JOURNAL OF BACTERIOLOGY, Sept. 1986, p. 792–798
0021-9193/86/090792-07$02.00/0

Vol. 167, No. 3

Regulatory Elements Common to the *Bacillus pumilus* and *Bacillus subtilis trp* operons

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Received 31 March 1986/Accepted 20 May 1986

The *trp* operon regulatory region of *Bacillus pumilus* was cloned and sequenced. The cloned *B. pumilus trp* promoter-leader region functioned in *Bacillus subtilis* to express the adjacent leukocyte interferon A gene on a multicopy transcriptional fusion plasmid, pBpIFI. In strains carrying this plasmid, anthranilate synthetase levels were elevated, possibly due to titration of a *B. subtilis trp* regulatory factor by multiple copies of the transcript of the plasmid-borne *B. pumilus trp* leader region. The *B. pumilus trp* promoter was recognized efficiently in vitro by *B. subtilis* σ^{43} RNA polymerase. Approximately 12% of the transcripts produced in vitro terminated in the leader region immediately following synthesis of a transcript structure resembling rho-independent terminators of enteric bacteria. An analogous terminator exists in the *B. subtilis trp* leader transcript. Nucleotide sequence comparison of the *B. pumilus* and *B. subtilis trp* leader regions revealed conservation of these and other sequences that could form transcript secondary structures postulated to regulate transcription termination in *B. subtilis* (H. Shimotsu, M. I. Kuroda, C. Yanofsky, and D. J. Henner, J. Bacteriol. 166:461–471, 1986). We propose that two elements implicated in *B. subtilis trp* operon regulation are conserved in the related organism *B. pumilus*: (i) alternative transcription antiterminator and terminator structures in the leader transcript, and (ii) a *trans*-acting factor present in limiting amounts that is required for transcription termination in the leader region.

We recently proposed a model in which a novel form of attenuation regulates transcription of the *Bacillus subtilis trp* operon (21). Our model is based on the following observations (20, 21). First, transcription initiation occurs at the *trp* promoter both in the presence and in the absence of exogenous tryptophan. Second, a palindrome in the transcribed leader region functions as a site of transcription termination both in vivo and in a purified in vitro transcription system. Third, deletion of sequences of the leader transcript that could form an upstream competing RNA structure, or antiterminator, results in loss of expression of the structural genes of the operon in vivo in response to starvation for tryptophan. This deletion increases the efficiency of transcription termination observed in vitro from about 10 to 100%. Fourth, multiple copies of the *trp* leader transcript appear to titrate a negative-acting regulatory element that normally reduces chromosomal *trp* operon expression in the presence of excess tryptophan.

Based on these observations and the earlier work of Hoch and others (8, 10), we have proposed a model for the control of transcription termination in the leader region of the *B. subtilis trp* operon in response to the availability of tryptophan (21). We postulated that when cellular tryptophan levels are low, RNA polymerase transcribes through the *trp* operon leader region and structural genes, yielding high levels of *trp* mRNA and the *trp* biosynthetic enzymes. Presumably, the antiterminator in the leader transcript forms efficiently under these conditions. We further postulated that when cellular tryptophan levels are high, a hypothetical *trp* regulatory factor, the product of the *mtr* locus (8, 10), facilitates formation of a transcription terminator during synthesis of *trp* leader RNA. The hypothetical factor presumably binds to a segment of leader RNA that would otherwise form the competing antitermination structure. Thus, the *trp* operon structural genes would not be transcribed when the intracellular concentration of tryptophan is high.

In this paper we ask whether features and properties of the *B. subtilis trp* leader region that support the above transcription attenuation model are conserved in the related species *Bacillus pumilus*. The *B. pumilus trp* operon is organized much like that of *B. subtilis*, and exogenous tryptophan appears to modulate its expression (9). We characterized the *B. pumilus trp* leader region and found that there are sequences specifying a transcription terminator and a potential antiterminator. We also showed that overproduction of the *B. pumilus trp* leader transcript in *trans* appeared to titrate a *trp* regulatory factor. These findings provide evidence that expression of the *trp* operons of both bacilli are regulated by the same novel form of transcription attenuation.

MATERIALS AND METHODS

Bacterial strains and transformations. *B. pumilus* wild-type strain NRRLB 3275 (13) was obtained from Paul Lovett. *B. subtilis* UOTO277 (*hisA1 metB5 recE4 nonB1*) (20) was the host for experiments with plasmid pIFI (20) and its derivatives. *B. subtilis* 1A72 (*mtr-264*) was obtained from the *Bacillus* Genetic Stock Center. Isogenic strains BG2086 (*mtr-264 argC4*) and BG2087 (*argC4*) were derived from BG15 (*trpC2 hisH argC4 ptm*) and 1A72 by PBS1 transduction (20a). JMB9 Δ*trpLD102* (*leu thi supE gal lac xyl ara hsdR* Δ*trpLD102*) was the *Escherichia coli* strain used for *trpD* complementation. *E. coli* JM83 (14) and MM294 (2) were used for plasmid constructions and preparations. JM101 (14) was used for preparation of M13 DNA templates for DNA sequencing. *E. coli* transformations were performed by the calcium shock procedure (4). Transformants were selected on LB plates supplemented with ampicillin at

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50 μg/ml or chloramphenicol at 12.5 μg/ml. *B. subtilis* was transformed by a published procedure (1) except that cells were diluted 2-fold into stage II medium instead of 10-fold. Transformants were selected on tryptose blood agar base (Difco Laboratories) plates containing chloramphenicol (5 μg/ml). Resistance to 5-methyltryptophan (5MeTrp) was examined by streaking strains on plates containing the tryptophan analog 5-fluorotryptophan as described by Hoch (8).

***trpD* complementation.** Chromosomal DNA was prepared from *B. pumilus* NRRLB 3275 as described by Lovett and Keggins (12). Chromosomal DNA (2 μg) and 1 μg of plasmid pUC13 (16) were digested with *Hin*dIII endonuclease; vector pUC13 was subsequently treated with calf intestinal phosphatase. Digested DNAs were phenol extracted, ethanol precipitated, and incubated in 100 μl of ligation buffer containing T4 DNA ligase for 18 h at 12°C. The ligation mix was used to transform *E. coli* JMB9 Δ*trpLD102* to Amp^r, and TrpD$^+$ colonies were selected on minimal agar containing 0.2% glucose, 0.2% acid-hydrolyzed casein (lacking tryptophan), and 10 μg of anthranilate per ml. Plasmids from 12 TrpD$^+$ isolates were examined. A plasmid, pVV60, carrying a 4.4-kilobase (kb) *Hin*dIII insert was selected for further study.

DNA sequencing. Specific restriction fragments and unidirectional deletion fragments generated by exonuclease III digestion (6) were cloned into M13mp10 and M13mp11 (15). DNA sequencing was performed by the dideoxynucleotide chain termination method of Sanger et al. (19).

Leukocyte interferon A assays. *B. subtilis* cells were grown in minimal medium containing 0.5% glucose, 0.5% acid-hydrolyzed casein (Difco), methionine (50 μg/ml), histidine (50 μg/ml), chloramphenicol (5 μg/ml), and minimal salts (23) in the presence or absence of tryptophan (20 μg/ml). Cells were harvested by centrifugation, lysed by treatment with lysozyme and detergent, and assayed for interferon activity by a bioassay described previously (3, 26).

Anthranilate synthetase assays. *B. subtilis* cells were grown in minimal medium containing 0.2% glucose, 0.2% acid-hydrolyzed casein, and minimal salts (23) in the presence or absence of tryptophan (20 μg/ml). Chloramphenicol (5 μg/ml) was added for cells containing plasmid pIFI (20) and its derivatives. Cells were grown to late log phase. Equivalent amounts of culture biomass were chilled, sedimented, washed once with saline, and suspended in 0.2 M Tris chloride, pH 7.8, on ice. Samples (10 to 40 μl) were assayed for anthranilate synthetase activity as previously described (5).

In vitro transcription. *Taq*I and *Eco*RI restriction fragments containing the *B. pumilus trp* promoter-leader region were used as templates. The restriction fragments were derived from pBp1, a plasmid containing a 700-base-pair (bp) *Taq*I fragment from pVV60 ligated into the *Acc*I site of pUC12 (16). [α-^{32}P]GTP was purchased from Amersham Corp. *B. subtilis* vegetative type (σ^{43}) RNA polymerase was the generous gift of J. Helmann and M. Chamberlin. Reactions were carried out for 30 min at 37°C in 120 mM KCl–4 mM $MgCl_2$–40 mM Tris chloride (pH 8.0)–10 mM β-mercaptoethanol–4 mM spermidine–2.5% glycerol–2.7 mM ATP–1.1 mM GTP–6 μCi of [α-^{32}P]GTP–1.4 mM UTP–0.7 mM CTP–10 to 20 nM restriction fragment–0.64 μg of RNA polymerase holoenzyme. The final volume of each reaction was 10 μl. Reactions were stopped by addition of an equal volume of 0.025% xylene cyanol–0.025% bromphenol blue–0.1% sodium dodecyl sulfate (SDS)–14 M urea and electrophoresed on a 6% polyacrylamide–7 M urea gel. The relative molar amounts of transcripts produced were determined by quantitating the Cherenkov radiation from ^{32}P-labeled excised bands as described previously (24).

RNase T_1 digestion of in vitro transcription products. RNase T_1 (Sankyo) was purchased from CalBiochem. Bands containing [α-^{32}P]GTP-labeled transcripts produced in vitro were visualized by autoradiography and excised from 6% polyacrylamide–7 M urea gels. The RNA was extracted in 0.5 M ammonium acetate–10 mM magnesium acetate–0.1% SDS–0.1 mM EDTA, ethanol precipitated twice, and suspended in sterile distilled water at 1,000 to 2,000 cpm/μl. A 4-μl amount of each transcript was reprecipitated in the presence of 10 μg of carrier tRNA, suspended in 5 μl of 10 mM Tris chloride (pH 7.5)–1 mM EDTA containing 2,000 U of RNase T_1 per ml, and incubated for 20 min at 37°C. Digestion products were analyzed on 40-cm 20% polyacrylamide–7 M urea sequencing gels.

Computer analyses. The Microgenie Sequence Analysis Program (17) from Beckman Instruments, Inc., was used to align DNA sequences for optimal homology (see Fig. 2). RNA secondary structures were predicted by using a program by Zuker and Stiegler (27) through the Bionet computer resource (Intelligenetics, Inc.). Prediction of the theoretically most stable RNA structures is based on published values of stacking and destabilizing energies as compiled by Salser (18).

RESULTS

Cloning the *B. pumilus trp* operon regulatory region. Keggins et al. (11) previously isolated a 2.3-megadalton *Eco*RI fragment from *B. pumilus* DNA that complements *B. subtilis trp* auxotrophs with mutations in *trpE*, *trpD*, *trpC*, or *trpF*. It was found that this fragment would also complement *trpD* mutants of *E. coli* (M. van Cleemput, V. Vania, and C. Yanofsky, unpublished). Preliminary sequence analysis indicated that the translation initiation codon and sequences upstream of *trpE*, the presumptive first gene of the operon, were not present on this *Eco*RI fragment (data not shown). A single *Hin*dIII site exists within the *Eco*RI fragment, and insertions into this *Hin*dIII site eliminated *trpC* complementation (12). To recover *B. pumilus* genomic sequences 5′ to the *trpE* gene, we digested DNA from *B. pumilus* NRRLB 3275 with *Hin*dIII and ligated the resulting fragments in the *Hin*dIII site of vector pUC13 (16). This ligation mix was used to transform *E. coli* JMB9 Δ*trpLD102* to Amp^r and TrpD$^+$ on minimal agar containing ampicillin (50 μg/ml), 0.2% glucose, 0.2% acid-hydrolyzed casein (lacking tryptophan), and anthranilate (10 μg/ml). A plasmid, pVV60, was isolated that carried a 4.4-kb *Hin*dIII fragment and would transform JMB9 Δ*trpLD102* to TrpD$^+$. pVV60 contained approximately 1.6 kb of DNA upstream from the *trpE* coding region of the previously isolated *Eco*RI fragment (Fig. 1). A 700-bp *Taq*I fragment containing 620 bp of DNA 5′ to the *trpE* coding region was subcloned from pVV60 in the *Acc*I site of vector pUC12 (16) to generate plasmid pBp1 (Fig. 1). *B. pumilus* genomic DNA contained a homologous *Taq*I fragment of identical size by Southern analysis (22; data not shown). The 700-bp *Taq*I fragment and deletion derivatives generated by restriction endonuclease or exonuclease III digestion of pBp1 were cloned into M13mp10 or M13mp11 (15) and sequenced by the dideoxynucleotide chain termination method of Sanger (19) as diagrammed in Fig. 1.

Nucleotide sequence comparison of the *B. pumilus* and *B. subtilis trp* operon regulatory regions. The nucleotide sequence of the *B. subtilis trp* operon and 5′ regulatory region

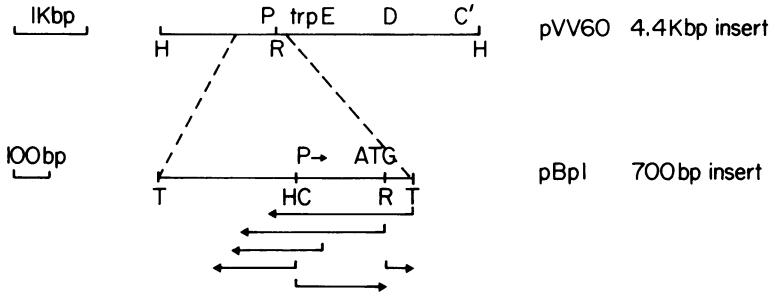

FIG. 1. Structure of the 4.4-kb *Hin*dIII insert of pVV60, and sequencing strategy for a subcloned *Taq*I fragment containing the *B. pumilus trp* promoter-leader region. The approximate positions of the promoter and first three genes of the operon are indicated above the fragments. Restriction endonuclease cleavage sites are indicated below each fragment. H, *Hin*dIII; R, *Eco*RI; T, *Taq*I; HC, *Hin*cII. Specific restriction fragments and unidirectional deletion fragments generated by exonuclease III digestion of pBp1 were cloned into M13mp10 and M13mp11 (15) and sequenced by the dideoxynucleotide chain termination method of Sanger et al. (19).

has been determined (7, 20). The transcription initiation site is 204 bp 5′ to the *trpE* coding region. Comparison of the *B. pumilus* sequence 5′ of *trpE* to the homologous sequence of *B. subtilis* (Fig. 2) revealed conservation of the −35 and −10 regions of the vegetative type (σ^{43}) promoter known to function in *B. subtilis* (21). Overall, the sequences displayed in Fig. 2 were 62% homologous. The transcripts of both sequences contained inverted repeats with the potential to form RNA hairpin structures beginning at positions +8, +62, +121, and +191. In addition, both transcripts would contain a series of seven or eight AGAG or UGAG direct repeats between positions +40 and +100. The *B. subtilis* and *B. pumilus trp* leader regions were not homologous to the *E. coli trp* leader region, nor did they encode leader peptides containing Trp residues as they do in enteric bacteria.

***B. pumilus trp* promoter functions in *B. subtilis*.** To determine whether a promoter existed on the 700-bp *Taq*I fragment derived from the *B. pumilus trp* operon 5′ flanking region, we constructed a transcriptional fusion plasmid, pBpIFI (Fig. 3). The *B. pumilus* insert DNA was removed from plasmid pBp1 by *Hin*dIII and partial *Eco*RI digestion, and the resulting *Eco*RI-*Hin*dIII fragment was used to replace the *B. subtilis trp* promoter located 5′ to the synthetic Shine-Dalgarno sequence and human leukocyte interferon A gene of plasmid ptrpIFI (20). pBpIFI and a control plasmid, pIFI, that lacked an insert 5′ to the interferon gene, were transformed into *B. subtilis* UOTO277. Interferon assays were performed on the resulting Cm^r transformants after culture in the presence or absence of tryptophan (20 μg/ml). The results showed an average of 9,500 U of interferon per

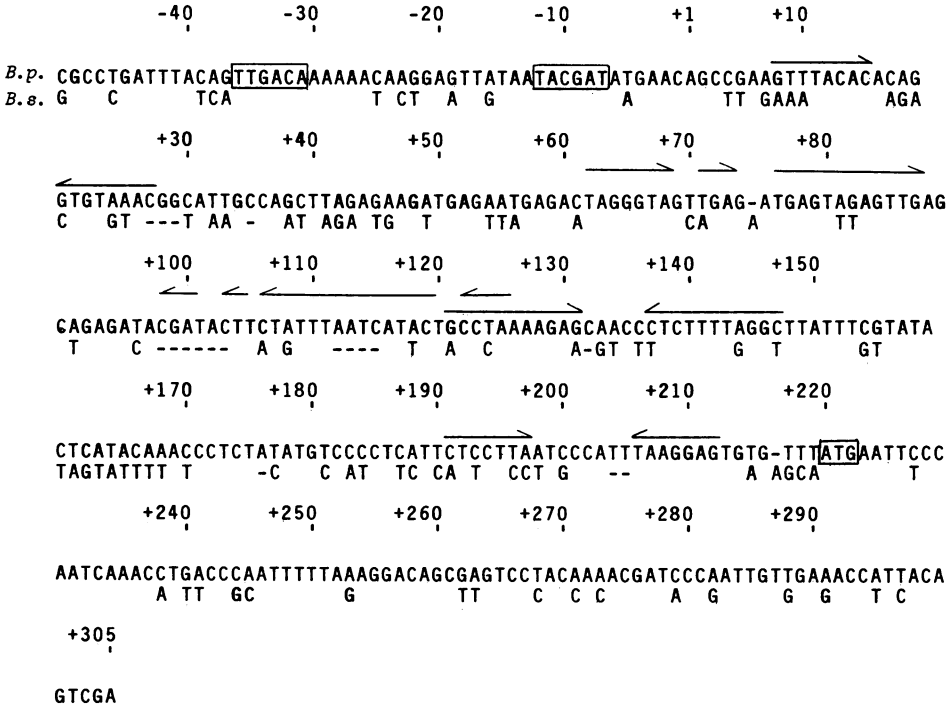

FIG. 2. Nucleotide sequence comparison of the *B. pumilus* (B.p.) and *B. subtilis* (B.s.) *trp* promoter-leader regions. The complete *B. pumilus* sequence is displayed; positions where the *B. subtilis* sequence differs are indicated below the *B. pumilus* sequence. Dashes represent gaps in the optimal sequence alignment. The −35 and −10 regions of the conserved promoter sequence and the *trpE* initiation codon (+220) are boxed. Inverted repeats are indicated by pairs of arrows above the nucleotide sequence. The sequence is numbered relative to the predicted transcription initiation site (+1).

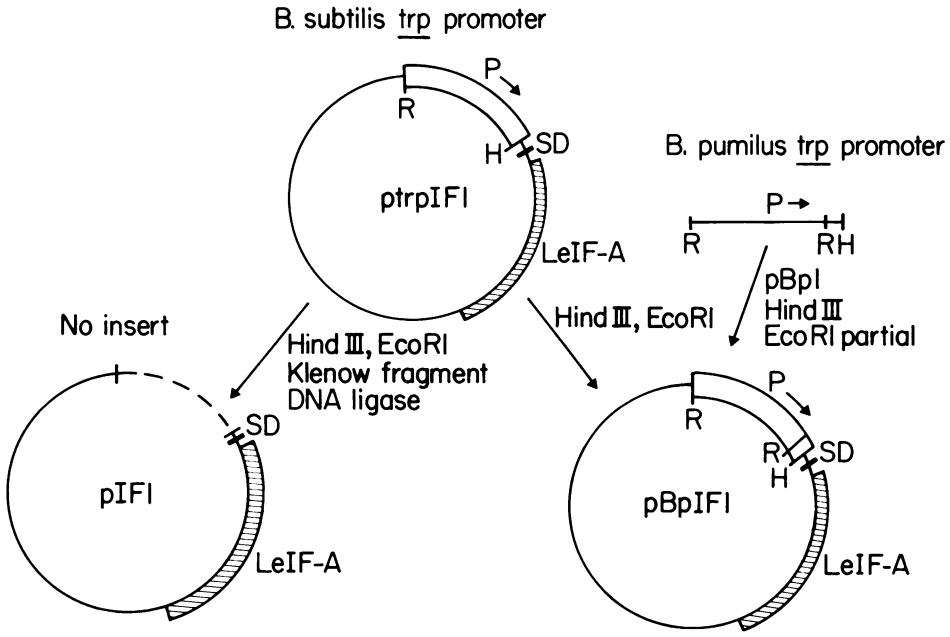

FIG. 3. Construction of pIFI and pBpIFI from ptrpIFI (20). To construct pIFI, ptrpIFI was digested with *Eco*RI and *Hin*dIII endonucleases, the digested ends were repaired by DNA polymerase I Klenow fragment, and the resulting blunt ends were ligated to produce a plasmid lacking an insert 5′ to the leukocyte interferon A gene. To construct pBpIFI, pBp1 (Fig. 1) was digested to completion with *Hin*dIII and partially with *Eco*RI. The desired 700-bp *Eco*RI-*Hin*dIII fragment was isolated from a 5% polyacrylamide gel and ligated into digested ptrpIFI to produce pBpIFI. R, *Eco*RI site; H, *Hin*dIII site; SD, Shine-Dalgarno or ribosome-binding site; LeIF-A, human leukocyte interferon A gene.

OD_{550} per ml in the strain with pBpIFI grown in the presence of tryptophan and 11,000 U per OD_{550} per ml in its absence. The strain with the control plasmid, pIFI, produced no detectable interferon activity. These results indicate that the *B. pumilus trp* promoter is functional in *B. subtilis*. As was observed with the cloned *B. subtilis trp* promoter-leader region (20), expression from the *B. pumilus trp* regulatory region cloned on a multicopy plasmid was not regulated significantly by exogenous tryptophan.

pBpIFI elevates anthranilate synthetase levels in *B. subtilis*. A portion of the *trp* leader region of *B. subtilis* confers 5MeTrp resistance to 5MeTrp-sensitive *B. subtilis* cells when cloned on a high-copy-number plasmid such as ptrpIFI (20). Analog resistance may be due to sequestering of a negative regulatory factor present in limiting amounts, leading to constitutive expression of the chromosomal *trp* genes (20, 21). The plasmid carrying the *B. pumilus trp* regulatory region, pBpIFI, conferred partial 5MeTrp resistance to strain UOTO277 (data not shown). To test whether resistance was due to increased expression of the chromosomal *trp* operon, we assayed the anthranilate synthetase activity of wild-type cells and of cells carrying plasmid pIFI and its derivatives, ptrpIFI and pBpIFI (Table 1). When strain UOTO277, with or without the control plasmid pIFI, was grown in the absence of tryptophan, anthranilate synthetase levels were elevated. Anthranilate synthetase levels dropped more than 100-fold when cells were grown in the presence of tryptophan (20 μg/ml). When UOTO277 carrying the *B. subtilis* (ptrpIFI) or *B. pumilus* (pBpIFI) *trp* leader region plasmid was grown in the absence of exogenous tryptophan, the elevated anthranilate synthetase levels were comparable to those of UOTO277 lacking a plasmid grown under the same conditions. However, addition of tryptophan (20 μg/ml) during growth lowered anthranilate synthetase levels only two- to threefold, respectively, in these strains. Another strain, BG2086, carrying a mutation at the *mtr* locus (8, 10) showed high-level expression in the presence and absence of exogenous tryptophan. The *mtr*$^+$ parent of BG2086, strain BG2087, showed normal regulation by tryptophan. These results demonstrate that the presence of multiple copies of the *B. subtilis* or *B. pumilus trp* leader regions, or a mutation in the *mtr* locus, causes elevated expression of the chromosomal *trpE* gene in *B. subtilis* despite the presence of excess tryptophan.

Transcription termination occurs in vitro within the *B. pumilus trp* leader region. When the 700-bp *Taq*I fragment carrying the *B. pumilus trp* promoter and leader region was transcribed in vitro by using *B. subtilis* σ^{43} RNA polymerase, two RNA bands were observed (Fig. 4A, lane 1). A prominent transcript (approximately 300 bases long) appeared at the position expected for a transcript that initiated at the

TABLE 1. Relative anthranilate synthetase activity in strains carrying pIFI and its derivatives[a]

Strain	Plasmid	Relative AS activity (%)	
		ACH	ACH Trp
UOTO277 (*hisA1 metB5 recE4*)		97	0.3
UOTO277	pIFI	89	0.6
UOTO277	ptrpIFI	79	45
UOTO277	pBpIFI	57	18
BG2086 (*mtr-264 argC4*)		100	102
BG2087 (*argC4*)		66	<0.1

[a] Anthranilate synthetase (AS) was measured as described in Materials and Methods. Equal numbers of late-log-phase cells were assayed for anthranilate synthetase activity. The data are expressed as relative values; activity for strain BG2086 grown in ACH medium (0.2% acid-hydrolyzed casein, 0.2% glucose, 1× minimal salts[23]) was arbitrarily set at 100%. ACH Trp, ACH medium supplemented with L-tryptophan (20 μg/ml). All media for strains carrying plasmids were supplemented with chloramphenicol (5 μg/ml).

predicted transcription initiation site and extended to the end of the restriction fragment (see Fig. 1). A fainter band also appeared, corresponding to an approximately 155-base transcript. When an *Eco*RI restriction fragment derived from pBp1 was used as a template, this 155-base band was still observed (Fig. 4A, lane 2). The larger run-off band present was approximately 80 bases shorter than in lane 1, corresponding to transcription from the *trp* promoter to the *Eco*RI site (Fig. 1). The relative molar amounts of each transcript were determined, and in each case the run-off transcript was about eight times more abundant than the 155-base transcript. To determine the origin of the 155-base transcript, we isolated the 300- and 155-base transcripts detected in lane 1 and the 220-base transcript detected in lane 2 and performed complete RNase T_1 digestion of the [α-^{32}P]GTP-labeled RNA (Fig. 4B). Inspection of the nucleotide sequence (Fig. 2) indicated that several characteristic oligonucleotides would be produced upon digestion after guanilate residues in RNA transcribed from this region (Table 2). Our analysis confirms that the large oligonucleotides predicted to occur in a 300-base run-off transcript that initiated near the predicted site of transcription initiation and extended to the *Taq*I site were detected (Fig. 4B, lanes 2 and 3). The oligonucleotides

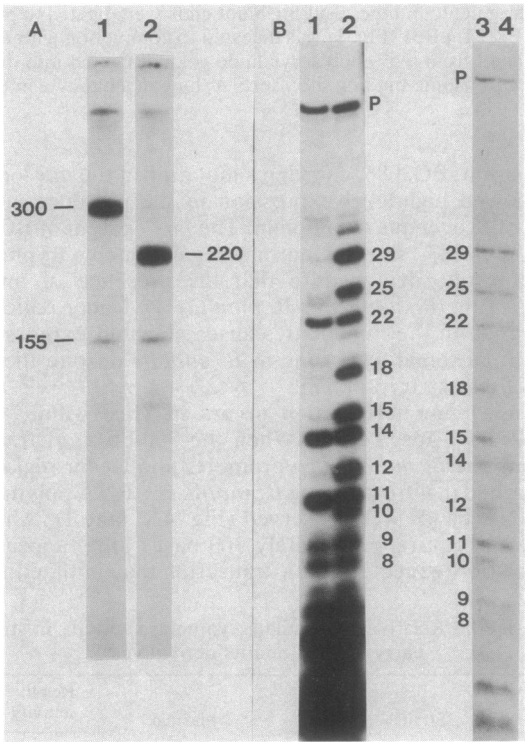

FIG. 4. (A) Autoradiogram of in vitro transcription products analyzed on a 6% polyacrylamide–7 M urea gel. In lane 1, the DNA template was a 700-bp *Taq*I fragment derived from pBp1 (Fig. 1). In lane 2, the DNA template was a 620-bp *Eco*RI fragment derived from pBp1. Approximate sizes of transcripts are indicated (in bases). (B) Autoradiogram of RNase T_1 digestion products of in vitro transcripts, run on 20% polyacrylamide–7 M urea gels. The 300-, 220-, and 155-base transcripts synthesized in vitro were purified and digested as described in Materials and Methods. Lane 1, digestion products of the 155-base transcript; lanes 2 and 3, digestion products of the 300-base transcript; lane 4, digestion products of the 220-base transcript. Sizes of oligonucleotides are indicated (in bases). P, Partial digestion product common to all three transcripts in this experiment.

TABLE 2. RNase T_1 digestion products of purified in vitro transcripts

Predicted from nucleotide sequence[a]		Observed after RNase T_1 digestion of purified transcripts[b]		
Position[c]	Size (nt)	300 nt[d]	220 nt[e]	155 nt[f]
+9	11	+	+	+
+100	22	+	+	+
+122	8	+	+	+
+132	14	+	+	+
+147	9	+	+	+
+156	25	+	+	−
+181	29	+	+	−
+224	18	+	−	−
+242	15	+	−	−
+266	12	+	−	−
+278	10	+	−	−
+291	12	+	−	−

[a] List of all predicted oligonucleotides, eight or more residues in length, derived from the sequence displayed in Fig. 2 (starting at +1, the predicted site of transcription initiation, and extending to the *Taq*I site at position +300) by cleavage after guanilate residues. nt, Nucleotide.

[b] Transcripts were purified and digested as described in Materials and Methods. The digestion products were analyzed on 20% polyacrylamide–7 M urea gels.

[c] +1, Predicted site of transcription initiation; see Fig. 2.

[d] See Fig. 4A, lane 1; Fig. 4B, lanes 2 and 3.

[e] See Fig. 4A, lane 2; Fig. 4B, lane 4.

[f] See Fig. 4A, lane 1; Fig. 4B, lane 1.

produced from the 220-base transcript (Fig. 4B, lane 4) were consistent with the digest profile of a run-off transcript that initiated near position +1 and extended to the *Eco*RI site. In addition, the oligonucleotides predicted to occur in the 155-base transcript if it initiated at the same promoter and was colinear with the two run-off transcripts were also detected (Fig. 4B, lane 1). The approximate size of the transcript and the presence of a 9-mer and absence of a 25-mer suggested that the 3′ end of the 155-base transcript lies about 10 nucleotides after a potential RNA hairpin extending from position +121 to position +147 (*B. pumilus* structure C:D, Fig. 5).

DISCUSSION

Potential *trp* regulatory signals are conserved in *B. subtilis* and *B. pumilus*. The DNA sequences of the *B. subtilis* and *B. pumilus trp* operon regulatory regions were compared and found to be 62% homologous (Fig. 2). The −35 and −10 regions of the σ^{43}-type promoters were identical. Comparison of the leader transcript sequences (from +1 to +220 relative to the presumed transcription initiation site in the *B. pumilus* sequence) showed that the ability to direct formation of RNA hairpin structures is conserved even when the sequences diverge. The significance of two conserved potential structures at positions +8 (5′ end of the mRNA) and +191 (encompassing the ribosome binding site for *trpE*) is unknown. A third potential structure, at position +121 of the RNA, appears to be a transcription terminator in *B. subtilis* (21). Sequence differences occur within this structure in the *B. pumilus* sequence (6 of 22 positions, Fig. 2), but due to complementary base changes, the ability to form a perfectly base-paired hairpin is conserved (Fig. 5). Sequences in the loops of the predicted hairpins are not conserved between the two species. As demonstrated in Fig. 4, *B. subtilis* RNA polymerase recognizes this segment of the *B. pumilus trp* leader transcript in vitro as a transcription terminator. We believe that the ability to form a base-paired transcription

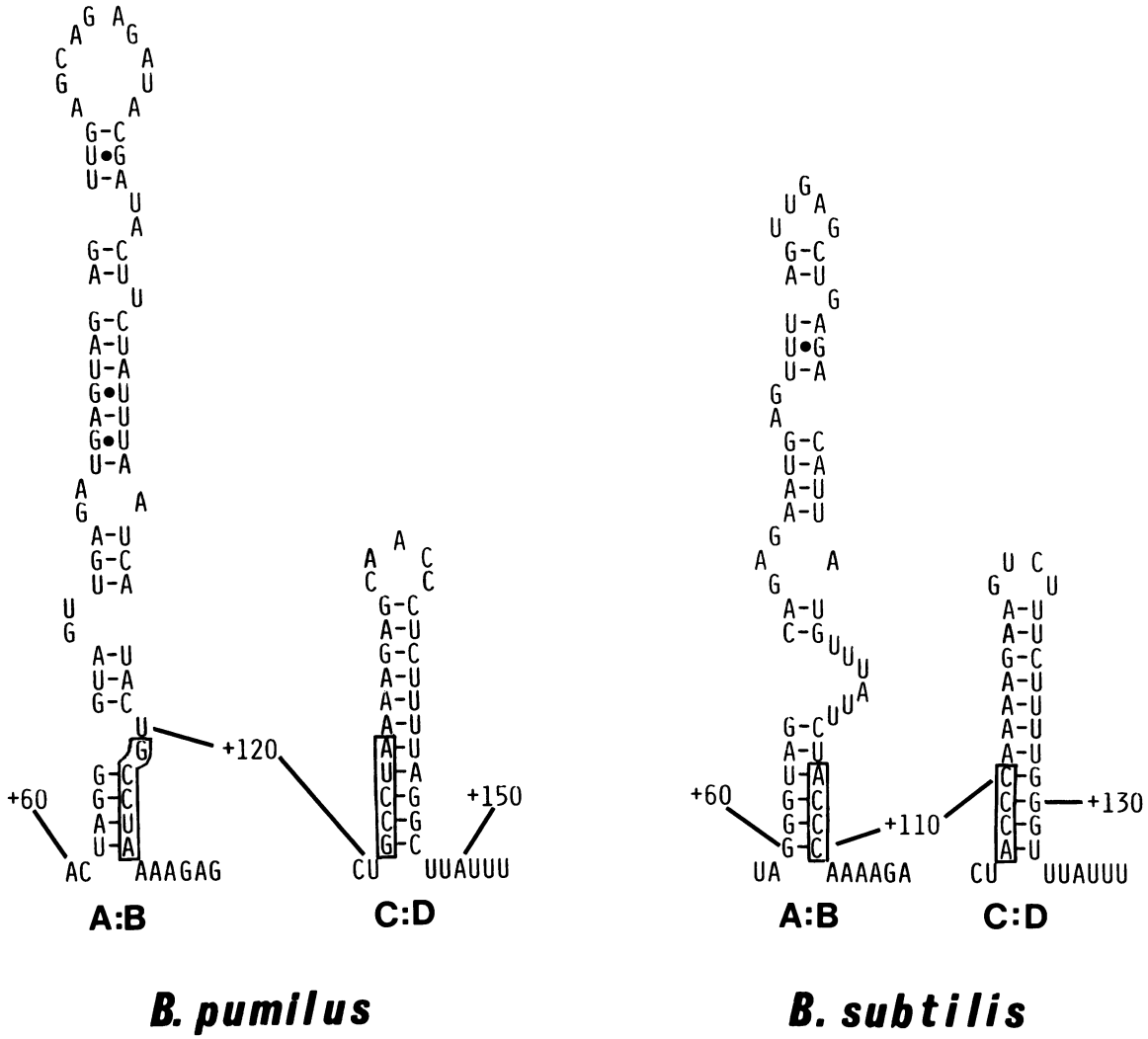

FIG. 5. RNA secondary structures predicted by computer analysis of the sequences of the *B. pumilus* and *B. subtilis trp* leader transcripts (27). Boxed nucleotides indicate the overlap between proposed antitermination (A:B) and termination (C:D) structures. The structures have the following calculated free energies of formation (18). *B. pumilus*: A:B, $\Delta G = -14.4$ kcal/mol; C:D, $\Delta G = -18.8$ kcal/mol. *B. subtilis*: A:B, $\Delta G = -14.3$ kcal/mol; C:D, $\Delta G = -15.3$ kcal/mol.

terminator at this position is a key regulatory feature of the two *Bacillus trp* operons. In *B. subtilis*, a predicted antitermination structure (structure A:B, Fig. 5) could preclude formation of this terminator. The competing segment of RNA overlaps a region (+47 to +86) previously implicated in negative regulatory factor binding (20). Homologous sequences that could form a comparable antiterminator exist in the *B. pumilus trp* leader transcript (Fig. 5).

A *B. subtilis trans*-acting factor interacts with the transcript of the *B. pumilus trp* leader region. Expression of the *B. subtilis trp* leader region on a high-copy-number plasmid confers resistance to the tryptophan analog 5MeTrp (20). Analysis of promoter and leader region deletions and point mutations led to the hypothesis that a segment of the nascent transcript binds a tryptophan-activated negative regulatory factor present in limiting amounts, resulting in increased expression of the chromosomal *trp* operon. In the studies described in this paper, we measured anthranilate synthetase synthesis directed by the chromosomal *trpE* gene and demonstrated that *trpE* expression was elevated in strains with plasmids carrying the *B. subtilis* (ptrpIFI) or *B. pumilus* (pBpIFI) *trp* leader regions. Expression was also elevated in a strain carrying a mutation in the *mtr* locus, but not in wild-type cells or cells carrying the control plasmid pIFI. Deletion analyses with the *B. subtilis trp* leader region suggest that the transcript segment necessary to confer 5MeTrp resistance overlaps segments of the hypothetical antiterminator (20, 21). Although we have not mapped the putative regulatory factor binding site within the *B. pumilus trp* leader region or demonstrated that transcription of the region is required to confer 5MeTrp resistance, *B. pumilus* has homology both in nucleotide sequence and possible structure to the *B. subtilis* leader RNA segment (+47 to +86), which has been implicated in negative regulatory factor binding (20, 21) (Fig. 2 and 5).

Attenuation control in the bacilli, as revealed by our studies with the *trp* operon, differs significantly from the well-understood mechanism of attenuation control of the amino acid biosynthetic operons of enteric bacteria (for a review, see reference 25). In the *trp* operons of the bacilli

there is no leader peptide coding region with tandem Trp codons that would allow translational control of transcription termination as seen in *E. coli*. Rather, it appears that a tryptophan-activated RNA-binding molecule is used to select between alternative secondary structures in the leader transcript, thereby regulating transcription termination. The objective of the two attenuation mechanisms is the same—to respond to changes in the availability of tryptophan by regulating formation of an RNA structure that serves as a transcription terminator.

ACKNOWLEDGMENTS

We thank Paul Lovett for sending strains and plasmids and John Helmann and Michael Chamberlin for the generous gift of *B. subtilis* σ^{43} RNA polymerase. Magda van Cleemput and Virginia Vania provided valuable assistance. We are grateful to Lisa Klig, Robert Landick, and Valley Stewart for critical reading of the manuscript. Computer analyses were performed through the Bionet computer resource (Intelligenetics, Inc., Palo Alto, Calif.).

These studies were supported in part by Public Health Service grant GM09738 from the National Institutes of Health. M.I.K. is a predoctoral trainee of the Public Health Service. C.Y. is a Career Investigator of the American Heart Association.

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