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Biochemistry

Characterization of the *Bacillus subtilis* tryptophan promoter region

(*trp* regulatory region/gene regulation/5-methyltryptophan resistance)

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ABSTRACT **The nucleotide sequence of the control region of the *trp* operon of *Bacillus subtilis* has been determined. The region was shown to contain the *trp* promoter by deletion analysis and by determination of the transcription start site. The *trp* promoter shows similarity to the consensus sequence for *Escherichia coli* and *B. subtilis* promoters. The presence of the *trp* control region on a high-copy-number plasmid confers resistance to the tryptophan analogue 5-methyltryptophan. It appears that an approximately 120-base-pair region comprising not only the *trp* promoter but also adjacent direct repeat sequences is necessary to confer 5-methyltryptophan resistance. We postulate that this region is involved in tryptophan regulation and confers 5-methyltryptophan resistance by titration of a *trp* regulatory protein. Removal of either the *trp* promoter or the adjacent direct repeat sequences abolished the 5-methyltryptophan-resistance phenotype. Placement of unrelated promoters adjacent to the direct repeat sequences restored 5-methyltryptophan resistance. This suggests that promoter activity is necessary for the regulatory function.**

The tryptophan (*trp*) operon of *Escherichia coli* has been a model system to investigate operon structure, gene function, and regulation of gene expression (1, 2). From such studies a detailed understanding of the regulation of the *trp* operon has emerged. The regulatory region of the *trp* operon consists of a promoter region that contains an operator that binds a tryptophan-activated repressor and regulates transcription (3, 4). Following the promoter region lies an additional regulatory element, the attenuator, that contains a site for regulated transcriptional termination (5). The tryptophan metabolism of *Bacillus subtilis* has been extensively studied (6) and the genes for the enzymes of the tryptophan pathway, *trpEDCFBA*, have been localized to a single area of the genetic map (7). However, for *B. subtilis*, or other Gram-positive bacteria, no transcriptional regulation system using repressor–operator interaction or attenuator–transcription termination control analogous to that found in *E. coli* has been described. Because *B. subtilis* has been the most studied Gram-positive organism, we felt that it would be of interest to determine whether such a transcriptional regulation system exists in the *trp* operon of *B. subtilis* and to compare its mechanisms of gene regulation with the corresponding mechanisms of *E. coli*. Recently, we have cloned several overlapping portions of the *trp* operon of *B. subtilis* into plasmid pBR322. The nucleotide sequence of the entire *trp* operon has been determined (ref. 8; unpublished data). In this paper, we report the nucleotide sequence of the control region of the *trp* operon of *B. subtilis*. The promoter was identified and the transcriptional start site was determined. In addition, we have identified a region that, when placed on a high-copy-number plasmid, confers resistance to the tryptophan analogue 5-methyltryptophan (5-MeTrp). We postulate that this region is involved in tryptophan regulation and confers 5-MeTrp resistance by titration of a *trp* regulatory protein.

MATERIALS AND METHODS

Strains and Plasmids. *E. coli* strain MM294 (F⁻ *supE44 endA1 thi-1 hsdR4*) was used as a host for plasmid constructions (9). *B. subtilis* strains UOTO277 (*hisA1 metB5 recE4 nonB1*) and 1A72 (*mtr-264*) were obtained from Hiuga Saito and the Bacillus Genetic Stock Center (Ohio State University), respectively. Plasmids pJH101-trpE2 (8, 10), pSPIFV (11), and pUC18 (12) were used for constructions of the derivative plasmids described here.

Plasmid Constructions. The procedures used for isolation of plasmid DNA, cleavage of restriction fragments, isolation of DNA fragments, ligation with T4 ligase, and transformation of *E. coli* and *B. subtilis* were carried out as described (13). BAL-31 exonuclease digestion was as described by Poncz *et al.* (14).

Assays. Cultures to be assayed for interferon expression were grown in minimal-glucose medium (15) containing chloramphenicol at 5 μg/ml in the presence or absence of tryptophan at 20 μg/ml. The cells were harvested by centrifugation, lysed by treatment with lysozyme and detergent, and assayed for interferon activity using a bioassay as described (11, 16).

Other Methods. DNA sequencing was conducted by the dideoxy chain-termination method of Sanger *et al.* (17). Specific restriction fragments were cloned into the M13mp8 or M13mp9 vectors for dideoxy sequencing (18). 5-MeTrp resistance was examined by streaking strains on plates containing the tryptophan analogue 5-fluorotryptophan as described by Hoch *et al.* (19). Preparation of RNA from *B. subtilis* 1A72 and nuclease S1 mapping experiments were carried out according to the method of Aiba *et al.* (20).

RESULTS

Nucleotide Sequence of the *trp* Operon Promoter Region. The promoter region of the *trp* operon was cloned as described (8) by using "plasmid rescue" of a plasmid that had been integrated into the chromosome of *B. subtilis*. The resulting plasmid, pJH101-trpE2, contained a 730-base-pair (bp) fragment that encoded the amino-terminal portion of the *trpE* gene and 615 bp of the 5′ flanking region. The entire nucleotide sequence of this 730-bp fragment was determined and the portion relevant to this study is shown in Fig. 1. The *trpE* structural gene sequence, including the position of the *trpE* initiation codon, is reported elsewhere (8).

Use of the *trp* Promoter to Express Leukocyte Interferon A in *B. subtilis*. To determine whether the 730-bp fragment derived from the 5′ flanking region of the *B. subtilis trp* operon is a functional promoter in *B. subtilis*, the fragment was placed on the 5′ side of the synthetic Shine–Dalgarno sequence of the leukocyte interferon A gene as constructed in plasmid pSPIFV (11). In this construction, designated ptrpIFI, the interferon gene follows a truncated *trpE* gene

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Abbreviations: 5-MeTrp, 5-methyltryptophan; bp, base pair(s).

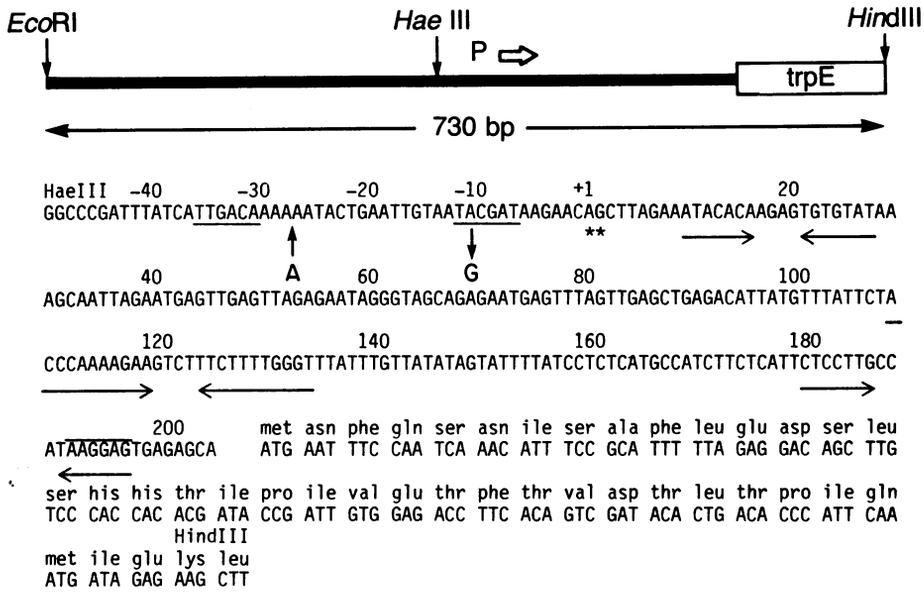

FIG. 1. Schematic DNA sequence of the *trp* control region. The *trpE* coding region is boxed and the *trp* promoter position is indicated by "P." The sequence shown is from the *Hae* III site to the *Hin*dIII of the 730-bp fragment in pJH101-*trpE2*, and it is numbered relative to the mRNA start site indicated by asterisks. Sequences constituting the −10 and −35 regions of the *trp* promoter are underlined. The arrow pointing up indicates the insertion of an adenosine; the arrow pointing down indicates the replacement of adenosine by guanosine. The possible Shine–Dalgarno sequence of the *trpE* gene is overlined. The deduced amino acid sequence of the *trpE* gene is also shown. Sequences exhibiting dyad symmetry are indicated by arrows.

(Fig. 2). This plasmid was transformed into *B. subtilis* strain UOTO277, and the transformant was assayed for interferon production after growth in the presence or absence of tryptophan. The results showed the production of 11,000 units per ml per OD_{550nm} unit in cultures grown in the presence of tryptophan and 23,000 units per ml per OD_{550nm} unit in cultures grown in the absence of tryptophan, indicating that a two-fold induction of interferon can be obtained by tryptophan limitation. A control plasmid, pIFI, lacking the 730-bp fragment but containing the interferon gene showed no detectable interferon activity. These results indicate that the 730-bp fragment is able to function as a promoter and that it responds to tryptophan limitation as expected for a promoter of the *trp* operon.

Localization of the *trp* Promoter. To determine the location of the *trp* promoter, a series of plasmids with various deletions within the *trp* promoter fragment was constructed. First, the sequence between the *Eco*RI and *Hae* III sites of ptrpIFI was deleted. This plasmid is designated ptrpIFId1 (Fig. 2). Deletion of this sequence in ptrpIFId1 had no detectable effect on expression of interferon. This indicates that the *trp* promoter is located between the *Hae* III and *Hin*dIII sites of ptrpIFI (Fig. 2).

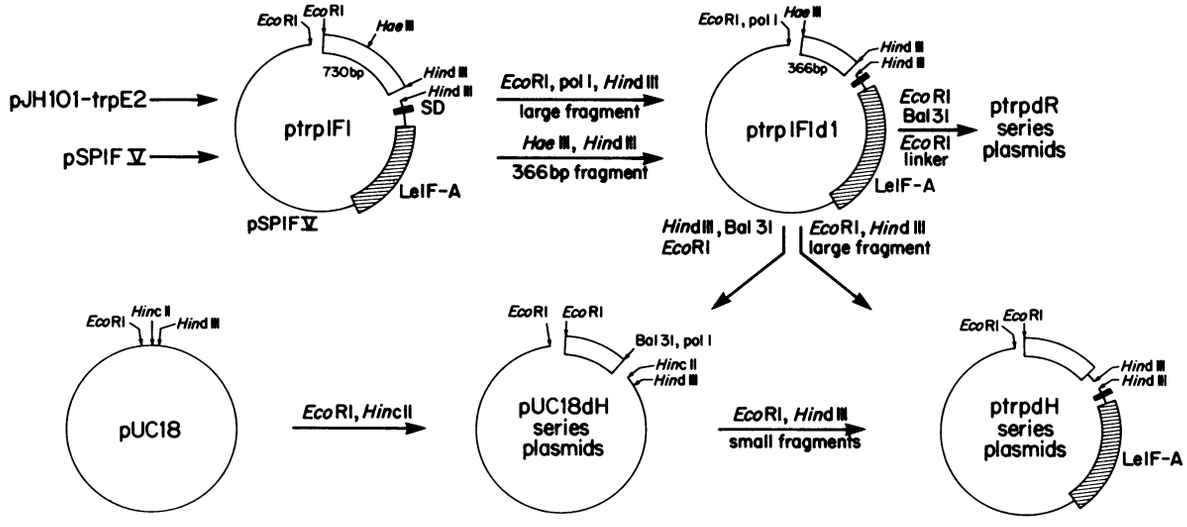

FIG. 2. Construction of ptrpIFI, ptrpIFId1, ptrpdR, and ptrpdH. ptrpIFI was constructed by ligating two fragments, one containing a synthetic Shine–Dalgarno sequence, the leukocyte interferon A gene, the pBR322 and pUB110 origins of replication, and the pC194 chloramphenicol acetyltransferase gene from pSPIFV (11) and a second containing the 730-bp *Eco*RI/*Hin*dIII fragment from pJH101-trpE2 (8). In this and following constructions, the *trpE* gene is not in the same reading frame as the interferon gene. ptrpIFId1 was constructed by ligating the blunt-ended *Eco*RI site to the *Hae* III site of ptrpIFI in the *trp* control region, reconstructing the *Eco*RI site for subsequent constructions. A series of deletion plasmids, ptrpdR, was constructed as follows. ptrpIFd1 was cleaved with *Eco*RI, digested with BAL-31 (0.5 unit, 25°C, 1–2 min), treated with the Klenow fragment of DNA polymerase I, and ligated with synthetic *Eco*RI linker. Excess polylinker was removed by digestion with *Eco*RI, and this was followed by self-ligation. The ptrpdH series of deletion plasmids was constructed as follows. ptrpIFId1 was cleaved with *Hin*dIII, digested with BAL-31 (0.5 unit, 25°C, 2–7 min), treated with the Klenow fragment of DNA polymerase I, cleaved with *Eco*RI, and ligated with the pUC18 vector, which had been digested with *Eco*RI/*Hin*dIII. The resulting plasmids, pUC18dH, were screened and the plasmids containing appropriate sizes of the insert were cleaved with *Eco*RI/*Hin*dIII and ligated to the vector portion of ptrpIFId1. The end points of all the deletions were determined by sequencing.

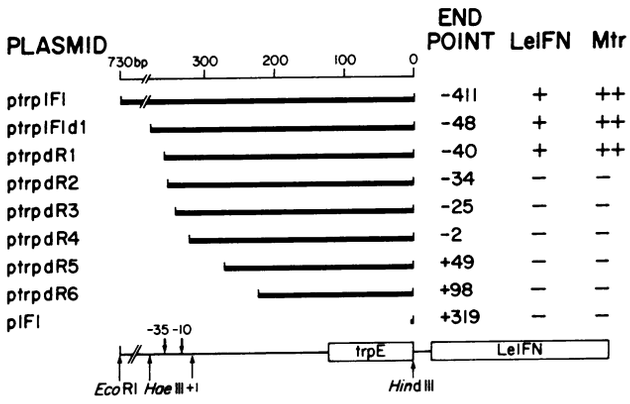

FIG. 3. Production of leukocyte interferon (LeIFN) and 5-MeTrp resistance of *B. subtilis* strains carrying various deletion plasmids. The top line is a scale in nucleotides. The bottom line represents the 730-bp fragment of the *trp* control region preceding the leukocyte interferon A gene. The heavy lines indicate remaining DNA. The locations of the ends of the deletions (end points) are shown relative to the mRNA start site at the +1 position. All of the deletions have an *Eco*RI linker at the site of the deletion. To eliminate the possibility that the deletion of the vector portion influences the property of the deletion plasmid, the 351-bp fragment of the *trp* control region in ptrpdR2 was recloned in the vector portion of ptrpIFI. This plasmid (ptrpdR2'; not shown in the figure) showed the same properties as the parental plasmid, ptrpdR2. 5-MeTrp resistance (Mtr) is indicated as follows: ++, growth after 24 hr; +, growth after 48 hr; −, no growth.

Next, a series of BAL-31 exonuclease deletions was constructed downstream from the *Hae* III site (Fig. 2). After transformation of these deletion plasmids, designated ptrpdR1–ptrpdR6, into *B. subtilis* strain UOTO277, transformants were assayed for interferon production. The extent of DNA removed in each of the deletion plasmids and the production of interferon in the *B. subtilis* strains carrying these deletion plasmids is shown in Fig. 3. The smallest insert upstream of the *trp* operon that retained interferon expression is carried by ptrpdR1. The largest insert that lost interferon expression is carried by ptrpdR2. Comparison of the DNA sequences of these two deletion plasmids revealed that ptrpdR1, which retains interferon expression, contains a consensus sequence similar to the "−35" region of prokaryotic promoters, T-T-G-A-C-A; however, in ptrpdR2, the first and second thymidines of this sequence have been deleted (Fig. 3). Also present was a "−10" region sequence T-A-C-G-A-T that shares four of six bases with the −10 consensus sequence (T-A-T-A-A-T) (21). To identify the transcription initiation site, a nuclease S1 mapping experiment was carried out (data not shown). The adenosine or guanosine residues located at positions +1 and +2 (Fig. 1) were identified as the transcription start sites. This result confirms the assignment of the promoter identified by the BAL-31 deletions.

Localization of a *trp* Regulatory Sequence Conferring 5-MeTrp Resistance. In *E. coli*, the tryptophan analogue 5-MeTrp inhibits bacterial growth by mimicking the action of tryptophan (22). This analogue has been reported to act as both a false feedback inhibitor (22, 23) and a false co-repressor in *E. coli* (24). Mutations in the *trp* repressor or operator in *E. coli* that constitutively synthesize the trp enzymes are resistant to 5-MeTrp (25, 26). We found that the *B. subtilis* strain carrying ptrpIFI is resistant to 5-MeTrp. To determine which portion of the plasmid confers the 5-MeTrp resistance, *B. subtilis* UOTO277 strains carrying a series of deletion plasmids were examined for 5-MeTrp resistance. The results indicated that *B. subtilis* strains carrying ptrpdR1 or plasmids whose inserts are larger than ptrpdR1 are resistant to 5-MeTrp (Fig. 3). However, *B. subtilis* strains carrying plasmids whose inserts are smaller than ptrpdR1 are sensitive to 5-MeTrp. Plasmids ptrpdR1 and ptrpdR2 define the left edge of the 5-MeTrp resistance. To define the right edge of the region necessary for conferring 5-MeTrp resistance on the cell, another series of BAL-31 exonuclease deletions was constructed from the *Hin*dIII site of ptrpIFId1. The construction of such deletion plasmids, designated ptrpdH1–ptrpdH8, is shown in Fig. 2. These plasmids were transformed into *B. subtilis* UOTO277, and transformants were examined for their 5-MeTrp resistance. The extent of DNA removed on the deletion plasmids and the sensitivity or resistance of the *B. subtilis* strains carrying these plasmids to 5-MeTrp are shown in Fig. 4*A*. The results indicated that *B. subtilis* strains carrying ptrpdH6 or plasmids whose inserts are smaller than ptrpdH6 are sensitive to 5-MeTrp. Transformants carrying deletion plasmids whose inserts are larger than ptrpdH4 were resistant to 5-MeTrp. The strain carrying ptrpdH5 was partially resistant to 5-MeTrp. A comparison of the DNA sequences of the deletion plasmids is shown in Fig. 4*B*. Several direct repeat sequences were identified in the DNA region that ptrpdH4 contained and that ptrpdH6 did not. The sequences T-G-A-G-T-T and A-G-A-G-A-A-T were found repeated three and two times, respectively (Fig. 4). The sequence A-G-A-A-T-G-A-G-T-T was also repeated. It is interesting to note that ptrpdH5, which was partially resistant to 5-MeTrp (Fig. 4), lost one of these direct-repeat sequences. To determine whether these direct-repeat sequences play a role in 5-MeTrp resistance, a deletion plasmid lacking only the direct-repeat region was constructed (Fig. 4). The *B. subtilis* cells carrying this plasmid (ptrpdH7-dR6) were sensitive to 5-MeTrp. This suggests that these direct-repeat sequences have a direct relationship to 5-MeTrp resistance.

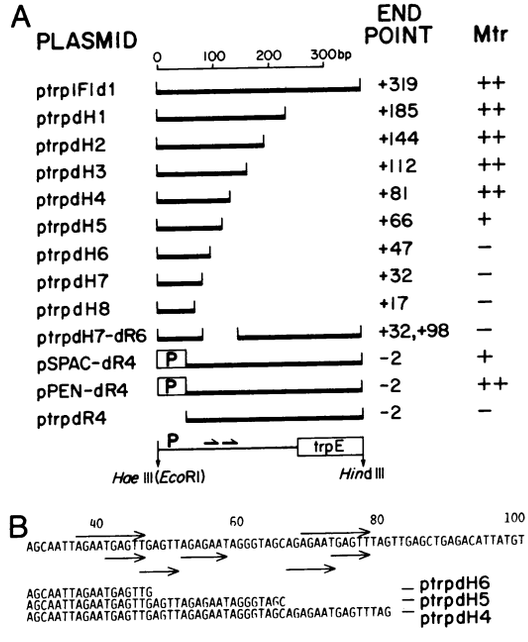

FIG. 4. (*A*) 5-MeTrp resistance of *B. subtilis* strains harboring deletion plasmids and plasmids in which the *trp* control region has been replaced by other promoters. The heavy lines indicate remaining DNA. The locations of the ends of the deletions (end points) are shown relative to the mRNA start site at the +1 position. All the ptrpdH series plasmids have a *Hin*dIII site derived from plasmid pUC18 at the site of deletion. 5-MeTrp resistance (Mtr) is as indicated in Fig. 3. (*B*) Sequences of the *trp* control region and deletion plasmids. Direct-repeat sequences are indicated by arrows.

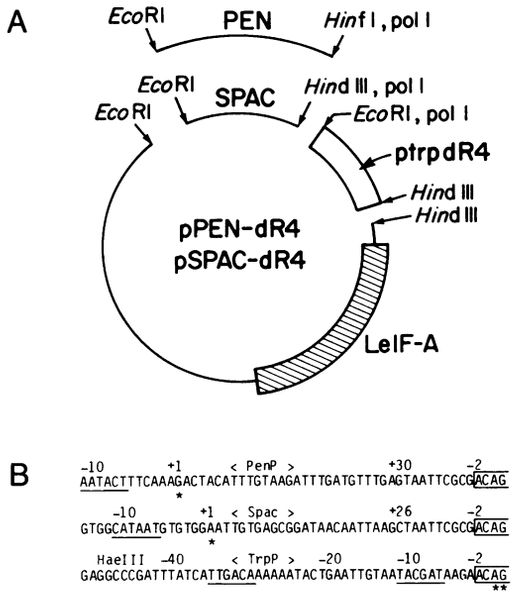

FIG. 5. Construction of pSPAC-dR4 and pPEN-dR4. (*A*) pSPAC-dR4 was constructed by ligating the *Hin*dIII site of the *spac* promoter to the *Eco*RI site of ptrpdR4. Before the ligation, both sites were made flush by treatment with the Klenow fragment of DNA polymerase I. pPEN-dR4 was constructed in a similar way. (*B*) Sequences of the junction region between the promoters and the *trp* control region in pSPAC-dR4 and pPEN-dR4.

5-MeTrp Resistance Requires an Active Promoter. It is interesting that the loss of 5-MeTrp resistance of the ptrpdR series of plasmids appeared to be associated with *trp* promoter activity (Fig. 3). This result is also supported by the finding that two promoter mutations abolished the 5-MeTrp-resistance phenotype. These promoter mutations (a base change in the −10 region and a one-base addition in the spacer region between the −10 and −35 regions, as shown in Fig. 1) were identified during sequencing of the *trp* promoter region. M13 phages with the *trp* promoter oriented opposite to the β-galactosidase gene were extremely unstable and generated mutations in the promoter region with a high frequency. The *B. subtilis* strains carrying plasmids that contained these mutations abolished not only promoter activity but also resistance to 5-MeTrp (data not shown). To determine whether the loss of 5-MeTrp resistance is due to the loss of promoter activity or to the removal of some specific sequences, the *trp* promoter was replaced with other promoters. Two *Bacillus* promoters, a *spac* promoter (16) and a *penP* promoter (27), were placed on the 5′ side of the deletion plasmid ptrpdR4 (Figs. 4 and 5). Although the strain carrying ptrpdR4 was sensitive to 5-MeTrp, the strains carrying pSPAC-dR4, which contained the *spac* promoter, or pPEN-dR4, which contained the *penP* promoter, became resistant to 5-MeTrp and expressed interferon (Fig. 4). These results suggest that promoter activity is necessary for conferring 5-MeTrp resistance.

DISCUSSION

The nucleotide sequences of at least 18 promoters that are utilized by *B. subtilis* σ55 RNA polymerase have been reported (28–30). All of these promoters display striking conformity in their −35 and −10 regions with the corresponding consensus sequences (T-T-G-A-C-A and T-A-T-A-A-T, respectively) for *E. coli* promoters (21). The distance between the −35 and −10 regions (17 or 18 bp) is also very well conserved (28). The *trp* promoter shows similarity to these other described promoters, with a −35 region sequence (T-T-G-A-C-A) and a −10 region sequence (T-A-C-G-A-T) separated by a spacing of 18 bp (Fig. 1). Deletion analysis and nuclease S1 mapping confirmed that this region was actually used as a promoter *in vivo*.

A human leukocyte interferon gene, placed under the control of the *trp* promoter on a high-copy-number plasmid, allowed the expression of interferon in *B. subtilis*. However, the expression was only minimally regulated by tryptophan, with an increase of only 2-fold in the level of interferon in the absence of tryptophan. This derepression level of the interferon is much lower than that of the chromosomal *trp* enzymes, which is approximately 20-fold (19). This lower depression ratio led us to suspect that the amount of *trp* regulatory protein might be limited and that a regulatory sequence present on the plasmid might titrate all the available regulatory protein molecules. If such was the case, we reasoned that the presence of the *trp* regulatory sequence on a high-copy-number plasmid might also derepress the chromosomal *trp* operon.

An indirect way to test for derepression of the *trp* operon is to measure resistance to the tryptophan analogue 5-MeTrp. In *E. coli*, mutations of either the *trp* repressor gene or the *trp* operator region can lead to derepression of the *trp* operon and 5-MeTrp resistance (25, 26). A 5-MeTrp-resistance locus has been reported in *B. subtilis* and mapped between *ser-1* and *aroF* in the chromosome (31). Since mutations of this locus, designated *mtr*, result in constitutive synthesis of the *trp* enzyme, the *mtr* locus has been assumed to code for a *trp* repressor protein or negative regulatory protein (31). As reported above, the presence of the *trp* control region on a high-copy-number plasmid confers 5-MeTrp resistance on the cells. We postulate that ptrpIFI contains a *trp* regulatory region and confers 5-MeTrp resistance by titration of a *trp* regulatory protein.

We found that the *trp* regulatory region consists of an ≈120-bp region comprising the *trp* promoter and adjacent direct-repeat sequences. Removal of the direct-repeat sequences abolished the 5-MeTrp phenotype. Thus we presume that these direct-repeat sequences are important to binding of a regulatory protein. However, the involvement of the *trp* promoter region in conferring 5-MeTrp resistance was unexpected. This involvement was shown by the ptrpdR series of deletion mutants, which coordinately abolished both promoter activity and 5-MeTrp resistance, and by single-base changes or insertions in the promoter, which also abolished both promoter activity and 5-MeTrp resistance. The ability to replace the *trp* promoter with unrelated promoters and restore both 5-MeTrp resistance and interferon expression strongly suggests that a functional promoter is necessary for the 5-MeTrp resistance.

It is possible that the *trp* regulatory protein might bind to the regulatory region only in the presence of RNA polymerase. However, the direct interaction between an RNA polymerase and a regulatory protein, binding cooperatively to the promoter and direct repeats, is unlikely because the distances between the promoter and direct repeats were very different for the natural *trp* promoter and each of the two different substituted promoters (Fig. 5). A model in which the regulatory protein interacts with the nascent transcript to cause regulated termination seems more probable. The regulatory protein might interact with the transcription complex in a similar manner to the N protein in bacteriophage λ (32). In this case, the direct repeats would be analogous to the *nut* site, which is an N-utilization or -recognition site (33). However, in this case the regulatory protein would aid in termination rather than in antitermination. A likely site for termination is the strong hairpin structure (nucleotides 108–133, Fig. 1) located downstream of the direct repeats. Alternatively, the regulatory protein might bind to the RNA transcript and favor the formation of a termination structure.

These models are highly speculative; however, they lead to testable hypotheses for directing future studies. We feel it is unlikely that termination is modulated translationally, as is the case for attenuation of the *trp* promoter in *E. coli*. Two potential leader peptides precede the *trpE* structural gene (ATGs at nucleotides 72–74 and 97–99, Fig. 1). However, neither of these are preceded by the stringent Shine–Dalgarno region usually found preceding Gram-positive translation initiation sites (34) nor are either rich in *trp* codons.

The *trpE* structural gene is preceded by a Shine–Dalgarno sequence "A-A-G-G-A-G," which appears to be involved in a stem–loop structure (Fig. 1). There are several cases in Gram-positive bacteria in which the Shine–Dalgarno region is within a stem–loop structure and that appear to be translationally regulated (35, 36). The best studied is the erythromycin-resistance gene of plasmid pE194, in which ribosome stalling appears to modulate the mRNA structure, leading to translational regulation (35). Further analysis of this region in the *trp* operon should allow us to determine whether this secondary RNA structure could function to control *trp* gene expression in *B. subtilis*, although it appears that any translational control that might be present must have a different mechanism than that found in *E. coli*.

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