Potential mechanisms by which TRAP could induce forward translocation of RNAP include: (i) by specific protein-protein interactions between TRAP and RNAP such as have been proposed for Mfd (29), or (ii) by nonspecific protein-protein interactions in which TRAP binding to the nascent transcript physically pushes RNAP forward or (iii) by interactions through the nascent RNA such as has been proposed for Rho (29). TRAP is not needed for termination when the GC content of the stem is increased (All GC, Figure 3), suggesting that the GC enriched hairpin induces hypertranslocation of RNAP without assistance from TRAP. Substitutions that remove the interruptions in the U-stretch also eliminate the need for TRAP to induce efficient termination at the attenuator (Figure 3D). In this case, without the interruptions, forward translocation of RNAP may not be required (35) allowing the AU-rich stem to cause termination via an alternative mechanism (36). The *trp* attenuator thus appears to represent a new type of bacterial transcription terminator that is neither truly intrinsic nor dependent on termination

factor rho (37), but instead relies on TRAP bound to the nascent RNA.

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REFERENCES

- Gollnick,P., Babitzke,P., Antson,A. and Yanofsky,C. (2005) Complexity in regulation of tryptophan biosynthesis in *Bacillus subtilis*. *Annu. Rev. Genet.*, **39**, 47–68.
- Gollnick,P., Babitzke,P., Merino,E. and Yanofsky,C. (2002) In Sonenshein,A.L., Hoch,J.A. and Losick,R. (eds), *Bacillus subtilis and its Closest Relatives*. ASM Press, Washington DC, pp. 233–244.
- Yakhnin,A.V. and Babitzke,P. (2010) Mechanism of NusG-stimulated pausing, hairpin-dependent pause site selection and intrinsic termination at overlapping pause and termination sites in the *Bacillus subtilis trp* leader. *Mol. Microbiol.*, **76**, 690–705.
- Gollnick,P., Ishino,S., Kuroda,M.I., Henner,D. and Yanofsky,C. (1990) The *mtr* locus is a two gene operon required for transcription attenuation in the *trp* operon of *Bacillus subtilis*. *Proc. Natl Acad. Sci. USA*, **87**, 8726–8730.
- Antson,A.A., Otridge,J., Brzozowski,A.M., Dodson,E.J., Dodson,G.G., Wilson,K.S., Smith,T.M., Yang,M., Kurecki,T. and Gollnick,P. (1995) The structure of *trp* RNA-binding attenuation protein. *Nature*, **374**, 693–700.
- Otridge,J. and Gollnick,P. (1993) MtrB from *Bacillus subtilis* binds specifically to *trp* leader RNA in a tryptophan dependent manner. *Proc. Natl Acad. Sci. USA*, **90**, 128–132.
- Babitzke,P., Bear,D.G. and Yanofsky,C. (1995) TRAP, the *trp* RNA-binding attenuation protein of *Bacillus subtilis* is a toroid shaped molecule that binds transcripts containing GAG or UAG repeats separated by two nucleotides. *Proc. Natl Acad. Sci. USA*, **92**, 7916–7920.
- Babitzke,P. and Yanofsky,C. (1993) Reconstitution of *Bacillus subtilis trp* attenuation *in vitro* with TRAP, the *trp* RNA-binding attenuation protein. *Proc. Natl Acad. Sci. USA*, **90**, 133–137.
- Santangelo,T.J. and Roberts,J.W. (2004) Forward translocation is the natural pathway of RNA release at an intrinsic terminator. *Mol. Cell*, **14**, 117–126.
- Barbolina,M.V., Kristoforov,R., Manfredo,A., Chen,Y. and Gollnick,P. (2007) The rate of TRAP binding to RNA is crucial for transcription attenuation control of the *B. subtilis trp* operon. *J. Mol. Biol.*, **370**, 925–938.
- Yang,M., de Saizieu,A., van Loon,A.P.G.M. and Gollnick,P. (1995) Translation of *trpG* in *Bacillus subtilis* is regulated by the *trp* RNA-binding Attenuation protein (TRAP). *J. Bacteriol.*, **177**, 4272–4278.
- Anagnostopoulos,C. and Spizizen,J. (1961) Requirements for Transformation in *Bacillus subtilis*. *J. Bacteriol.*, **81**, 741–746.

13. Vogel,H.J. and Bonner,D.M. (1956) Acetylornithinase of *Escherichia coli*: partial purification and some properties. *J. Biol. Chem.*, **218**, 97–106.
14. Perego,M. (1993) Integrational vectors for genetic manipulation in *Bacillus subtilis* and other gram-positive bacteria. In Hoch,J.A., Sonenshein,A.L. and Losick,R. (eds), *Bacillus subtilis and other gram-positive bacteria*. American Society for Microbiology, Washington, DC.
15. Shimotsu,H. and Henner,D.J. (1986) Construction of a single-copy integration vector and its use in analysis of regulation of the *trp* operon of *Bacillus subtilis*. *Gene*, **43**, 85–94.
16. Antson,A.A., Brzozowski,A.M., Dodson,E.J., Dauter,Z., Wilson,K.S., Kurecki,T. and Gollnick,P. (1994) Eleven-fold symmetry of the *trp* RNA-binding attenuation protein (TRAP) from *Bacillus subtilis* determined by X-ray analysis. *J. Mol. Biol.*, **244**, 1–5.
17. King,K., Benkovic,S.J. and Modrich,P. (1989) Glu-111 is required for activation of the DNA cleavage center of EcoRI endonuclease. *J. Biol. Chem.*, **264**, 11807–11815.
18. Miller,J.H. (1972) *Experiments in Molecular Genetics*. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
19. Babitzke,P., Schaak,J., Yakhnin,A.V. and Bevilacqua,P.C. (2003) Role of RNA structure in transcription attenuation in *Bacillus subtilis*: the *trpEDCFBA* operon as a model system. *Methods Enzymol.*, **371**, 392–404.
20. Barbolina,M.V., Li,X. and Gollnick,P. (2005) *Bacillus subtilis* TRAP binds to its RNA target by a 5' to 3' directional mechanism. *J. Mol. Biol.*, **345**, 667–679.
21. d'Aubenton Carafa,Y., Brody,E. and Thermes,C. (1990) Prediction of rho-independent *Escherichia coli* transcription terminators. A statistical analysis of their RNA stem-loop structures. *J. Mol. Biol.*, **216**, 835–858.
22. Yarnell,W.S. and Roberts,J.W. (1999) Mechanism of intrinsic transcription termination and antitermination. *Science*, **284**, 611–615.
23. Gusarov,I. and Nudler,E. (1999) The mechanism of intrinsic transcription termination. *Mol. Cell*, **3**, 495–504.
24. Shimotsu,H., Kuroda,M.I., Yanofsky,C. and Henner,D.J. (1986) Novel form of transcription attenuation regulates expression of the *Bacillus subtilis* tryptophan operon. *J. Bacteriol.*, **166**, 461–471.
25. Merino,E., Babitzke,P. and Yanofsky,C. (1995) *trp* RNA-binding attenuation protein (TRAP)-*trp* leader RNA interactions mediate translational as well as transcriptional regulation of the *Bacillus subtilis trp* operon. *J. Bacteriol.*, **177**, 6362–6370.
26. Yakhnin,A.V. and Babitzke,P. (2002) NusA-stimulated RNA polymerase pausing and termination participates in the *Bacillus subtilis trp* operon attenuation mechanism in vitro. *Proc. Natl Acad. Sci. USA*, **99**, 11067–11072.
27. Grundy,F.J., Yousef,M.R. and Henkin,T.M. (2005) Monitoring uncharged tRNA during transcription of the *Bacillus subtilis glyQS* gene. *J. Mol. Biol.*, **346**, 73–81.
28. Pavco,P.A. and Steege,D.A. (1990) Elongation by *Escherichia coli* RNA polymerase is blocked in vitro by a site-specific DNA binding protein. *J. Biol. Chem.*, **265**, 9960–9969.
29. Roberts,J. and Park,J.S. (2004) Mfd, the bacterial transcription repair coupling factor: translocation, repair and termination. *Curr. Opin. Microbiol.*, **7**, 120–125.
30. de Hoon,M.J., Makita,Y., Nakai,K. and Miyano,S. (2005) Prediction of transcriptional terminators in *Bacillus subtilis* and related species. *PLoS Comput. Biol.*, **1**, e25.
31. Yanofsky,C. (2000) Transcription attenuation: once viewed as a novel regulatory strategy. *J. Bacteriol.*, **182**, 1–8.
32. Gutierrez-Preciado,A., Henkin,T.M., Grundy,F.J., Yanofsky,C. and Merino,E. (2009) Biochemical features and functional implications of the RNA-based T-box regulatory mechanism. *Microbiol. Mol. Biol. Rev.*, **73**, 36–61.
33. Landick,R., Turnbough,C.L. and Yanofsky,C. (1996) In Neidhardt,F.C. (ed.), *Escherichia coli and Salmonella Cellular and Molecular Biology*, 2nd edn. ASM Press, Washington DC, pp. 1263–1286.
34. Szigeti,R., Milescu,M. and Gollnick,P. (2004) Regulation of the tryptophan biosynthetic genes in *Bacillus halodurans*: common elements but different strategies than those used by *Bacillus subtilis*. *J. Bacteriol.*, **186**, 818–828.
35. Larson,M.H., Greenleaf,W.J., Landick,R. and Block,S.M. (2008) Applied force reveals mechanistic and energetic details of transcription termination. *Cell*, **132**, 971–982.
36. Touloukhanov,I. and Landick,R. (2003) The flap domain is required for pause RNA hairpin inhibition of catalysis by RNA polymerase and can modulate intrinsic termination. *Mol. Cell*, **12**, 1125–1136.
37. Ciampi,M.S. (2006) Rho-dependent terminators and transcription termination. *Microbiology*, **152**, 2515–2528.