JOURNAL OF BACTERIOLOGY, Feb. 1995, p. 839–842
0021-9193/95/$04.00+0
Copyright © 1995, American Society for Microbiology
Vol. 177, No. 3

The *mtrB* Gene of *Bacillus pumilus* Encodes a Protein with Sequence and Functional Homology to the *trp* RNA-Binding Attenuation Protein (TRAP) of *Bacillus subtilis*

ROBERT J. HOFFMAN AND PAUL GOLLNICK*

Department of Biological Sciences, State University of New York at Buffalo, Buffalo, New York 14260

Received 3 October 1994/Accepted 28 November 1994

The *mtrB* gene from *Bacillus pumilus* encodes a 76-amino-acid polypeptide with 77% identity to the *trp* RNA-binding attenuation protein (TRAP) from *Bacillus subtilis*. *B. pumilus* TRAP binds *trp* leader RNA from either *B. subtilis* or *B. pumilus* in a tryptophan-dependent manner. Altering threonine 52 to alanine eliminated RNA-binding activity of *B. pumilus* TRAP.

The *Bacillus subtilis trp* operon is regulated by transcription attenuation involving the RNA-binding protein TRAP (*trp* RNA-binding attenuation protein) (7, 8, 11, 17). TRAP, which is the product of the *mtrB* gene (8), is activated by L-tryptophan to bind to a target site in the 204-nucleotide *trp* leader RNA transcript upstream of the start codon of the first structural gene (5, 15). This binding influences the secondary structure of the leader RNA to form a transcription terminator, thereby halting transcription in the leader and preventing expression of the operon (6). In the absence of tryptophan, the operon is expressed because TRAP does not bind, and an alternative antiterminator structure forms in the leader transcript.

Kuroda et al. (12) showed that the *cis*-acting features of the *Bacillus pumilus trp* operon are similar to those in *B. subtilis* and that expression of the cloned *B. pumilus trp* leader transcript in *B. subtilis* resulted in deregulated overexpression of the *trp* operon, presumably by titrating the *trp* regulatory factor. They therefore proposed that the *B. pumilus trp* operon is also regulated by an attenuation mechanism similar to that which controls the *B. subtilis* operon (12).

We have cloned and sequenced the *B. pumilus mtrB* gene and found it to encode a 76-amino-acid polypeptide (molecular weight, 8,301) with 77% identity to *B. subtilis* TRAP. Purified *B. pumilus* TRAP binds to both *B. pumilus* and *B. subtilis trp* leader RNAs in a tryptophan-dependent manner. We also show that a mutation which changes threonine 52 to alanine in *B. pumilus* TRAP eliminates the ability of this protein to bind RNA, apparently by disrupting tryptophan binding.

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1. *B. subtilis* was transformed by using natural competence (1) as modified in reference 12. Resistance to 5-fluorotryptophan (Ftrp) was tested as described elsewhere (12).

Cloning and sequencing the *mtrB* gene from *B. pumilus*. PCR using oligonucleotide primers based on nucleotides 800 to 823 within *mtrA* and nucleotides 1390 to 1410 downstream of the *mtrB* gene in *B. subtilis* (8) resulted in amplification of a 580-bp fragment from *B. pumilus* genomic DNA which contained the *mtrB* gene.

Purification of TRAP. Purification of *B. subtilis* TRAP has been described previously (15). *B. pumilus* TRAP was purified similarly, from overexpressing *Escherichia coli* SG62052/pGP1-2/pTZ18BpmtrB, except that the immunoaffinity column contained antibodies prepared against *B. pumilus* TRAP.

Gel mobility shift assays. Uniformly ^{32}P-labelled *trp* leader RNA transcripts were prepared as described previously (15) by using T7 RNA polymerase to transcribe pTZ18dR4 or pTZ19Bptrpl cut with *Hin*dIII or *Eco*RI, respectively. Labelled RNAs terminated at the attenuator were purified from denaturing polyacrylamide gels (14). The *B. subtilis* transcript contained nucleotides 2 to 138 (17), and the *B. pumilus* RNA contained nucleotides 1 to 153 (12).

Gel mobility shift assays were performed as described previously (15) using 150,000 dpm of gel-purified ^{32}P-labelled RNA (1 ng $\approx$ 0.022 pmol). *B. subtilis* TRAP concentrations were determined with the A_{280} by using an extinction coefficient of 1,250 M^{-1}, which was determined by amino acid analysis. *B. pumilus* TRAP concentrations were determined by Bio-Rad protein assay using *B. subtilis* TRAP standards.

Nucleotide sequence accession number. The sequence of the *B. pumilus mtrB* gene has been deposited in GenBank (accession no. L37879).

Characteristics of the *B. pumilus mtrB* gene. The nucleotide sequence of the *B. pumilus mtr* fragment contains one partial open reading frame with homology to the final 46 amino acids of MtrA (GTP cyclohydrolase I [4]) from *B. subtilis* (8) and a second open reading frame highly homologous to TRAP from *B. subtilis* (4, 6, 8, 15). The two open reading frames are separated by 24 bp, which contain a putative ribosome binding site for *mtrB*.

The deduced amino acid sequences of the *B. pumilus* and *B. subtilis* TRAP proteins are highly homologous, with 58 of the 76 residues being identical. Homology is strongest in the middle portion of the proteins, where residues 32 to 59 are identical. The amino-terminal region shows the least homology, particularly residues 2 to 6. In the recently completed three-dimensional X-ray crystal structure of tryptophan-activated *B. subtilis* TRAP (3), the first 7 residues are not ordered in the crystal. Moreover, preliminary data from a mutant in which residues 2 to 6 were deleted suggest that these amino acids are not essential for TRAP function (6a).

***B. pumilus* TRAP binds to *trp* leader RNA from *B. pumilus* and *B. subtilis*.** *B. subtilis* TRAP has been shown to specifically bind *trp* leader RNA in a tryptophan-dependent manner (15) and to cause transcription termination at the *trp* attenuator in vitro (6). We found that *B. pumilus* TRAP also binds to *trp* leader RNA from either *B. pumilus* or *B. subtilis* in vitro (Fig.

* Corresponding author. Mailing address: Department of Biological Sciences, State University of New York at Buffalo, Buffalo, NY 14260. Phone: (716) 645-2887. Fax: (716) 645-2975. Electronic mail address: CAMGOLLP@UBVMS.CC.BUFFALO.EDU.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid(s)	Relevant characteristic(s)	Source or reference
Strains		
B. pumilus NRRLB 3275	Prototroph	13
B. subtilis BG4233	*argC4* Δ*mtrB*	D. Henner
E. coli SG62052/pGP1-2		18
Plasmids		
pTZ18U, pTZ19U	Ap^r T7 promoter plasmids	U.S. Biochemical
pTZ18mtrAB	Ap^r T7 expression plasmid for *B. subtilis mtrA* and *mtrB*	4
ptrpdR4	*B. subtilis trp* leader from −2 to +318	16
pTZ18dR4	320-bp *Eco*RI-*Hin*dIII *trp* leader fragment from ptrpdR4 subcloned in pTZ18U	This study
pTZ19Bptrpl	*B. pumilus trp* leader sequences from +1 to +246 in pTZ19U	This study
pBPmtr1	*B. pumilus mtrB* in pUC118(19)	This study
pBPmtr1A	*B. pumilus mtrB* (T52A) in pUC118(19)	This study
pTZ18BpmtrB1	*B. pumilus mtrB* from pBPmtr1 subcloned in pTZ18U	This study
pTZ18BpmtrB1A	*B. pumilus mtrB* (T52A) from pBPmtr1A subcloned in pTZ18U	This study
pJBO87	*B. subtilis* expression vector using the *spac* promoter (20); 1.3-kb *Pvu*I fragment containing the alpha peptide from pUB110 (10) subcloned into pDH87 (20)	This study
pXP1	*B. pumilus mtrB* in pJBO87	This study

1A and B). In both cases, RNA binding depends on tryptophan with little or no binding in the absence of added tryptophan (Fig. 1, lanes marked with asterisks). The binding of *B. pumilus trp* leader RNA and *B. pumilus* TRAP resulted in several shifted bands, labelled C and C′ in Fig. 1A. However, when *B. pumilus* TRAP bound to *B. subtilis trp* leader RNA, only a single shifted band (C) corresponding to the protein-RNA complex was observed (Fig. 1B). In this case, a small amount of tryptophan-independent binding was seen (Fig. 1B, compare lanes 10 and 10*). *B. pumilus* TRAP did not bind to several nonspecific RNAs tested with this assay (data not shown). These results indicate that *B. pumilus* TRAP binds to both *trp* leader RNAs; however, the complexes formed may be slightly different.

As we have shown previously (15), *B. subtilis* TRAP binds to *trp* leader RNA from *B. subtilis* and forms a single complex seen as one shifted band, labelled C in Fig. 1D. *B. subtilis* TRAP also binds *B. pumilus trp* leader RNA, and as was seen when this RNA binds to *B. pumilus* TRAP, several shifted bands are observed (Fig. 1C, bands C and C′).

It is curious that when either TRAP binds to the *B. pumilus trp* leader RNA, several shifted bands are seen, presumably corresponding to alternative TRAP-RNA complexes. The different complexes may contain different amounts of TRAP, or

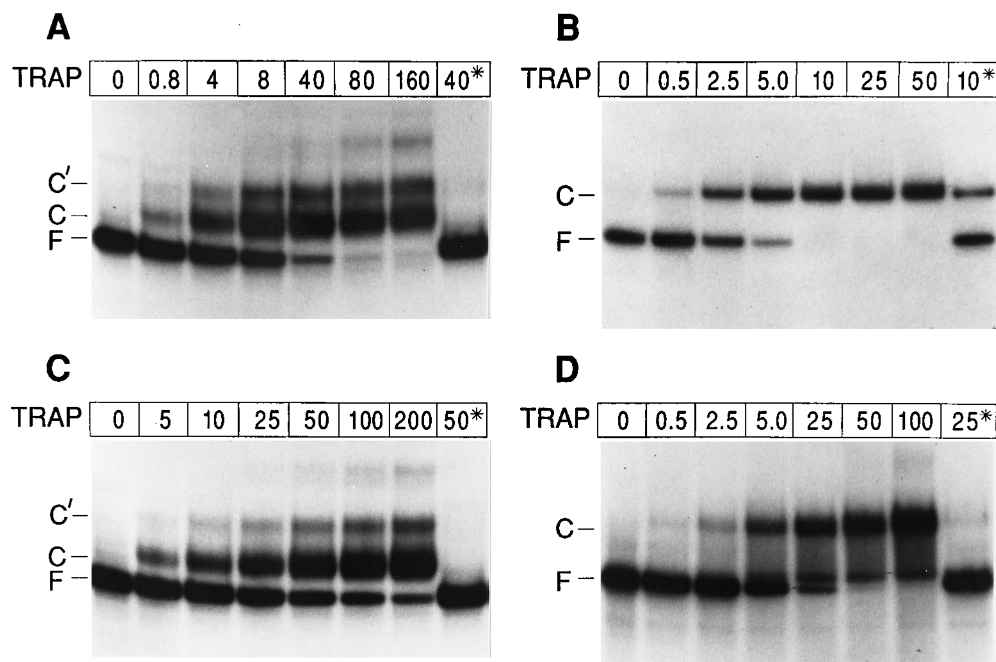

FIG. 1. RNA mobility shift assay of *B. pumilus* and *B. subtilis* TRAP with *trp* leader transcripts from either *B. pumilus* or *B. subtilis*. In vitro-transcribed ^{32}P-labelled *trp* leader RNA was incubated with various amounts (as indicated) of purified TRAP in the presence of 1.2 mM L-tryptophan and electrophoresed on native 6% polyacrylamide gels. (A) *B. pumilus* TRAP (0 to 160 ng) with *B. pumilus trp* leader RNA; (B) *B. pumilus* TRAP (0 to 50 ng) with *B. subtilis trp* leader RNA; (C) *B. subtilis* TRAP (0 to 200 ng) with *B. pumilus trp* leader RNA; (D) *B. subtilis* TRAP (0 to 100 ng) with *B. subtilis trp* leader RNA. Lanes with an asterisks contain the TRAP levels indicated with RNA in the absence of tryptophan. F, free RNA; C and C′, complex of RNA with TRAP.

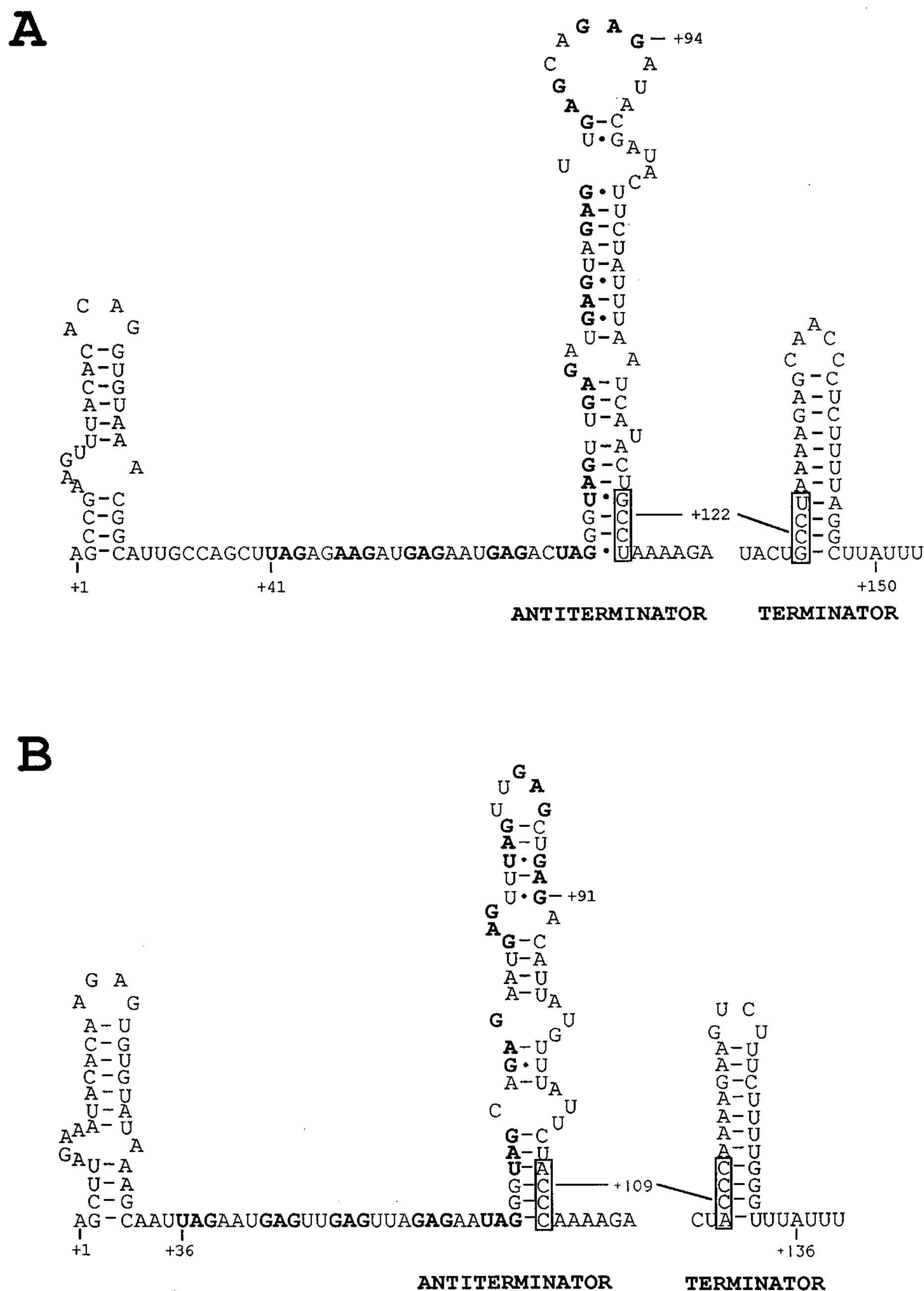

FIG. 2. RNA secondary structures of the *trp* leader transcripts from *B. pumilus* (A) and *B. subtilis* (B) predicted by using the Fold program of the University of Wisconsin Genetics Computer Group, version 7.3. Boxed nucleotides indicate the overlaps between the proposed antiterminator and terminator structures. The 11 G/UAG trinucleotide repeats of the proposed TRAP recognition sites are indicated in boldface letters.

the bound RNA may adopt alternative conformations in each complex. Recently, Babitzke et al. (5) presented evidence that TRAP recognizes a series of 11 G/UAG repeats found in the *trp* leader region from either *Bacillus* species (Fig. 2). Examination of the proposed binding sites in either leader shows several subtle differences, including an unusual AAG in the second repeat of *B. pumilus* and several differences in the spacing between repeats. The positions of the repeats are also slightly different with relation to the antiterminator stem-loop. It is not clear what difference between these RNAs is responsible for the differences seen in the mobility shift gels when complexes are formed with either TRAP.

T52A mutant *B. pumilus* TRAP does not bind *trp* leader RNA. We discovered one clone of *B. pumilus mtrB* in which nucleotide 319 was changed from an A to a G, resulting in threonine 52 changing to alanine (T52A). Purified T52A TRAP did not bind *trp* leader RNA in a gel mobility shift assay at any amount of protein tested up to 1,000 ng (data not shown). We then compared the tryptophan dependency of *B. pumilus* TRAP binding to *trp* leader RNA for both the wild-type and mutant proteins. The wild-type protein was activated by between 1 and 10 μM L-tryptophan (Fig. 3A). In contrast, T52A was not activated at any concentration of tryptophan up to 1,200 μM (Fig. 3B). Most significantly, while the wild-type

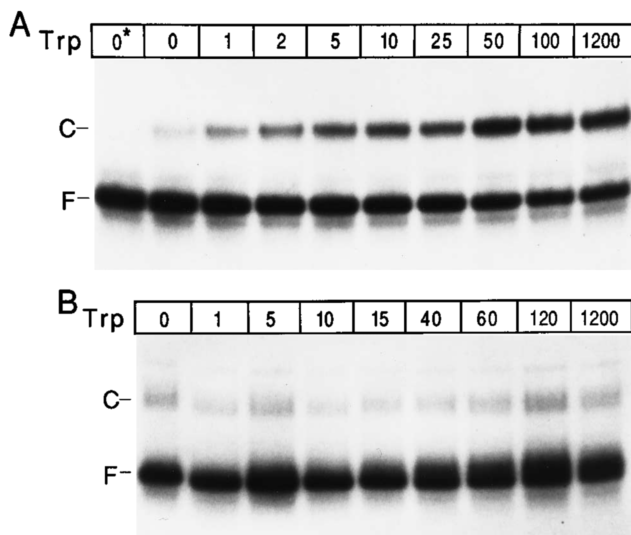

FIG. 3. RNA mobility shift assay of tryptophan dependency of wild-type and T52A mutant *B. pumilus* TRAP binding to *trp* leader RNA. (A) Purified wild-type TRAP (4 ng; compare with Fig. 1B, lanes 2.5 and 5.0) was incubated with ^{32}P-labelled *B. subtilis trp* leader RNA in the presence of 0 to 1,200 μM L-tryptophan as indicated. Lane 0*, RNA in the absence of protein. (B) Purified T52A mutant *B. pumilus* TRAP (30 ng) incubated with *trp* leader RNA in the presence of 0 to 1,200 μM L-tryptophan.

TRAP bound L-tryptophan in an equilibrium dialysis assay, T52A TRAP did not (data not shown). These results indicate that changing threonine 52 to alanine eliminates the ability of this protein to be activated by L-tryptophan to bind *trp* leader RNA.

Changing threonine 52 to alanine could prevent proper folding of the protein. We therefore compared the sizes of the purified *B. pumilus* wild-type and T52A mutant TRAP proteins with that of *B. subtilis* TRAP, which has recently been shown to be an 11-mer of identical subunits (2, 3, 5). All three proteins ran similarly on a Phenomenex SEC-2000 size exclusion column (data not shown), indicating that they all have similar sizes. These results indicate that the T52A mutant polypeptide folds well enough to assemble into an 11-mer resembling the *B. subtilis* protein. This conclusion supports the interpretation that the loss of tryptophan binding and RNA binding of the T52A mutant reflects a specific alteration in the tryptophan binding site of this protein and does not simply result from a drastic effect on folding of the protein.

We also examined in vivo regulation of the *trp* operon by both wild-type and T52A *B. pumilus* TRAP by expressing them in *B. subtilis* and testing the effect of Ftrp on the resulting strains. Growth of *mtrB*$^+$ strains is inhibited by Ftrp, whereas *mtrB* mutant strains continue to express the *trp* operon and grow (9). *B. subtilis* BG4233 contains a deletion of *mtrB* and is resistant to Ftrp. Expressing *B. pumilus mtrB* from the *spac* promoter (20) rendered this strain sensitive to Ftrp, indicating that *B. pumilus* TRAP is capable of regulating the *B. subtilis trp* operon in vivo. In contrast, BG4233 remains resistant to Ftrp when the T52A mutant *mtrB* is expressed, confirming that T52A TRAP does not function to regulate the *trp* operon.

In the X-ray crystal structure of tryptophan-activated *B. subtilis* TRAP, the side chain hydroxyl of the homologous threonine 52 makes a hydrogen bond with one of the carboxylic acid oxygens of tryptophan (3). Threonine 52 is also involved in forming a hydrophobic pocket that surrounds the indole ring of the bound tryptophan. Changing this residue to alanine is predicted to disrupt both of these interactions with tryptophan. We therefore suspect that the loss of RNA binding seen with the *B. pumilus* T52A protein is due to the inability of this protein to bind and be activated by L-tryptophan, indicating that threonine 52 is part of the tryptophan binding domain of this protein.

This work was supported by National Science Foundation grant MCB-9118654, and R. Hoffman was supported by an REU supplement to this grant. P. Gollnick is a Pew Scholar in the Biomedical Sciences.

Xiao-Ping Chen and Bruce Fernandez provided valuable technical assistance. We thank Mike McGarry of Roswell Park Springville Laboratories for preparing the anti-*B. pumilus* TRAP antibodies. We also thank Tomasz Kurecki for help with high-performance liquid chromatography. Special thanks go to John Otridge for much generous assistance with this work. We thank Chris Baumann, Jerry Koudelka, John Otridge, and Charles Yanofsky for critical reading of the manuscript. We are indebted to Jim Stamos for his excellent photographic services.

REFERENCES

1. **Anagnostopoulos, C., and J. Spizizen.** 1961. Requirements for transformation in *Bacillus subtilis*. J. Bacteriol. **81:**741–746.
2. **Antson, A. A., A. M. Brozozwski, E. J. Dodson, Z. Dauter, K. S. Wilson, T. Kurecki, J. Otridge, and P. Gollnick.** 1994. Eleven-fold symmetry of the *trp* RNA-binding attenuation protein from *Bacillus subtilis* determined by X-ray analysis. J. Mol. Biol. **244:**1–5.
3. **Antson, A. A., E. J. Dodson, Z. Dauter, K. S. Wilson, T. Kurecki, and P. Gollnick.** The three-dimensional structure of the *trp* RNA-binding attenuation protein from *B. subtilis* determined by X-ray diffraction. Submitted for publication.
4. **Babitzke, P., P. Gollnick, and C. Yanofsky.** 1992. The *mtrAB* operon of *Bacillus subtilis* encodes GTP cyclohydrolase I (MtrA), an enzyme involved in folic acid biosynthesis, and MtrB, a regulator of tryptophan biosynthesis. J. Bacteriol. **174:**2059–2064.
5. **Babitzke, P., J. T. Stults, S. J. Shire, and C. Yanofsky.** 1994. TRAP, the *trp* RNA-binding attenuation protein of *Bacillus subtilis*, is a multisubunit complex that appears to recognize G/UAG repeats in the *trpEDCFBA* and *trpG* transcripts. J. Biol. Chem. **269:**16597–16604.
6. **Babitzke, P., and C. Yanofsky.** 1993. Reconstitution of *Bacillus subtilis trp* attenuation *in vitro* with TRAP, the *trp* RNA-binding attenuation protein. Proc. Natl. Acad. Sci. USA **90:**133–137.

6a. **Baumann, C., and P. Gollnick.** Unpublished data.

7. **Gollnick, P.** 1994. Regulation of the *B. subtilis trp* operon by an RNA binding protein. Mol. Microbiol. **11:**991–997.
8. **Gollnick, P., S. Ishino, M. I. Kuroda, D. Henner, and C. Yanofsky.** 1990. The *mtr* locus is a two gene operon required for transcription attenuation in the *trp* operon of *Bacillus subtilis*. Proc. Natl. Acad. Sci. USA **87:**8726–8730.
9. **Hoch, S. O., C. W. Roth, I. P. Crawford, and E. W. Nester.** 1971. Control of tryptophan biosynthesis by the methyltryptophan resistance gene in *Bacillus subtilis*. J. Bacteriol. **105:**38–45.
10. **Jalanko, A., I. Palva, and J. Söderlund.** 1981. Restriction maps of plasmids pUB110 and pBD9. Gene **14:**325–328.
11. **Kuroda, M. I., D. Henner, and C. Yanofsky.** 1988. *cis*-acting sites in the transcript of the *Bacillus subtilis trp* operon regulate expression of the operon. J. Bacteriol. **170:**3080–3088.
12. **Kuroda, M. I., H. Shimotsu, D. J. Henner, and C. Yanofsky.** 1986. Regulatory elements common to the *Bacillus pumilus* and *Bacillus subtilis trp* operons. J. Bacteriol. **167:**792–798.
13. **Lovett, P. S., and F. E. Young.** 1970. Genetic analysis in *Bacillus pumilus* by PBS-1-mediated transduction. J. Bacteriol. **101:**603–608.
14. **Milligan, J. F., and O. C. Uhlenbeck.** 1989. Determination of RNA-protein contacts using thiophosphate substitutions. Biochemistry **28:**2849–2855.
15. **Otridge, J., and P. Gollnick.** 1993. MtrB from *Bacillus subtilis* binds specifically to *trp* leader RNA in a tryptophan dependent manner. Proc. Natl. Acad. Sci. USA **90:**128–132.
16. **Shimotsu, H., and D. J. Henner.** 1984. Characterization of the *Bacillus subtilis* tryptophan promoter region. Proc. Natl. Acad. Sci. USA **81:**6315–6319.
17. **Shimotsu, H., M. I. Kuroda, C. Yanofsky, and D. J. Henner.** 1986. Novel form of transcription attenuation regulates expression of the *Bacillus subtilis* tryptophan operon. J. Bacteriol. **166:**461–471.
18. **Tabor, S., and C. C. Richardson.** 1985. A bacteriophage T7 RNA polymerase/promoter system for controlled exclusive expression of specific genes. Proc. Natl. Acad. Sci. USA **82:**1074–1078.
19. **Vieira, J., and J. Messing.** 1987. Production of single-stranded plasmid DNA. Methods Enzymol. **153:**3–11.
20. **Yansura, D. G., and D. J. Henner.** 1984. Use of the *Escherichia coli lac* repressor and operator to control gene expression in *Bacillus subtilis*. Proc. Natl. Acad. Sci. USA **81:**439–443.