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Expression of the *Bacillus subtilis trpEDCFBA* Operon Is Influenced by Translational Coupling and Rho Termination Factor

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The *trp* RNA-binding attenuation protein (TRAP) regulates expression of the *Bacillus subtilis trpEDCFBA* operon by transcription attenuation and translational control mechanisms. Both mechanisms require binding of tryptophan-activated TRAP to 11 (G/U)AG repeats in the *trp* leader transcript. *trpE* translational control involves formation of a TRAP-dependent RNA structure that sequesters the *trpE* Shine-Dalgarno (SD) sequence (the SD blocking hairpin). By comparing expression levels from *trpE′-′lacZ* translational fusions controlled by the wild-type leader or by a leader that cannot form the SD blocking hairpin, we found that translational control requires a tryptophan concentration higher than that required for transcription attenuation. We also found that inhibition of *trpE* translation by the SD blocking hairpin does not alter the stability of the downstream message. Since the coding sequences for *trpE* and *trpD* overlap by 29 nucleotides, we examined expression levels from *trpED′-′lacZ* translational fusions to determine if these two genes are translationally coupled. We found that introduction of a UAA stop codon in *trpE* resulted in a substantial reduction in expression. Since expression was partially restored in the presence of a tRNA suppressor, our results indicate that *trpE* and *trpD* are translationally coupled. We determined that the coupling mechanism is TRAP independent and that formation of the SD blocking hairpin regulates *trpD* translation via translational coupling. We also constructed a *rho* mutation to investigate the role of Rho-dependent termination in *trp* operon expression. We found that TRAP-dependent formation of the SD blocking hairpin allows Rho access to the nascent transcript, causing transcriptional polarity.

Organisms utilize a wide range of regulatory mechanisms to control gene expression. Bacteria have developed several sophisticated regulatory mechanisms that allow the organisms to modulate gene expression after transcription has initiated. In addition, several subtle mechanisms allow the organisms to fine tune the final level of any particular gene product. Expression of the *Bacillus subtilis trpEDCFBA* operon is regulated in response to changes in the intracellular concentration of tryptophan by the *trp* RNA-binding attenuation protein (TRAP). TRAP regulates expression of the *trp* operon by transcription attenuation and translational control mechanisms (reviewed in references 4 and 12). TRAP exists as a complex consisting of 11 identical subunits arranged in a single ring (3). Tryptophan cooperatively activates TRAP by binding between adjacent TRAP subunits (3, 6). When TRAP is activated by tryptophan, 11 KKR motifs that outline the periphery of the TRAP complex bind to 11 (G/U)AG repeats present in the nascent *trp* leader transcript, thereby wrapping the RNA around the periphery of the TRAP complex (2, 7, 33). In the transcription attenuation mechanism, TRAP binding prevents formation of an antiterminator structure, since six of the (G/U)AG repeats are present within this RNA structure (5, 7). In this case, TRAP binding promotes the formation of an overlapping intrinsic terminator, resulting in transcription termination before RNA polymerase reaches the structural genes. In the absence of TRAP binding, formation of the antiterminator permits transcription of the entire operon (5). A third stem-loop structure that forms at the extreme 5′ end of the *trp* leader transcript also plays a role in the transcription attenuation mechanism. TRAP-5′ stem-loop interaction increases the affinity of TRAP for *trp* leader RNA and reduces the number of (G/U)AG repeats that are required for stable TRAP-*trp* leader RNA association. Thus, TRAP-5′ stem-loop interaction may increase the likelihood that TRAP will bind to the (G/U)AG repeats in time to block antiterminator formation (10, 30).

In addition to regulating transcription of the *trp* operon, TRAP also regulates translation of *trpE* (11, 17, 20). RNA structural studies of the *trp* operon readthrough transcript indicated that the most thermodynamically stable conformation of the leader RNA contains a large secondary structure that includes the last six (G/U)AG repeats in the 5′ half of the stem (Fig. 1). TRAP binding to these repeats prevents or disrupts formation of this large secondary structure, thereby promoting the formation of a structure that sequesters the *trpE* Shine-Dalgarno (SD) sequence (the *trpE* SD blocking hairpin) (11, 17, 20). Formation of the *trpE* SD blocking hairpin inhibits TrpE synthesis by blocking ribosome access to the *trpE* ribosome binding site (11). In vivo and in vitro studies have established that multiple nucleotide substitutions that destabilize the *trpE* SD blocking hairpin, without altering the SD sequence itself, reduce the ability of TRAP to regulate TrpE synthesis (11, 20). In this study, we examined the effect of tryptophan concentration on transcription attenuation and translational control and found that translational regulation requires a tryp-

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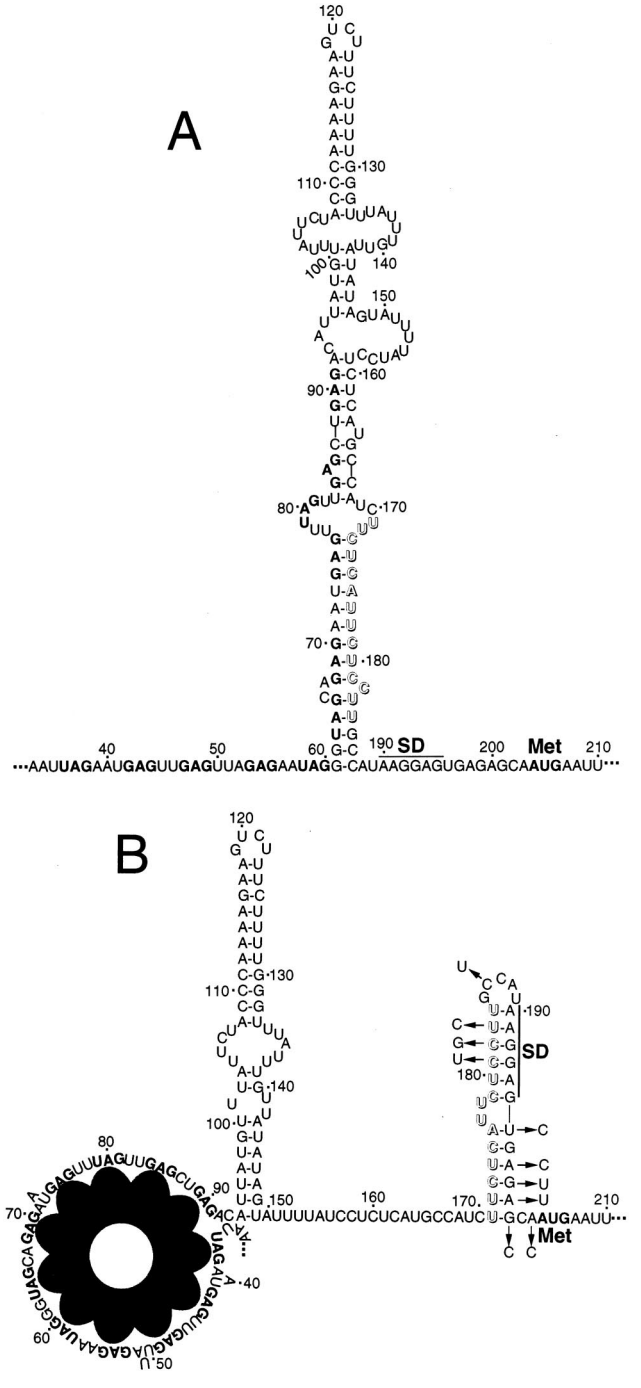

FIG. 1. Model of the *trpE* translational control mechanism. (A) Under tryptophan-limiting conditions, TRAP is not activated and is unable to bind to the leader of *trp* operon readthrough transcripts. In this case the *trpE* SD sequence is single stranded, allowing efficient translation. (B) Under excess-tryptophan conditions, tryptophan-activated TRAP binds to the (G/U)AG repeats present between nucleotides 36 and 91 in the leader of a *trp* operon readthrough transcript. In this case, the secondary structure of the downstream RNA is altered such that the *trpE* SD sequence is sequestered in a stable RNA hairpin (*trpE* SD blocking hairpin). This RNA secondary structure inhibits TrpE synthesis by preventing 30S ribosomal subunit interaction with the *trpE* message (11). The nucleotides between 171 and 184 that are responsible for sequestering the *trpE* SD sequence are shown in outline type in both diagrams. In the absence of bound TRAP, these nucleotides basepair with a segment of the TRAP binding site. The (G/U)AG repeats and the *trpE* AUG initiation codon are indicated in boldface. The nucleotide substitutions in the SD *trpL* that prevent formation of the *trpE* SD blocking hairpin are indicated with arrows. The nucleotides that make up the mutually exclusive antiterminator and terminator structures extend from positions 60 to 111 or 108 to 133, respectively. Periods indicate that sequence information has been omitted. Numbering is from the start of transcription.

tophan concentration higher than that required for transcription attenuation.

The sequence of the *trpEDCFBA* operon revealed that the coding sequences of all but two of the genes (*trpC* and *trpF*) overlap by several nucleotides (Fig. 2). This gene organization suggested that the *trp* genes might be regulated by translational coupling (14), a process in which translation of a gene is partially dependent on translation of the gene immediately upstream (19, 24). The first two genes of the *trp* operon, *trpE* and *trpD*, have the most extensive overlap in the operon (29 nucleotides). We found that these two genes are translationally coupled and that formation of the *trpE* SD blocking hairpin regulates TrpD synthesis via translational coupling. Since inhibition of translation can result in reduced mRNA stability (18) and/or transcriptional polarity (16), we performed experiments to determine if *trpE* translational control influences *trp* operon expression by reducing the stability of the message or by allowing Rho access to the nascent *trp* transcript. Our results establish that formation of the *trpE* SD blocking hairpin promotes Rho-mediated transcriptional polarity but does not influence the stability of the downstream mRNA.

MATERIALS AND METHODS

Plasmids and bacterial strains. The genotypes of the *B. subtilis* strains used in this study are listed in Table 1. Plasmid pPB20 containing the wild-type (WT) *B. subtilis trp* promoter, the leader, and the first 40 *trpE* codons was previously described (5). pINT-SDtrpL contains several *trp* leader point mutations (SD*trpL*) that destabilize the *trpE* SD blocking hairpin without disrupting the *trpE* SD sequence itself (20). Plasmid pHD15 contains the *trp* promoter, the leader, the entire *trpE* coding sequence, and the N-terminal coding sequence of *trpD*, while pHD22 contains a *trpE'-'lacZ* translational fusion that is controlled by the *trp* promoter and leader (11). The integration plasmids ptrpBGI (WT *trpL trpE'-'lacZ*) (29) and pHD24 (SD *trpL trpE'-'lacZ*) were previously described (11). The integration vector, ptrpBGI-PLK, used for generation of *trpE'-'lacZ* translational fusions, was described previously (20). The plasmid constructions used to generate *trpED'-'lacZ* translational fusions were made in *Escherichia coli*. Integrative plasmids containing various translational fusions were linearized with *Pst*I and separately integrated into the *amyE* locus of the *B. subtilis* chromosome by homologous recombination. For example, to construct strains containing *trpE-'lacZ* translational fusions in a tryptophan auxotrophic strain, we integrated ptrpBGI or pHD24 into strain 168 (*trpC2*) to yield strain PLBS176 or PLBS201, respectively. Transformation was by natural competence (1). Selection was for chloramphenicol resistance (5 μg/ml). Integration into *amyE* was confirmed by screening for the absence of amylase activity on starch plates by using iodine staining (28). In each case, the correct plasmid construction was confirmed by automated DNA sequencing.

Construction of the *trpED'-'lacZ* translational fusions used in this study was done in several steps. First, a double-stranded DNA linker was ligated into the *Hin*dIII site of pPB20. The resulting plasmid, pYH2, contains four restriction sites (*Xba*I, *Bam*HI, *Nhe*I, and *Hin*dIII) derived from the DNA linker. The DNA containing the overlapping *trpED* region in pHD15 was amplified by PCR. In one case, an in-frame lysine codon (AAA) was retained immediately downstream from an *Xba*I site. In the other case, we introduced a TAA termination codon in place of the natural lysine codon. The TAA- or AAA-containing PCR products were ligated into pYH2 to produce pYH3 or pYH7, respectively. DNA fragments containing the *trp* promoter, leader, and portions of *trpE* and *trpD* from pYH3 or pYH7 were subcloned into ptrpBGI-PLK to produce pYH5 or pYH10,

FIG. 2. Schematic representation of the *B. subtilis trpEDCFBA* operon highlighting the overlapping reading frames. With the exception of the 4-nucleotide intercistronic region between *trpC* and *trpF*, all of the open reading frames overlap by several residues. The length of each overlap (in nucleotides) is shown in parentheses. The positions of the *trp* promoter (p>) and leader (*trpL*) are shown (not drawn to scale).

respectively. Note that pYH10 contains the WT *trp* promoter and leader followed by an in-frame *trpED′-′lacZ* translational fusion in which the central region of *trpE* (codons 41 through 483) was deleted. pYH5 is identical to pYH10 except that the TAA stop codon replaces the lysine codon. Importantly, this stop codon would uncouple translation of *trpD* from translation of *trpE*. These translational fusions were separately integrated into the *amyE* locus of strain CB312 or CB313 as described above. Similar *trpED′-′lacZ* translational fusions were constructed in which the SD *trpL* region from pINT-SDtrpL replaced the WT *trpL*. The SD *trpL* fusions were separately integrated into strain CB312 or CB313. TRAP-deficient (Δ*mtrB*) strains were constructed by transforming various strains with chromosomal DNA from strain BG4233 (Δ*mtrB*) (15). Selection was for 5-fluorotryptophan resistance (200 μg/ml).

A null *rho* mutation was constructed by replacing nucleotides +155 to +165 relative to the *rho* initiation codon with the *neo* (Km^r) gene from plasmid pMK3 (31). The resulting plasmid, pYH14, was linearized with *Sca*I and subsequently used to replace the WT *rho* allele in several strains by transformation-mediated homologous recombination. Selection was for kanamycin resistance (10 μg/ml). Proper allelic replacement in each strain was confirmed by PCR amplification of chromosomal DNA.

β-Galactosidase assay. *B. subtilis* cultures were grown in minimal-acid casein hydrolysate medium in the presence of 5 μg of chloramphenicol/ml. Tryptophan prototrophs were grown in the presence of 0 or 200 μM tryptophan. Tryptophan auxotrophs were grown in the presence of 20, 50, 100, 200, or 400 μM tryptophan. The cells were harvested during late exponential growth (110 Klett units; filter no. 54; Klett Manufacturing Co., Inc.). Aliquots were then assayed for β-galactosidase activity as previously described (11).

mRNA half-life and steady-state level determinations. *B. subtilis* cultures were grown in minimal-acid casein hydrolysate medium in the presence of 20 or 200 μM tryptophan (*trpC2* auxotrophic strains) or 0 or 200 μM tryptophan (*trp* prototrophic strains). The growth medium was supplemented with appropriate antibiotics (5 μg of chloramphenicol/ml or 10 μg of kanamycin/ml). When the cultures reached late exponential phase (110 Klett units; filter no. 54), 100 μg of rifampin/ml was added to inhibit transcription initiation. Six-milliliter aliquots were removed 0, 1, 2, 4, 8, and 16 min after the addition of rifampin and added to an equal volume of frozen killing buffer (8.5 mM Tris-HCl, pH 7.2, 5 mM $MgCl_2$, 25 mM sodium azide, and 500 μg of chloramphenicol/ml). Total RNA was isolated using the RNeasy protocol (Qiagen), and genomic DNA was eliminated using RNase-free DNase I (Promega). RNA was extracted with an equal volume of phenol-chloroform, precipitated with ethanol, and suspended in Tris-EDTA. Quantification of mRNA was performed by slot blot hybridization. Ten micrograms of total RNA from each sample was mixed with an equal volume of denaturing solution (0.55 ml of formamide, 0.2 ml of 37% formaldehyde, 0.2 ml of morpholinepropanesulfonic acid, pH 7.0) and incubated for 15 min at 65°C. Samples were subsequently chilled on ice and spotted onto Hybond-N+ membranes (Amersham Pharmacia Biotech) using a Minifold I dot blot apparatus (Schleicher & Schuel). After filtration, RNA was covalently linked to the membrane using a UV Stratalinker (Strategene). Prehybridization was performed at 65°C for 1 h in a buffer containing 7% sodium dodecyl sulfate (SDS), 0.5 M sodium phosphate buffer, pH 7.2, and 10 mM EDTA (9). Hybridization was carried out at 65°C for 12 h by adding *lacZ*, *trpE*, or *trpD* DNA probes that were radiolabeled using the random-primer DNA-labeling kit (Boehringer Mannheim Biochemical). The filters were washed twice for 30 min each time with 1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) containing 0.1% SDS at 62°C followed by two 30-min washes with 0.1× SSC containing 0.1% SDS at the

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

Strain	Genotype[a]	Source
168	*trpC2*	BGSC[b]
BG4233	Δ*mtrB argC4*	15
CB312	*his met*	C. Stewart[c]
CB313	*sup-3*	C. Stewart
PLBS127	*rpoB18 amyE*::[*trpp*(−412 to +203) *trpE′-′lacZ* Cm^r]	11
PLBS129	*rpoB18 amyE*::[*trpp*(−412 to +203) SD^{mod} *trpE′-′lacZ* Cm^r]	11
PLBS138	*his met amyE*::[*trpp*(−412 to +203) *trpED′-′lacZ* (*trpE* stop) Cm^r]	This study
PLBS139	*sup-3 amyE*::[*trpp*(−412 to +203) *trpED′-′lacZ* (*trpE* stop) Cm^r]	This study
PLBS142	*his met amyE*::[*trpp*(−412 to +203) *trpED′-′lacZ* Cm^r]	This study
PLBS143	*sup-3 amyE*::[*trpp*(−412 to +203) *trpED′-′lacZ* Cm^r]	This study
PLBS144	*his met amyE*::[*trpp*(−412 to +203) SD^{mod} *trpED′-′lacZ* (*trpE* stop) Cm^r]	This study
PLBS145	*sup-3 amyE*::[*trpp*(−412 to +203) SD^{mod} *trpED′-′lacZ* (*trpE* stop) Cm^r]	This study
PLBS146	*his met amyE*::[*trpp*(−412 to +203) SD^{mod} *trpED′-′lacZ* Cm^r]	This study
PLBS147	*sup-3 amyE*::[*trpp*(−412 to +203) SD^{mod} *trpED′-′lacZ* Cm^r]	This study
PLBS176	*trpC2 amyE*::[*trpp*(−412 to +203) *trpE′-′lacZ* Cm^r]	This study
PLBS201	*trpC2 amyE*::[*trpp*(−412 to +203) SD^{mod} *trpE′-′lacZ* Cm^r]	This study
PLBS283	Δ*mtrB argC4 his met amyE*::[*trpp*(−412 to +203) *trpED′-′lacZ* (*trpE* stop) Cm^r]	This study
PLBS284	Δ*mtrB argC4 sup-3 amyE*::[*trpp*(−412 to +203) *trpED′-′lacZ* (*trpE* stop) Cm^r]	This study
PLBS285	Δ*mtrB argC4 his met amyE*::[*trpp*(−412 to +203) *trpED′-′lacZ* Cm^r]	This study
PLBS286	Δ*mtrB argC4 sup-3 amyE*::[*trpp*(−412 to +203) *trpED′-′lacZ* Cm^r]	This study
PLBS289	*rpoB18 rho*::*neo amyE*::[*trpp*(−412 to +203) *trpE′-′lacZ* Cm^r]	This study
PLBS291	*rho*::*neo his met amyE*::[*trpp*(−412 to +203) *trpED′-′lacZ* (*trpE* stop) Cm^r]	This study
PLBS292	*rho*::*neo sup-3 amyE*::[*trpp*(−412 to +203) *trpED′-′lacZ* (*trpE* stop) Cm^r]	This study
PLBS293	*rho*::*neo his met amyE*::[*trpp*(−412 to +203) *trpED′-′lacZ* Cm^r]	This study
PLBS294	*rho*::*neo sup-3 amyE*::[*trpp*(−412 to +203) *trpED′-′lacZ* Cm^r]	This study
PLBS321	*rho*::*neo his met*	This study
PLBS322	*rho*::*neo trpC2*	This study
W168	*rpoB18*	BGSC

[a] *trpp* denotes the *trp* promoter; a prime indicates truncation of the gene; −412 to +203 is the DNA fragment containing *trpp* and neighboring regions that was incorporated, relative to the transcription start site; (*trpE* stop) indicates that the strain contains an engineered UAA stop codon in the *trpE* coding sequence. *trpED′-′lacZ* contains an in-frame deletion of *trpE* as well as a translational fusion between *trpD* and *lacZ*. *sup-3* encodes a UAA tRNA suppressor.

[b] BGSC, *Bacillus* Genetic Stock Center, Ohio State University, Columbus, Ohio.

[c] These strains were obtained from Charles R. Stewart, Department of Biochemistry and Cell Biology, Rice University, Houston, Tex.

TABLE 2. Effect of various tryptophan concentrations on expression of *trpE'-'lacZ* fusions

Strain	Relevant genotype	β-Gal activity[a]						β-Gal ratio[e]	
		0 μM[b]	20 μM	50 μM	100 μM	200 μM	400 μM	0 μM/200 μM	20 μM/400 μM
PLBS127	WT *trpL*	42 ± 11	ND[d]	ND	ND	0.1 ± 0.02	ND	420	ND
PLBS129	SD *trpL*	39 ± 9	ND	ND	ND	5.3 ± 0.2	ND	7	ND
PLBS176	WT *trpL trpC2*	NA[c]	44 ± 12	7.4 ± 2.1	2.6 ± 0.2	0.8 ± 0.1	0.2 ± 0.06	NA	220
PLBS201	SD *trpL trpC2*	NA	43 ± 11	12 ± 1.0	8.7 ± 1.0	5.0 ± 0.4	4.7 ± 0.2	NA	9

[a] The β-galactosidase (β-Gal) activity expressed from the *trpE'-'lacZ* fusion is given in Miller units (21). The values are averages from at least three independent experiments ± standard deviation.
[b] Concentration of tryptophan in the growth media.
[c] NA, not applicable. Tryptophan auxotrophic strains require tryptophan for growth.
[d] ND, not determined.
[e] Ratio of activities at the indicated concentrations.

same temperature. mRNA levels were quantified with a PhosphorImager (Molecular Dynamics, Inc.) and the ImageQuant software package. Determination of the steady-state levels of *trpE* and *trpD* mRNAs was performed as described above except that only 0-min time points were analyzed and rifampin was omitted.

RESULTS

The *trpE* translational control mechanism requires a concentration of tryptophan higher than that required for transcription attenuation. Expression of the *trpEDCFBA* operon is regulated by TRAP-mediated transcription attenuation and translational control mechanisms. In the transcription attenuation mechanism, only transient TRAP interaction with the (G/U)AG repeats would be necessary to block formation of the antiterminator. Once transcription has terminated, it would be advantageous for the TRAP-*trp* leader RNA complex to dissociate to prevent titration of TRAP. However, for translational control it appears that TRAP must remain associated with the triplet repeats to maintain formation of the *trpE* SD blocking hairpin because the *trpE* SD sequence is single stranded in the absence of bound TRAP (11). It was also shown that the affinity of TRAP for *trp* leader RNA increases with increasing tryptophan concentrations (25, 32) and that the dissociation rate of TRAP-*trp* leader RNA complexes decreases as the concentration of tryptophan increases (8). Because of these findings, we reasoned that the tryptophan concentration required for translational control would be higher than that required for transcription attenuation.

In a previous study, we compared expression levels from *trpE'-'lacZ* translational fusions containing the WT *trpL* (transcription attenuation and *trpE* translational control mechanisms functioning) or the SD *trpL* (only transcription attenuation mechanism functioning) (11). Although it was not appreciated at the time, our previously published results (11) suggested that TRAP-mediated translational control required an intracellular tryptophan concentration higher than that required for transcription attenuation. To further explore this possibility, we repeated our previously published expression studies. Since the effect of exogenous tryptophan on expression of the *trp* operon can be assessed from the −Trp/+Trp ratio, we examined β-galactosidase activity in prototrophic strains containing *trpE'-'lacZ* translational fusions controlled by either the WT *trpL* (PLBS127) or the SD *trpL* (PLBS129). The levels of expression in both strains were similar when the cells were grown in the absence of exogenous tryptophan (Table 2). However, when the cells were grown in the presence of 200 μM tryptophan, we observed 50-fold higher expression in the SD *trpL* strain (Table 2).

To allow more precise control of the intracellular tryptophan concentrations, we integrated the translational fusions into a *trpC2* auxotrophic strain. Expression was examined over a wide range of tryptophan concentrations in the growth media. Since the β-galactosidase activity of the auxotrophic strains grown in the presence of 20 μM tryptophan was similar to the activity of prototrophic strains grown in the absence of tryptophan (Table 2), we used 20 μM as the lower limit for subsequent expression studies. As the tryptophan concentration was gradually increased from 20 to 400 μM, expression from the WT *trpL* strain (PLBS176) continued to decrease (Fig. 3 and Table 2). However, the decrease in expression in the SD *trpL* strain (PLBS201) was much more gradual and

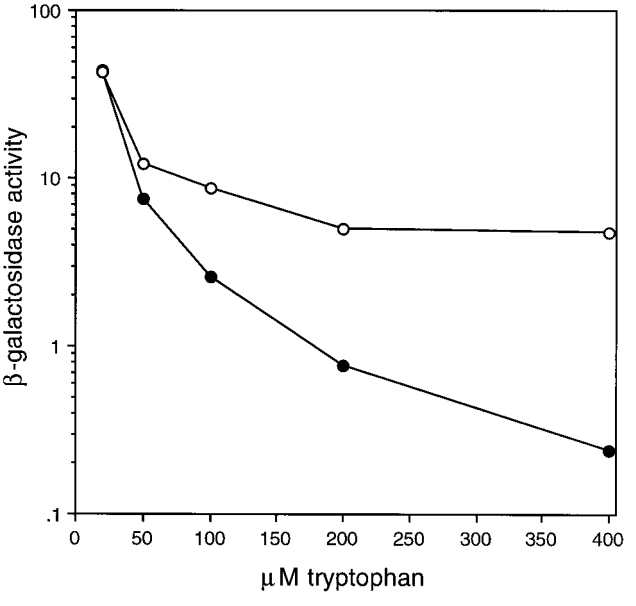

FIG. 3. β-Galactosidase activity from *trpC2* auxotrophic strains as a function of tryptophan concentration in the growth media. *trpE'-'lacZ* translational fusions were under control of the WT *trpL* (solid circles) or SD *trpL* (open circles). Both the transcription attenuation and *trpE* translational control mechanisms function in the WT *trpL* strain (PLBS176), whereas only the transcription attenuation mechanism functions in the SD *trpL* strain (PLBS201). β-Galactosidase activity is shown in Miller units (21). This figure is derived from the data presented in Table 2.

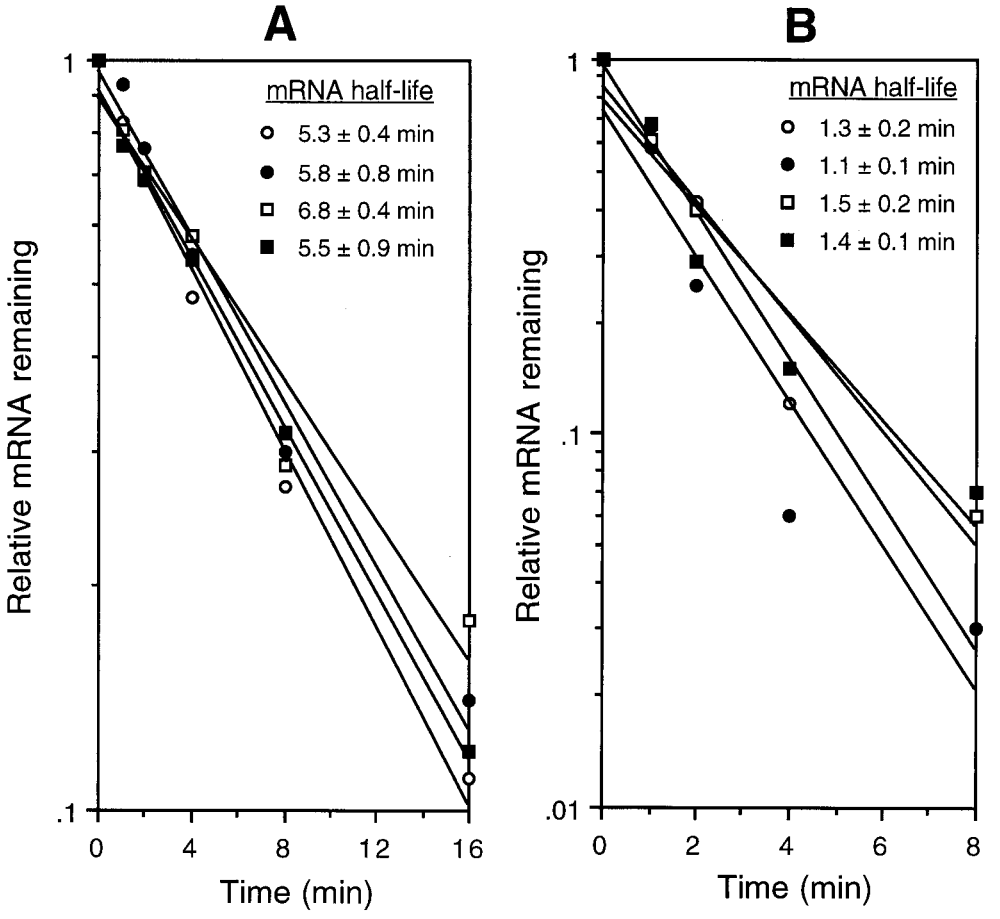

FIG. 4. Time course of *lacZ*, *trpE*, and *trpD* mRNA decay. (A) *lacZ* mRNA half-life experiments in strains containing *trpE'-'lacZ* translational fusions that were controlled by either the WT *trpL* (PLBS176) or the SD *trpL* (PLBS201) when each strain was grown in the presence of 20 or 200 μM tryptophan. Open circles, WT *trpL* with 20 μM tryptophan; solid circles, WT *trpL* with 200 μM tryptophan; open squares, SD *trpL* with 20 μM tryptophan; solid squares, SD *trpL* with 200 μM tryptophan. (B) *trpE* and *trpD* mRNA half-life experiments from the natural *trp* operon when strain 168 was grown in the presence of 20 or 200 μM tryptophan. Open circles, *trpE* with 20 μM tryptophan; solid circles, *trpE* with 200 μM tryptophan; open squares, *trpD* with 20 μM tryptophan; solid squares, *trpD* with 200 μM tryptophan. The relative levels of mRNA remaining at 0, 1, 2, 4, 8, and 16 min after the addition of rifampin were determined by dot blot analysis using the corresponding probe. Each value is an average from at least two independent experiments. The mRNA level corresponding to each 0-min time point was set to 1. The mRNA half-life ± the standard deviation for each strain and growth condition is shown next to the corresponding symbol.

reached a lower limit at 200 μM tryptophan. Although levels of expression were similar in the two strains when grown in the presence of 20 or 50 μM tryptophan, the difference in expression reached a factor of 3, 6, or 24 when the tryptophan concentration was increased to 100, 200, or 400 μM, respectively. Since these two strains have only the transcription attenuation mechanism in common, the difference in expression at the higher tryptophan concentrations is due to the functional translational control mechanism in the WT *trpL* strain. These results suggest that the transcription attenuation mechanism is primarily responsible for controlling expression at low tryptophan concentrations and that the translational control mechanism does not play a significant role until the concentration of tryptophan in the growth media is raised above 50 μM.

Inhibition of *trpE* translation mediated by the SD blocking hairpin does not alter stability of downstream mRNA. Inhibition of translation has been shown to increase the rate of mRNA decay in some cases (reviewed in reference 18). This presumably results from decreased ribosomal protection of the downstream message from ribonucleases. We tested the possibility that formation of the *trpE* SD blocking hairpin destabilized the downstream mRNA. We determined the *lacZ* mRNA half-life in strains containing *trpE'-'lacZ* fusions controlled by either the WT *trpL* (PLBS176) or the SD *trpL* (PLBS201). Since formation of the *trpE* SD blocking hairpin is dependent on tryptophan-activated TRAP, we expected that the mRNA half-life would be reduced in the WT *trpL* strain when it was grown in the presence of tryptophan. We were surprised to find that the *lacZ* mRNA half-lives were essentially identical in both strains when grown under limiting or excess tryptophan conditions (Fig. 4A). To ensure that the *lacZ* mRNA half-life data accurately reflected *trp* mRNA stability, we determined the half-life of *trpE* and *trpD* mRNAs in the presence of limiting or excess tryptophan. While the decay of *trpE* and *trpD* mRNAs was considerably more rapid than that of the *lacZ* message, formation of the *trpE* SD blocking hairpin did not influence the stability of the transcripts (Fig. 4B). Thus,

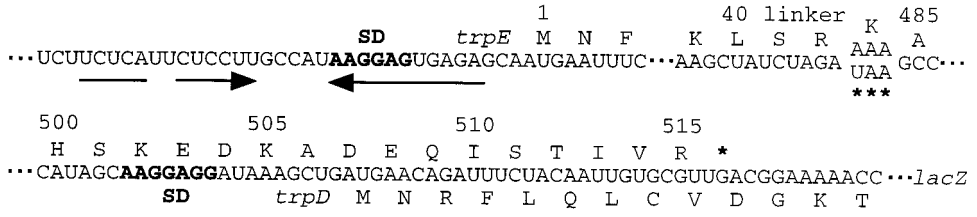

FIG. 5. Nucleotide and amino acid sequences of the *trpED′-′lacZ* translational fusion transcripts used in the translational coupling studies. Only the crucial features of the *trpE* and *trpD* coding regions are shown. The *trpE* and *trpD* amino acid sequences are shown above and below the nucleotide sequence, respectively. The *trpE* and *trpD* coding sequences overlap by 29 nucleotides. Two fusions were constructed that contained either an AAA lysine codon or an engineered UAA stop codon (***). The tRNA suppressor encoded by the *sup-3* allele can place a lysine residue at the UAA stop codon with approximately 15% efficiency (22). The position of the natural *trpE* UGA termination codon is shown (*). The *trpE* codons 41 to 483 were replaced by a short linker. Thus, there are 31 codons between the engineered stop codon and the natural *trpE* stop codon. The *trpE* and *trpD* SD sequences are shown in boldface. The inverted arrows indicate the nucleotides involved in *trpE* SD blocking hairpin formation. Periods indicate that sequence information has been omitted.

trpE translational control does not lead to destabilization of *trp* mRNA.

trpE and *trpD* are translationally coupled. The coding regions of *trpE* and *trpD* overlap by 29 nucleotides (Fig. 2). To determine if this overlap allows translational coupling of these two genes, we constructed two *trpED′-′lacZ* translational fusions in which the central region of *trpE* was deleted (Fig. 5). In one of the fusions, we introduced a UAA termination codon in *trpE*. This stop codon would disrupt translational coupling unless it was translated by a tRNA suppressor. The other fusion contained the natural lysine codon (AAA) instead of the engineered stop codon. In both cases, the reading frame of the internal *trpE* deletion was maintained (Fig. 5). The translational fusions were integrated into the *amyE* locus of WT or *sup-3 B. subtilis* strains. It was previously shown that the *sup-3* allele encodes a tRNA suppressor that can place a lysine residue at UAA stop codons approximately 15% of the time (22). Thus, placement of a lysine residue at this position by the tRNA suppressor would result in a *trpE* polypeptide that is identical in sequence to that encoded by the other fusion. We examined expression from the *trpED′-′lacZ* fusion and found that the levels of regulation in response to tryptophan for all four strains were comparable (compare the −Trp/+Trp β-galactosidase ratios for the first four strains in Table 3). Introduction of the engineered UAA stop codon reduced expression of the fusion sevenfold in the absence of tryptophan and fourfold in its presence (compare expression levels from PLBS142 and PLBS138 [Table 3]). Importantly, when expression was examined in the *sup-3* strain, we found that expression was partially restored in the strain that contained the engineered stop codon; expression increased threefold in the absence of tryptophan and twofold in its presence (compare expression levels from PLBS138 and PLBS139 [Table 3]). The *sup-3* allele had little effect on expression when the engineered stop codon was not present (compare expression levels from PLBS142 and PLBS143 [Table 3]). The finding that suppression of the UAA stop codon within the *trpE* coding sequence partially restored expression of the *trpED′-′lacZ* translational fusion demonstrates that *trpE* and *trpD* are translationally coupled.

We also examined expression from TRAP-deficient (Δ*mtrB*) strains that were otherwise identical to those just described. This allowed us to examine the extent of translational coupling in the absence of TRAP-mediated control of the *trp* operon. As expected, expression was not regulated in response to tryptophan (compare −Trp/+Trp β-galactosidase ratios for the strains corresponding to lines 5 through 8 in Table 3). However, the same general pattern of expression indicative of

TABLE 3. Translational coupling of *trpE* and *trpD*

Strain	Relevant genotype[a]	UAA stop codon in *trpE*[b]	β-Gal activity[c]		β-Gal ratio (−Trp/+Trp)
			−Trp	+Trp	
PLBS142	WT *trpL*	No	194 ± 25	2.3 ± 0.4	84
PLBS138	WT *trpL*	Yes	27 ± 4	0.6 ± 0.1	45
PLBS143	WT *trpL sup-3*	No	198 ± 28	3.2 ± 0.3	62
PLBS139	WT *trpL sup-3*	Yes	79 ± 7	1.1 ± 0.1	72
PLBS285	WT *trpL* Δ*mtrB*	No	1,570 ± 90	1,660 ± 90	1
PLBS283	WT *trpL* Δ*mtrB*	Yes	230 ± 20	240 ± 20	1
PLBS286	WT *trpL* Δ*mtrB sup-3*	No	1,570 ± 130	1,510 ± 60	1
PLBS284	WT *trpL* Δ*mtrB sup-3*	Yes	460 ± 40	460 ± 30	1
PLBS146	SD *trpL*	No	169 ± 15	18 ± 2	9
PLBS144	SD *trpL*	Yes	18 ± 3	2.2 ± 0.2	8
PLBS147	SD *trpL sup-3*	No	223 ± 25	23 ± 4	10
PLBS145	SD *trpL sup-3*	Yes	50 ± 4	4.2 ± 0.3	12

[a] WT *trp* leader (WT *trpL*) or SD *trp* leader (SD *trpL*). UAA stop codons are recognized by the tRNA suppressor encoded by the *sup-3* allele.
[b] Presence (Yes) or absence (No) of the engineered stop codon within the coding sequence of *trpE*.
[c] β-Galactosidase (β-Gal) activity expressed from the *trpED′-′lacZ* fusions is given in Miller units (21). Cells were grown in the absence (−Trp) or presence (+Trp) of tryptophan (200 μM). The values are averages from six independent experiments ± standard deviation.

TABLE 4. Effect of Rho termination factor on *trp* operon expression

Strain	Relevant genotype[a]	Translational fusion	UAA stop codon in *trpE*[b]	β-Gal activity[c]		β-Gal ratio (−Trp/+Trp)
				−Trp	+Trp	
PLBS127	WT *trpL*	*trpE'-'lacZ*	NA[d]	26 ± 5	0.2 ± 0.03	130
PLBS289	WT *trpL rho*::*neo*	*trpE'-'lacZ*	NA	39 ± 7	0.6 ± 0.03	65
PLBS142	WT *trpL*	*trpED'-'lacZ*	No	190 ± 20	2.1 ± 0.5	90
PLBS138	WT *trpL*	*trpED'-'lacZ*	Yes	24 ± 6	0.5 ± 0.1	48
PLBS143	WT *trpL sup-3*	*trpED'-'lacZ*	No	240 ± 50	2.9 ± 0.5	83
PLBS139	WT *trpL sup-3*	*trpED'-'lacZ*	Yes	80 ± 8	1.0 ± 0.2	80
PLBS293	WT *trpL rho*::*neo*	*trpED'-'lacZ*	No	210 ± 14	14 ± 1	15
PLBS291	WT *trpL rho*::*neo*	*trpED'-'lacZ*	Yes	27 ± 3	12 ± 2	2
PLBS294	WT *trpL rho*::*neo sup-3*	*trpED'-'lacZ*	No	316 ± 20	21 ± 2	15
PLBS292	WT *trpL rho*::*neo sup-3*	*trpED'-'lacZ*	Yes	114 ± 10	14 ± 2	8

[a] WT *trp* leader (WT *trpL*). UAA stop codons are recognized by the tRNA suppressor encoded by the *sup-3* allele. *rho* is disrupted by a *neo* insertion.
[b] Presence (Yes) or absence (No) of the engineered stop codon within the coding sequence of *trpE*.
[c] β-Galactosidase (β-Gal) activity expressed from the *trpE'-'lacZ* and the *trpED'-'lacZ* fusions is given in Miller units (21). Cells were grown in the absence (−Trp) or presence (+Trp) of tryptophan (200 μM). The values are averages from at least four independent experiments ± standard deviation.
[d] NA, not applicable.

translational coupling was retained. We observed a sevenfold reduction in expression from the *trpED'-'lacZ* fusion when the stop codon was introduced into the *trpE* coding sequence (compare expression levels from PLBS285 and PLBS283 [Table 3]), while expression was restored twofold when the tRNA suppressor (*sup-3*) was present (compare expression levels from PLBS283 and PLBS284 [Table 3]). As was observed for the *mtrB*$^+$ strains, the *sup-3* allele had little affect on expression when the engineered stop codon was not present (compare expression levels from PLBS285 and PLBS286 [Table 3]).

The *trpE* SD blocking hairpin regulates translation of *trpD* via translational coupling. Since *trpE* and *trpD* are translationally coupled, it was possible that formation of the *trpE* SD blocking hairpin would also regulate translation of *trpD*. To test this possibility, we compared expression levels from *trpED'-'lacZ* fusions containing WT *trpL* or SD *trpL trp* leaders. We found that the −Trp/+Trp ratios were similar in all four SD *trpL* strains and that the SD *trpL* reduced regulation of the *trpED'-'lacZ* fusion in response to tryptophan (compare the −Trp/+Trp β-galactosidase ratios for the last four strains in Table 3 with those of the first four strains). This reduction in regulation was primarily due to elevated expression of the SD *trpL*-containing strains when they were grown in the presence of tryptophan. As expected, we found that the translational coupling expression patterns were retained in the SD *trpL* strains. In this case, introduction of the engineered stop codon reduced expression eight- to ninefold (compare expression levels from PLBS146 and PLBS144 [Table 3]), while expression was restored two- to threefold when the *sup-3* allele was present (compare expression levels from PLBS144 and PLBS145 [Table 3]). Again, the tRNA suppressor (*sup-3*) had little effect on expression when the UAA stop codon was not present (compare expression levels from PLBS146 and PLBS147 [Table 3]). Taken together, these results demonstrate that the *trpE* SD blocking hairpin regulates TrpD synthesis via translational coupling of *trpE* and *trpD*.

Rho termination factor influences expression of the *trp* operon. Polarity in operons can be caused by Rho-dependent termination within transcripts that are not being translated (reference 16 and references therein). Thus, inhibition of *trpE* translation caused by the SD blocking hairpin could result in Rho-dependent transcriptional polarity. Since we determined that *trpE* translational control only occurred at relatively high concentrations of tryptophan (see above), we expected Rho to have its greatest effect when cells were grown in the presence of excess tryptophan. To test this possibility, we engineered a Rho-deficient strain by disrupting *rho* with a kanamycin resistance gene. As was previously reported (16, 26), Rho is not essential for the viability of *B. subtilis*.

We first compared expression from a *trpE'-'lacZ* fusion in WT (PLBS127) and *rho* mutant (PLBS289) strains when the cells were grown in the presence or absence of tryptophan. While expression was only slightly higher in the *rho* mutant when grown in the absence of tryptophan, we observed threefold-higher expression when the cells were grown in the presence of excess tryptophan (Table 4). We then examined the effect of *rho* on expression from the *trpED'-'lacZ* fusion. The *rho* mutation caused only a slight increase in expression when each strain was grown in the absence of tryptophan (compare expression levels from PLBS142 with those from PLBS293, those from PLBS138 with those from PLBS291, those from PLBS143 with those from PLBS294, and those from PLBS139 with those from PLBS292 [Table 4]). Note that translational coupling was retained in the *rho* strains when they were grown in the absence of tryptophan. The expression pattern was dramatically different when the same strains were grown in the presence of excess tryptophan. In this case, the expression levels of the four *rho* mutant strains were similar and did not exhibit the translational coupling pattern. Moreover, expression in all four strains was 7- to 24-fold higher than that observed for their respective isogenic WT strains (Table 4).

To ensure that expression from the translational fusions was indicative of the effect that Rho had on expression from the natural *trp* operon, we examined *trpE* and *trpD* transcript levels in WT and *rho* strains. We found that both *trpE* and *trpD* transcript levels were substantially higher in *rho* mutants. This was particularly evident when each strain was grown in the presence of excess tryptophan (Table 5). The finding that *rho* null strains had elevated *trp* transcript levels is consistent with the translational fusion studies (Table 4). Taken together, our results establish that Rho causes transcriptional polarity of the *trp* operon under conditions that promote translational control (tryptophan excess).

TABLE 5. Effect of Rho on *trpE* and *trpD* transcript levels

Strain	Relevant genotype	Tryptophan (μM)	mRNA level (%)[a]	
			trpE	*trpD*
168	*trpC2*	20	51 ± 6	53 ± 6
168	*trpC2*	200	5.0 ± 0.3	4.9 ± 0.4
PLBS322	*rho*::*neo trpC2*	20	100	100
PLBS322	*rho*::*neo trpC2*	200	43 ± 2	37 ± 7
CB312	WT	0	14 ± 1	19 ± 5
CB312	WT	200	5.0 ± 1.0	3.4 ± 0.6
PLBS321	*rho*::*neo*	0	100	100
PLBS321	*rho*::*neo*	200	48 ± 1	44 ± 4

[a] The highest level of both *trpE* and *trpD* mRNAs was present in *rho* mutant strains grown under tryptophan-limiting conditions and was set at 100%. The values are averages of two independent experiments ± standard deviation.

DISCUSSION

Expression of the tryptophan biosynthetic operon (*trpEDCFBA*) is regulated by TRAP-mediated transcription attenuation and translational control mechanisms. In the transcription attenuation mechanism, only transient TRAP interaction with the (G/U)AG repeats would be necessary to block formation of the antiterminator. However, for translational control it is probably necessary for TRAP to remain associated with the triplet repeats to maintain formation of the *trpE* SD blocking hairpin. Although the intracellular concentration of free tryptophan was not determined in this study, the results of our expression experiments indicate that the translational control mechanism requires a tryptophan concentration higher than that required for attenuation (Fig. 3 and Table 2). It was recently shown that *yhaG* encodes a putative transmembrane protein involved in tryptophan transport and that TRAP regulates translation of this gene (27). Since *B. subtilis* is a soil bacterium, it is reasonable to assume that the organism would encounter environments in which the amounts of available tryptophan differ. Thus, the ability of *B. subtilis* to utilize different regulatory mechanisms to modulate *trp* operon expression in response to changes in the environmental supply of tryptophan may provide a growth advantage.

It is generally assumed that inhibition of translation can lead to a decrease in mRNA half-life by increasing the susceptibility of the message to nucleolytic attack. While this has been substantiated in several instances, there are examples where it has been shown that inhibition of translation did not alter the mRNA half-life or even had a stabilizing affect on the message (18). In this study, we found that inhibition of translation by the *trpE* SD blocking hairpin did not alter the stability of the downstream mRNA (Fig. 4).

The importance of translational coupling in maintaining stoichiometric production of biosynthetic pathway enzymes and ribosomal proteins is well established in *E. coli* (19, 24). In the majority of cases, the translation termination codon of the upstream cistron overlaps the initiation codon of the downstream cistron. In the case of the *B. subtilis trpEDCFBA* operon, the lengths of the overlaps are far more extensive (8 to 29 nucleotides) (Fig. 2). Our expression studies establish that *trpE* and *trpD* constitute a translationally coupled gene pair (Table 3). In addition, since we still observed substantial translation of *trpD* when we uncoupled translation by introducing the stop codon in *trpE*, our results indicate that TrpD synthesis is not dependent on translational coupling. Moreover, our coupling studies demonstrated that the *trpE* translational control mechanism, relying on TRAP-dependent formation of the *trpE* SD blocking hairpin, regulates TrpD synthesis via translational coupling (Table 3). However, since we observed the same general pattern of translational coupling in TRAP-deficient (Δ*mtrB*) strains, our results indicate that the coupling mechanism itself is independent of TRAP (Table 3).

Translational coupling is generally thought to occur by one of two mechanisms (19, 24). In one case, the translating ribosome disrupts an RNA hairpin that can sequester the SD sequence of the downstream cistron. As a consequence, a second ribosome can gain access to the now single-stranded SD sequence and initiate translation. Since our computer predictions have not detected any evidence for a *trpD* SD blocking hairpin, we do not believe that this mechanism is involved in our case. In other instances, it is thought that the same ribosome that translates the upstream cistron can initiate translation of the downstream cistron because the respective stop and start codons overlap or are close to one another. While it is possible that coupling of *trpE* and *trpD* relies on this type of mechanism, it is difficult to envision how such an extensive overlap would be accommodated.

With the exception of *trpC* and *trpF*, all of the genes in the *B. subtilis trp* operon overlap by several nucleotides (Fig. 2). In the case of these two genes, the intercistronic region is only 4 nucleotides. Since it is known that genes with short intercistronic gaps can be coupled (19), it is possible that the entire *B. subtilis trpEDCFBA* operon is translationally coupled. Thus, it is conceivable that formation of the *trpE* SD blocking hairpin regulates expression of the entire operon via translational coupling. This possibility remains to be tested. Since overlapping genes are particularly common in *B. subtilis*, it is likely that translational coupling plays a significant role in the coordinate regulation of several genes in this organism (12).

Transcriptional polarity can occur when Rho is allowed access to a nascent transcript when translation is inhibited. Our results establish that formation of the *trpE* SD blocking hairpin leads to transcriptional polarity of the *trp* operon (Tables 4 and 5). Thus, it appears that Rho binds downstream from this RNA structure. It was previously shown that *rho* is a nonessential gene in *B. subtilis* (16, 26), in contrast to *E. coli*, *Rhodobacter sphaeroides* (13), and *Micrococcus luteus* (23), in which it is an essential gene. Interestingly, it was determined that *B. subtilis* Rho regulates expression of its own gene (16). To the best of our knowledge, this is the only other known example of Rho-dependent regulation in *B. subtilis*.

When cells are growing under conditions of tryptophan excess, TRAP would be activated and most likely bind to the message as it is being synthesized. In most cases this would promote Rho-independent (intrinsic) termination in the *trp* leader (transcription attenuation). However, in some instances, RNA polymerase will escape termination despite TRAP binding, since transcription termination is never 100% efficient. In other instances, TRAP might bind prior to transcription of the *trpE* SD sequence but not in time to promote termination. Both of these scenarios would result in a nascent TRAP-bound readthrough transcript that would contain the *trpE* SD sequence in the SD blocking hairpin. Rho could then bind to the

nascent transcript and cause transcriptional polarity by promoting premature transcript release from RNA polymerase.

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