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Genetics

The *mtr* locus is a two-gene operon required for transcription attenuation in the *trp* operon of *Bacillus subtilis*

(transcription regulator/regulatory mutants/RNA binding protein/*in vivo* regulation)

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ABSTRACT **We have cloned and characterized the *mtr* operon of *Bacillus subtilis*. This operon encodes a presumed RNA-binding regulatory protein that is required for attenuation control of the *trp* operon. We have shown that the *mtr* operon consists of two structural genes, *mtrA* and *mtrB*, predicted to encode 22-kDa and 8-kDa polypeptides, respectively. MtrB shows homology with RegA, an RNA-binding regulatory protein of bacteriophage T4. The lesions in several *mtr* mutants were localized to *mtrB* or the putative *mtr* promoter. Several *mtrB* alleles were dominant to *mtr*$^+$, suggesting that the regulatory factor is a multimeric protein. The *in vivo* action of the *mtrA* and *mtrB* gene products was analyzed in an *E. coli* strain containing a *trpE–lacZ* gene fusion under control of the *B. subtilis trp* promoter/attenuator region. Both MtrA and MtrB were necessary for regulation of β-galactosidase production.**

Expression of the *Bacillus subtilis* tryptophan (*trp*) operon is regulated over 100-fold by attenuation in response to changes in the intracellular level of L-tryptophan (1). Attenuation is mediated by a trans-acting regulatory factor that appears to function by binding to the leader transcript and, when bound, promote formation of a leader RNA hairpin structure that causes transcription termination (1, 2). Overexpression of the *trp* leader transcript in trans results in constitutive expression of the operon (1, 2), presumably by titrating out the regulatory factor. Deletion analyses localized the leader RNA target sites of the regulatory factor to a 10-base sequence, AGAAUGAGUU, repeated 22 bases apart in the leader transcript (2). Mutations in the *mtr* (*m*ethyl*t*ryptophan *r*esistance) locus eliminate transcription termination in the *trp* leader region, leading to elevated synthesis of the tryptophan biosynthetic enzymes (1, 2). This overexpression is responsible for resistance to tryptophan analogs such as 5-methyltryptophan.

To explore the role of the *mtr* gene products in *trp* operon regulation, we have cloned and sequenced the *mtr* locus¶ and have examined its function. This locus was found to be a two-gene operon. One of these genes encodes a polypeptide with homology to RegA, an RNA-binding regulatory protein of bacteriophage T4. Both Mtr polypeptides appear to be required for regulation of *trp* operon expression.

MATERIALS AND METHODS

Bacterial Strains and Plasmids. The bacterial strains and plasmids used in this study are described in the text or appropriate legends. CYBS12, a strain with a single copy *trpPL trpE–lacZ* gene fusion integrated into the *amyE* locus, has been described (2). BG4231 and BG4232 contain plasmids pMY46 and pMY47, respectively, integrated into the *amyE* locus of W168. These plasmids express *mtrA* plus *mtrB* or *mtrB*, respectively, from the *spac* promoter (3).

Plasmids pSI10, pSI16, pSI18, and pSI29 were constructed by inserting polymerase chain reaction (PCR)-amplified products prepared from *mtr*$^+$ genomic DNA into pJM102 or pJM103 (4). The 1.1-kilobase (kb) *Hin*dIII–*Nde* I *mtr*-containing fragment from λ7A (*mtr3164*) was subcloned into pJM103 to create pPDG47. Plasmids pNS10 and pND7 were derived by ligating *Nde* I–*Spl* I and *Nde* I–*Dra* I fragments of pPDG47 into pJM103.

To construct a *B. subtilis/Escherichia coli* shuttle vector containing the *mtr* operon, a PCR product was generated from BG2087 (*mtr*$^+$) chromosomal DNA using one primer complementary to nucleotides 288–310 (see Fig. 2) in which G-306 was changed to C creating a *Sph* I site, in combination with a second primer complementary to nucleotides 1575–1552, downstream of the *Hin*dIII site at position 1489. The resulting PCR product was digested with *Sph* I and *Hin*dIII and ligated into the polylinker of pJM103. The *mtr* operon-containing fragment was then excised with *Eco*RI and *Hin*dIII and ligated into pHY300-PLK (5) to yield the plasmid pSI45. Plasmids pTTmtrAB and pTTmtrB, which express MtrA plus MtrB or MtrB, respectively, using the *tac* promoter, were constructed by ligating the *Nde* I–*Hin*dIII or *Dra* I–*Hin*dIII fragments, respectively (see Fig. 1), into pTac-Term (6). Plasmid ptrpE-lacZ contains the first 40 codons of *trpE* fused in frame to *lacZ* in pHY300-PLK (5).

Transformation and Screening Procedures. *E. coli* transformations were performed using the calcium-shock procedure (7). *aroB*$^+$ transformants were selected on minimal agar containing 0.2% glucose, 0.2% acid-hydrolyzed casein, and ampicillin (50 μg/ml). *B. subtilis* was transformed using natural competence (8). Selection was on tryptose blood agar containing chloramphenicol (5 μg/ml) or tetracycline (20 μg/ml). Mtr function was tested on minimal agar supplemented with 0.2% acid-hydrolyzed casein, 0.2% glucose, and 5-fluorotryptophan (200 μg/ml). *mtr* strains grow well in the presence of 5-fluorotryptophan, whereas *mtr*$^+$ strains are inhibited by this analog. With *B. subtilis* strains containing the *trpE–lacZ* fusion, Mtr function was determined using plates containing minimal agar plus 0.2% acid-hydrolyzed casein, L-tryptophan (20 μg/ml), and 5-bromo-4-chloro-3-indolyl β-D-galactoside (50 μg/ml). On these plates wild-type colonies are white whereas *mtr* colonies are blue.

DNA Preparation. Chromosomal DNA was prepared from *B. subtilis* by standard procedures (9). *Sau*3A partial digestion and size-fractionation of DNA fragments on sucrose

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Abbreviation: PCR, polymerase chain reaction.

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¶The sequence reported in this paper has been deposited in the GenBank data base (accession no. M37320).

gradients were according to Maniatis *et al.* (10). λEMBL3 arms and λZAP DNA were purchased from Stratagene.

Library Construction and Screening. The λEMBL3 library was constructed by ligating *Bam*HI/*Eco*RI-digested λEMBL3 DNA with ≈15-kb BG2087 (*mtr*$^+$) *Sau*3A fragments. The λZAP libraries were created by ligating *Eco*RI-digested and alkaline phosphatase-treated λZAP DNA with ≈7-kb *Eco*RI fragments of BG2087 (*mtr*$^+$) or WB3164 (*mtr3164*) chromosomal DNA. GigaPak Gold (Stratagene) was used for *in vitro* packaging.

The libraries were screened by plaque hybridization using ^{32}P-labeled fragments (11) containing either *aroB* (λEMBL3 library) or a portion of *mtr* (λZAP library), according to Benton and Davis (12).

PCR reactions (13) were carried out using an Ericomp Thermocycler and *Thermus aquaticus* polymerase from Perkin–Elmer/Cetus using the recommended reaction conditions. Oligonucleotides were synthesized using an Applied Biosystems model 380B DNA synthesizer. Southern analyses were performed by standard procedures (10) and DNA sequencing was performed using Sequenase from United States Biochemical. Sequence analysis was performed using the University of Wisconsin Genetics Computer Group package (14).

In vitro protein synthesis in *E. coli* S30 extracts (15) and SDS/urea polyacrylamide gel electrophoresis (16) were performed as described. β-Galactosidase assays were performed as described by Miller (17).

RESULTS

Cloning the *mtr* Locus from *B. subtilis*. Initial attempts to clone the *mtr* locus based on its close linkage to the selectable marker *aroB* (18) failed to yield any clones capable of complementing *mtr* mutants in trans. However, DNA from one isolate, λ1A, transformed CYBS11 (*aroB mtr264*) to *aroB*$^+$ *mtr*$^+$, but only by homologous recombination, indicating that it contained a portion of the *mtr* locus.

Southern analyses demonstrated that λ1A had the segment of the *mtr* region ending within a *Bcl* I site in the genome (Fig. 1). These analyses also indicated that the *mtr* locus should reside on a 7-kb *Eco*RI fragment. To clone this fragment we prepared libraries using *Eco*RI-digested *mtr*$^+$ genomic DNA ligated into λZAP (Stratagene) and probed these libraries with a fragment of *mtr* obtained from λ1A (Fig. 1). No positive clones were obtained. We then examined an *Eco*RI library prepared with DNA from an *mtr* mutant (*mtr3164*), reasoning that our inability to clone the intact *mtr* locus from *B. subtilis* might be due to toxicity of the *mtr* gene product to *E. coli*. (This hypothesis was subsequently shown to be incorrect.) Several positive clones were obtained. Attempts to obtain *in vivo* excision of the pBlueScript plasmid in λZap using R408 helper phage (Stratagene) were unsuccessful. Apparently, the *mtr*-containing fragment either interfered with *in vivo* excision or was lethal in *E. coli* when in a pBlueScript plasmid. DNA was prepared from one slow-growing *mtr* clone, λ7A, that contained the expected 7-kb *Eco*RI insert (Fig. 1). Attempts to subclone the entire insert into a variety of low-copy- and high-copy-number *E. coli* plasmids also failed. Accordingly, we subcloned fragments of the 7-kb insert and focused on the 1.5-kb *Nde* I–*Eco*RV fragment (Fig. 1), which extends ≈1 kb beyond the *Bcl* I site that marked the end of the λ1A insert.

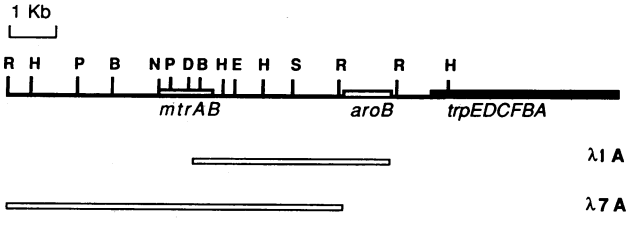

FIG. 1. Partial restriction map of the chromosomal region surrounding *mtr*, *aroB*, and *trpEDCFBA*. The inserts in the λ clones λ1A and λ7A are indicated below the map. R, *Eco*RI; H, *Hin*dIII; P, *Pvu* I; B, *Bcl* I; N, *Nde* I; E, *Eco*RV; S, *Sac* I; D, *Dra* I.

Nucleotide Sequence of the *Eco*RV–*Nde* I Fragment. The nucleotide sequence of the *Nde* I–*Eco*RV fragment was determined on both strands (Fig. 2). This fragment overlapped the *mtr*$^+$ segment of λ1A and contained two open reading frames, which we designated *mtrA* and *mtrB*. Wild-type *mtrA* and *mtrB* are predicted to encode 22-kDa and 8-kDa polypeptides, respectively. We found that the *mtr3164* mutation deleted one of eight adenines between base pairs (bp) 1207–1214 (Fig. 2); this deletion resulted in a read-through 26 codons beyond the *mtrB* stop codon. Immediately downstream of the *mtrB* stop codon is an inverted repeat capable of forming a stem and loop structure followed by a run of thymines, typical of factor-independent transcription terminators.

The *Nde* I site at one end of the subcloned fragment was only 45 bp upstream of the AUG start codon of *mtrA* and homologous integration of this fragment demonstrated that it lacked the *mtr* promoter (see below). To clone the region upstream of the *Nde* I site, we used the inverse PCR technique (19), exploiting the presence of a *Bcl* I site ≈2 kb upstream of the *Bcl* I site within *mtrB* (Fig. 1) and the *Pvu* I site within *mtrA*. The nucleotide sequence of 360 bp immediately upstream of the *Nde* I site was then determined (Fig. 2).

Localization of the *mtr* Operon. To localize the functional boundaries of the *mtr* operon, we used plasmid-mediated homologous integration of overlapping DNA fragments of the *mtr* region (Fig. 3). If both ends of the fragment were entirely within the *mtr* transcriptional unit, then homologous integration would disrupt the operon and only mutant progeny would be recovered. However, if the donor fragment contained either the promoter or the 3′ end of the *mtr* functional unit, then one intact *mtr* unit would be generated by integration, and all the progeny would be *mtr*$^+$. By integrating a number of plasmids containing inserts extending various lengths upstream of the *Nde* I site, we demonstrated that the *mtr* promoter is within 300 bp of the *Nde* I site. The 3′ end of the *mtr* functional unit was similarly located between the *Spl* I site and its proximal *Hin*dIII site.

On the basis of the above data, we reconstructed the entire *mtr* operon (bp 301–1488; Fig. 2) in a *B. subtilis*/*E. coli* shuttle vector. This plasmid, pSI45, complemented all *mtr* mutants in trans. Both *E. coli* and *B. subtilis* tolerate pSI45 containing the reconstructed operon.

Characterization of *mtr* Mutants. The *mtr* locus from several mutant strains was amplified by PCR, subcloned into pJM103 (4), and sequenced. Multiple clones from independent amplifications were analyzed. In addition, we confirmed that each change was responsible for the mutant phenotype by integrating the respective fragment into the wild-type chromosome. The changes detected are indicated in Table 1. Previously isolated (18) *mtr* alleles 222, 3159, 3160, and 3164 contained the same mutation, a deletion of an A·T base pair near the end of *mtrB*. Allele 264 has a mutation that converts the *mtrB* stop codon to a lysine codon; this extends the *mtrB* open reading frame 6 codons. Mutants 102, 109, 301, 302, and 303 have missense changes in *mtrB*. Two *mtr* mutants, 110 and 140, have changes that we believe decrease the activity of the *mtr* promoter (Fig. 2). In allele 110, a guanine is added at position 341, altering the spacing between the proposed −10 and −35 regions of the putative *mtr* promoter. Mutation 140 reduces homology to the consensus −35 promoter sequence.

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   1 GATTGACTCTGTTTTTGATACGATCTTAGATGCACTTAAAAACGGTGATA
  51 AAATCCAACTGATCGGTTTTGGTAACTTCGAGGTGCGTGAACGTTCTGCA
 101 CGTAAAGGACGCAATCCTCAAACAGGTGAAGAAATCGAAATTCCAGCAAG
 151 CAAAGTACCTGCTTTCAAACCAGGTAAAGCGCTTAAAGACGCAGTTGCCG
 201 GAAAATAATTGTGAATATAGATCGTGTATGCATCTAGCTTACATACACTT
 251 TATTTCTTCACAGAAAAGCCCCTTTCTAAGGGGCTTTTCATATTTCAAGA
                          TTGACA
 301 GCATGGGCTTCCTGACAGGGCATTCACTTTGCTTTTAGCGGGGCATATGT

       TATAAT
 351 GCTAGAATCGAAATTAAATGTATTCATTGGTGGAGGACATAGAACATGAA
                                                    MetLy

 401 AGAAGTTAATAAAGAGCAAATCGAACAAGCTGTTCGTCAAATTTTAGAAG
     sGluValAsnLysGluGlnIleGluGlnAlaValArgGlnIleLeuGluA

 451 CGATCGGAGAAGACCCGAATAGAGAAGGGCTTCTTGATACTCCGAAAAGA
     laIleGlyGluAspProAsnArgGluGlyLeuLeuAspThrProLysArg

 501 GTCGCAAAGATGTATGCCGAAGTATTCTCCGGCTTGAATGAAGATCCAAA
     ValAlaLysMetTyrAlaGluValPheSerGlyLeuAsnGluAspProLy

 551 AGAACATTTCCAGACTATCTTCGGTGAAAACCATGAGGAGCTTGTTCTTG
     sGluHisPheGlnThrIlePheGlyGluAsnHisGluGluLeuValLeuV

 601 TAAAAGATATAGCGTTTCATTCTATGTGTGAGCATCACCTTGTTCCCTTT
     alLysAspIleAlaPheHisSerMetCysGluHisHisLeuValProPhe

 651 TATGGAAAAGCACATGTTGCATATATCCCGCGAGGCGGAAAGGTCACAGG
     TyrGlyLysAlaHisValAlaTyrIleProArgGlyGlyLysValThrGl

 701 ACTCAGCAAACTGGCACGTGCCGTTGAAGCCGTTGCAAAGCGCCCGCAGC
     yLeuSerLysLeuAlaArgAlaValGluAlaValAlaLysArgProGlnL

 751 TTCAGGAACGCATCACTTCTACAATTGCAGAAAGCATCGTAGAAACGCTT
     euGlnGluArgIleThrSerThrIleAlaGluSerIleValGluThrLeu

 801 GATCCGCATGGCGTAATGGTAGTGGTTGAAGCGGAACACATGTGCATGAC
     AspProHisGlyValMetValValValGluAlaGluHisMetCysMetTh

 851 GATGCGCGGTGTAAGAAAACCGGGTGCGAAAACTGTGACTTCAGCAGTCA
     rMetArgGlyValArgLysProGlyAlaLysThrValThrSerAlaValA

 901 GAGGCGTTTTTAAAGATGATGCCGCTGCCCGTGCAGAAGTATTGGAACAT
     rgGlyValPheLysAspAspAlaAlaAlaArgAlaGluValLeuGluHis

 951 ATTAAACGCCAGGACTAATAAAGATAGAGGGTGCTATAAATGAACCAAAA
     IleLysArgGlnAspEnd                          MetAsnGlnLy

1001 GCATTCAAGTGATTTTGTCGTCATTAAAGCCGTTGAGGACGGAGTGAATG
     sHisSerSerAspPheValValIleLysAlaValGluAspGlyValAsnV

1051 TGATCGGCCTGACAAGAGGAACAGACACAAAGTTTCACCATTCCGAAAAA
     alIleGlyLeuThrArgGlyThrAspThrLysPheHisHisSerGluLys

1101 CTCGACAAGGGAGAAGTGATCATCGCTCAGTTTACAGAGCATACTTCTGC
     LeuAspLysGlyGluValIleIleAlaGlnPheThrGluHisThrSerAl

1151 CATTAAAGTCAGAGGAGAGGCACTGATTCAAACCGCGTACGGAGAAATGA
     aIleLysValArgGlyGluAlaLeuIleGlnThrAlaTyrGlyGluMetL

1201 AAAGCGAAAAAAAATAAGCTGGCTGTCCCCGCTGTTAAAAGCAAATGCTC
     ysSerGluLysLysEnd

1251 GCAAACAGCGGGAGGATACAGCCAATTTCTTTTTTTTATGCTATAATGAT

1301 ACCTGCGTAAGCGAATGAAAAGATAAAACTCATACATAGATTAAGAAATA
1351 AATGTGAATTTGGGGACAAGGGTGATATTTTTGCAAGACATCTACGGAAC
1401 TTTAGCCAATCTGAACACGAAATTAAAACAAAAGCTGTCTCATCCTTATT
1451 TAGCGAAGCATATTTCTGCGCCGAAAATTGATGAGGATAAGCTTCTTCTT
1501 TTTCATGCTTTATTTGAAGAAGCCGACATAAAAAACAACGACAGAGAAAA
1551 TTATATTGTAACAGCGATGCTTGTACAAAGCGCCCTTGATACCCATGATG
1601 AAGTGACGACAGCTAGAGTCATAAAACGAGACGAAAACAAAAACCGCCAA
1651 TTGACTGTTCTCGCGGGCGATTATTTCAGCGGGCTGTACTACTCTTTACT
1701 ATCTGAAATGAAGGATATCTACATGATTCGGACGCTTGCTACAGCCATTA
1751 AAGAAATCAACGAACATAAAATTCGTCTGTATGACCGTTCTTTCAAGGAC
1801 GAAAACGATTTTTTCGAAAGTGTCGGCGTCGTTGAATCAGCTTTATTCCA
1851 TCGTGTGGCGGAACACTTCAACCTCCCGCGCTGGAAAAAGCTGTCGAGTG
1901 ATTTTTTTGTATTTAAGCGGCTTATGAACGGAAATGATGCATTTCTGGAT
1951 GTGATCGGCAGTTTTATACAGCTGGGAAAAACAAAAGAAGAG  1992
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FIG. 2. Nucleotide sequence of the *B. subtilis mtr* operon and surrounding regions. The derived amino acid sequences of *mtrA* and *mtrB* are indicated and the consensus promoter sequence is shown directly above the proposed −10 and −35 regions. The stems of a putative transcription terminator sequence are underlined. Lines are above proposed ribosome binding sites for *mtrA* and *mtrB*.

None of the above mutations was in *mtrA*. We attempted to isolate mutations in *mtrA* by selecting for 5-fluorotryptophan resistance in strains (BG4232 and BG4231) diploid for *mtrB* or diploid for both *mtrA* and *mtrB*. Several *mtr* mutants were isolated and characterized but none had changes in *mtrA* (Table 2). Some of these mutants were shown to have missense changes in one or the other copy of *mtrB* and to confer a dominant mutant phenotype when both copies of *mtrA* and *mtrB* were expressed. The changes in three mutants of this type, 301, 302, and 303, were determined (Table 1). These findings suggest that the Mtr protein is oligomeric, possibly containing two copies of the MtrB polypeptide.

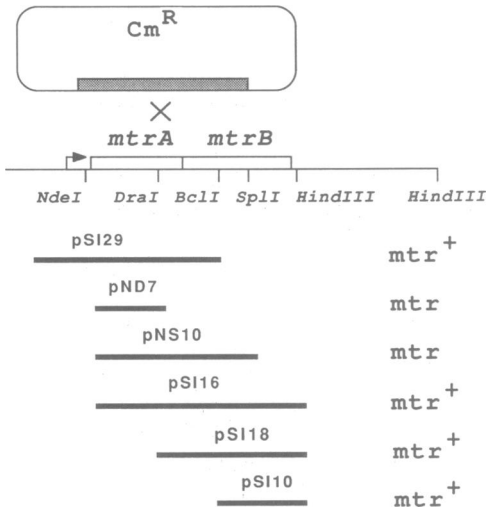

FIG. 3. Mapping the extent of the *mtr* operon by homologous integration into CYBS12. The inserts present in the plasmids used are indicated below the abbreviated restriction map of the *mtr* operon. The phenotype of progeny resulting from integration is indicated.

Insertion Analysis. To analyze the roles of *mtrA* and *mtrB* in greater detail, transformants of CYBS12 were prepared by homologous integration of either of two plasmids, one containing only the beginning of *mtrA* and the other containing the end of *mtrA* and the beginning of *mtrB*, driven by the inducible *spac* promoter (3). Integration yielded the two arrangements shown in Fig. 4: In transformant type A, *spac* controls both *mtrA* and *mtrB*, while in transformant type B, *mtrA* is intact and is driven by the *mtr* promoter and *spac* controls *mtrB*. In both cases the resulting transformants were *mtr* in the absence of isopropyl β-D-thiogalactoside and *mtr*$^+$ in its presence. These results confirm that *mtrB* plays a role in regulating the *trp* operon. Deleting *mtrB* from the chromosome also gives the Mtr phenotype. Attempts to delete *mtrA*, or both *mtrA* and *mtrB*, were unsuccessful, as were efforts to disrupt *mtrA* by homologous integration.

Expression of *mtrA* and *mtrB*. Expression of *mtrB* or of *mtrA* and *mtrB* was achieved using *tac* promoter fusions in an

Table 1. Characterization of *mtr* mutations

mtr allele	Change	Result of change
264	T-1215 → A	*mtrB* TAA (stop) → AAA (Lys)
222, 3159	Delete one A	Read-through at *mtrB* stop
3160, 3164	(1207–1214)	
102	T-1120 → A	Ile-44 → Asn in MtrB
109	C-1101 → T	Leu-38 → Phe in MtrB
110	Add one G at 341	Alters spacing in promoter
140	G-331 → A	Alters proposed −35 region
301*	T-1060 → C	Leu-24 → Pro in MtrB
302*	C-1089 → T	His-34 → Tyr in MtrB
303*	G-1057 → A	Gly-23 → Asp in MtrB

*Negative complementing mutant.

Table 2. β-Galactosidase production in *E. coli* strains containing a *trpE–lacZ* fusion under control of the *B. subtilis trp* promoter–leader region

Plasmids present	Relative β-galactosidase activity	
	− Trp	+ Trp
ptrpE-lacZ + ptacterm	1.00 ± 0.06	0.92 ± 0.07
ptrpE-lacZ + pTTmtrB	1.08 ± 0.18	0.98 ± 0.16
ptrpE-lacZ + pTTmtrAB	0.25 ± 0.02	0.10 ± 0.02

Values shown are the average (± SD) of three experiments. The value obtained with ptrpE-lacZ + ptacterm in the absence of tryptophan was set at 1.0.

in vitro transcription/translation system (15). pTTmtrB directed the synthesis of a labeled MtrB polypeptide of the approximate predicted mass (8 kDa), and pTTmtrAB directed the synthesis of both this polypeptide and the expected 20-kDa MtrA polypeptide (Fig. 5). These polypeptides also were synthesized *in vivo* in *E. coli*, using a T7 promoter expression system (20) (data not shown).

Regulation of the *B. subtilis trp* Operon in *E. coli*. To determine whether MtrA is required for *trp* operon regulation and whether MtrA and MtrB are sufficient for this regulation, we constructed a plasmid with the *B. subtilis trp* promoter-leader-*trpE* region (the first 40 codons of *trpE*) fused in-frame to *E. coli lacZ*. We introduced this plasmid into *E. coli* and measured β-galactosidase production directed by this fusion, in the presence or absence of *mtr*-expressing plasmids. In the absence of *mtr*-expressing plasmids, β-galactosidase production by the recipient strain was not regulated by tryptophan (Table 2). Expressing *mtrB* alone in this strain had no effect on the β-galactosidase levels. However, expressing both *mtrA* and *mtrB* decreased the β-galactosidase level to one-third in minimal medium and to one-tenth in the presence of tryptophan. These results suggest that both *mtrA* and *mtrB* are required for attenuation control of the *B. subtilis trp* operon.

DISCUSSION

We have cloned and analyzed the *mtr* locus of *B. subtilis*. This locus is required for attenuation control of the *trp* operon of this organism (1, 2). *mtr* was found to be an operon consisting of two genes, *mtrA* and *mtrB*. Homologous integration studies and mutant analyses indicate that the *mtr* promoter lies immediately upstream of *mtrA*. The proposed −10 region of the *mtr* promoter matches the consensus sequence at five of six positions; however, the proposed −35 region matches the consensus at only three of six positions (Fig. 2). An apparent transcription terminator is located just beyond *mtrB*. An autonomously replicating *B. subtilis* plasmid containing bp 301–1488 (Fig. 2) complemented all classes of *mtr* mutants, suggesting that this fragment contains the entire sequence responsible for *mtr* expression and function.

Several lines of evidence indicate that *mtrB* expression is required for *trp* operon regulation. We have characterized seven mutations in *mtrB* that result in constitutive expression of the *trp* operon. Three of these are trans-dominant, suggesting that the regulatory protein is oligomeric. Additionally, when *mtrB* is placed under control of the inducible *spac* promoter, regulation of the *trp* operon becomes isopropyl β-D-thiogalactoside-dependent.

The evidence that *mtrA* is required for regulating the *trp* operon is not as clear as that for *mtrB*. To date, *mtrA* involvement in *trp* operon regulation has only been demonstrated in *E. coli*, where expression of *mtrB* alone was insufficient to achieve regulation of a *B. subtilis trpE–lacZ* fusion. These *in vivo* experiments also suggest that no *B. subtilis* protein besides MtrA and MtrB is necessary for Mtr regulation of the *trp* operon in *E. coli*.

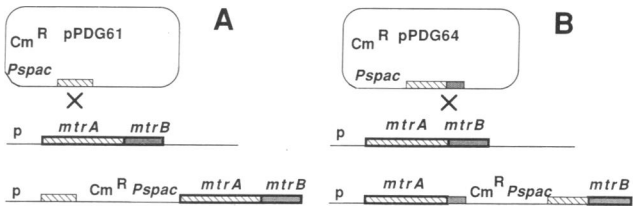

FIG. 4. Homologous integration that places subregions of the *mtr* operon under control of the regulated *spac* promoter. pPDG61 contains the *Nde* I–*Dra* I fragment of *mtrA*, and pPDG64 contains nucleotides 800 (near the end of *mtrA*) through 1117 (within *mtrB*). Arrangements after integration of pPDG61 and pPDG66 are indicated in *A* and *B*, respectively. Intact genes are indicated by heavily outlined boxes.

Attempts to obtain point mutations in *mtrA*, to disrupt it, or to delete it were unsuccessful. Even when we selected *mtr* mutants in a strain diploid for *mtrB*, we obtained only dominant *mtrB* mutations. Perhaps *mtrA* is an essential gene in *B. subtilis*. If this is the case, then the putative promoter mutants *mtr110* and *mtr140* must decrease expression of the *mtr* operon only partially, so that enough MtrA is synthesized to provide this essential function. Similarly, in the integrants in which Pspac drives expression of *mtrA* (see Fig. 3), cells survive in the absence of inducer. This would be explained if there is a low level of uninduced expression of *mtrA*. Expressing *mtrB* in trans in the *mtr110* strain restored regulation of the *trp* operon (data not shown), indicating that the Mtr phenotype of *mtr110* is due to insufficient production of MtrB.

Computer searches of the EMBL (May 1990; release 23.0) and National Biomedical Research Foundation (June 1990; release 25.0) protein bases did not reveal any significant homologies to MtrA or MtrB. However, when we compared MtrA and MtrB directly with known RNA-binding proteins, we observed some homology between MtrB and RegA from bacteriophage T4 (21) (Fig. 6). The RegA protein is a translational repressor that acts by binding to ribosome binding sites and preventing initiation of translation (22). Although MtrB and RegA are only 20% identical overall, there are many conservative amino acid differences, indicating that overall similarity is 50%. The homology between MtrB and RegA extends uniformly throughout these polypeptides and only two small gaps must be introduced to obtain optimal alignment. MtrB and RegA may, therefore, use similar structural features in RNA recognition. Neither Mtr polypeptide

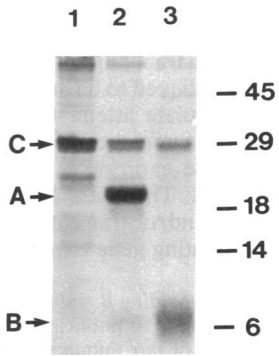

FIG. 5. SDS/urea/polyacrylamide gel electrophoresis (16) in 20% gels of ^{35}S-labeled polypeptides produced in an *in vitro*-coupled transcription–translation system used the following plasmids as templates. Lanes: 1, pTacTerm (control); 2, pTTmtrAB; 3, pTTmtrB. A and B mark the MtrA and MtrB polypeptides; C denotes β-lactamase. Molecular size standards (in kDa) are indicated.

```
                .         .    *DP*   .    Y* F        N
MtrB  1 ...MNQKHSSDFVVIKAVEDGVNVIGLTRGTDTKFHHSEKL..DKGEVII 45
          :. |...||: :|   :.:. :|:....|. :.:| .:  .||  .|
RegA  1 MIEITLKKPEDFLKVK...ETLTRMGIANNKDKVLYQSCHILQKKGLYYI 47

                .         .         .         .
MtrB 46 AQFTEHTSAIKVRGEALIQTAYGEMKSEKN..................... 75
        .:|.|    ::: | .:  |. :|::.:.
RegA 48 VHFKE...MLRMDGRQVEMTEEDEVRRDSIAWLLEDWGLIEIVPGQRTFM 94
```

FIG. 6. Amino acid sequence homology between MtrB and RegA as determined using the BestFit program in the sequence analysis software package of the University of Wisconsin Genetics Computer Group. Missense changes in the characterized *mtrB* mutants are indicated above the MtrB sequence. Asterisks mark the changes in negative complementing mutants. | indicates identical amino acids, : indicates highly homologous amino acids, and . indicates somewhat homologous amino acids.

shows homology to known members of two component regulatory systems (4, 23–25).

Studies with the related organism *Bacillus pumilus* suggest that its *trp* operon is regulated by a similar mechanism of transcription attenuation (26). A fragment of the *mtr* locus from *B. pumilus* has been cloned, and its sequence suggests that this bacillus contains an operon with genes highly homologous (>80%) to *B. subtilis mtrA* and *mtrB* (data not shown). Thus not only is *trp* operon expression regulated by a similar mechanism of attenuation in both bacilli, but the trans-acting regulator is highly conserved.

A potential Mtr binding site has been located in the tryptophan-regulated *trpG* gene of *B. subtilis* (Donald Stahly, personal communication). *trpG* is the only tryptophan biosynthetic gene of *B. subtilis* not in the *trp* operon (27); it is located within an apparent folic acid biosynthetic operon. It was proposed that Mtr represses *trpG* translation by binding at the *trpG* ribosome-binding site (Donald Stahly, personal communication). *mtr* expression also has been shown to affect translation of the *trp* operon transcript (2). This effect is presumed to be due to bound Mtr inducing the formation of an RNA secondary structure that sequesters the *trpE* ribosome binding site (2). Thus it appears that Mtr regulates expression of a number of genes in response to tryptophan availability and does so by several mechanisms. These properties illustrate the versatility of this type of RNA-binding regulatory protein.

Attenuation control by a trans-acting RNA-binding factor may be a common regulatory mechanism. The *pur* operon of *B. subtilis* is regulated by availability of adenine and guanine nucleotides (28). A hypothetical guanine nucleotide-activated regulator is proposed to bind to the leader RNA and promote formation of a terminator structure, causing transcription termination (29). SacY of *B. subtilis*, a putative antitermination protein, regulates expression of the levansucrase (*sacB*) gene in response to the presence of sucrose (4, 30). In this case, the presumed RNA-binding protein is believed to activate transcription by masking a transcription terminator. The product of *bglG* of *E. coli* is believed to be an RNA-binding protein that acts similarly to regulate attenuation in the *bgl* operon (31). Thus Mtr may be a member of a class of regulatory proteins that control gene expression by binding to specific target sequences in RNA. This class may include regulators that affect transcription and/or translation and are capable of either inhibiting or activating gene expression.

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