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Regulation of Expression of the *Escherichia coli* K-12 *mtr* Gene by TyrR Protein and Trp Repressor

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The *Escherichia coli* K-12 *mtr* gene, which encodes a tryptophan-specific permease, was cloned, and its nucleotide sequence was determined. The precise location of the *mtr* gene at 69 min on the *E. coli* chromosome was determined. The *mtr* gene product was identified as a 414-amino-acid residue protein with a calculated molecular weight of 44,332. The protein is very hydrophobic, consistent with its presumed location spanning the cytoplasmic membrane. The initiation sites of transcription and translation were identified. Construction of an *mtr-lacZ* transcriptional fusion facilitated investigation of the molecular basis of *mtr* regulation. The TyrR protein in association with phenylalanine or tyrosine is responsible for the activation of *mtr* expression, whereas the Trp repressor in conjunction with tryptophan serves to repress expression of this gene. Site-directed mutagenesis confirmed that sequences in the *mtr* regulatory region homologous to TyrR protein and to Trp repressor-binding sites were involved in the activation and repression of *mtr* expression, respectively. Sequences homologous to σ^{70}- and σ^{54}-dependent promoters were identified upstream of the transcription start point of *mtr*. It was determined that transcription of *mtr* occurs only via a σ^{70}-dependent promoter.

In *Escherichia coli* the uptake of tryptophan is known to occur via at least three transport systems: a general aromatic amino acid transport system encoded by the *aroP* gene (mapping at 3 min [11, 17]); a low-affinity tryptophan permease encoded by a gene in the same transcription unit as *tnaA* (at 84 min [21, 26]), which is variously designated as *trpP* or *tnaB* (5, 15, 64); and the high-affinity tryptophan transport system encoded by the *mtr* gene (at 69 min [35, 48, 69]). Strains harboring a mutation in this latter gene are resistant to growth inhibition by 5-methyltryptophan (35), as they are unable to accumulate this analog of tryptophan at a level at which it interferes with protein synthesis.

Transport assays have indicated that expression of the *mtr* gene is enhanced when cells are grown in medium supplemented with phenylalanine, but that this effect is negated if tryptophan is also present (68). Induction mediated by phenylalanine requires functional TyrR protein, the product of the *tyrR* gene (20), making *mtr* a part of the TyrR regulon (49). Little is known about the molecular events which mediate the repression by tryptophan.

In this communication we describe the cloning of the *mtr* gene and identify the initiation sites of transcription and translation. The molecular basis of the activation and repression of *mtr* expression is also investigated. In addition to the effects of the TyrR protein, the role of the Trp repressor, known to regulate transcription initiation of the *trp* operon (72), *aroH* (29, 73), and its own structural gene, *trpR* (30, 39), is evaluated in terms of the regulation of the *mtr* gene.

(A preliminary report of this work has been presented [59].)

MATERIALS AND METHODS

Bacterial strains, plasmids, and phages. The bacterial strains used in this study were all derivatives of *Escherichia coli* K-12, and their relevant genotypes are described in Table 1. The plasmids used are also listed in Table 1. The bacteriophages M13mp18 and M13mp19 have been described previously (43).

Media and chemicals. The minimal medium used was the half-strength buffer 56 of Monod et al. (45), supplemented with 0.2% glucose and appropriate growth factors. To study regulation, we added phenylalanine, tyrosine, and tryptophan to the minimal medium at a final concentration of 1 mM each. Trimethoprim was used in nutrient and minimal medium at final concentrations of 40 and 10 μg/ml, respectively. Ampicillin and kanamycin were used at final concentrations, in all media, of 25 and 10 μg/ml, respectively. [α-^{35}S]dATP (1,200 Ci/mmol; 10 mCi/ml) and [γ-^{32}P]ATP (ca. 2,000 to 3,000 Ci/mmol; 10 Ci/ml) for use in DNA sequencing and primer extension analysis were obtained from Amersham International, Amersham, United Kingdom. L-[3-^{14}C]tryptophan (52 Ci/mmol; 0.02 mCi/ml) for use in transport assays was obtained from New England Nuclear Research Products, Boston, Mass. Oligonucleotides were synthesized on a Gene Assembler Plus (Pharmacia-LKB, Uppsala, Sweden).

Recombinant DNA techniques. Standard recombinant DNA procedures were used essentially as described by Sambrook et al. (56).

Nucleotide sequence determination. The DNA fragment to be sequenced was cloned in both orientations into the *Sma*I site of M13mp18, and sets of overlapping deletions were generated with exonuclease III, following digestion of the DNA with *Bam*HI and *Pst*I (34). DNA sequencing was carried out by using the dideoxy-chain termination method of Sanger et al. (57), with T7 DNA polymerase (Pharmacia). The complete sequence of both strands was determined, and areas of compression were resolved by substituting dITP for dGTP in the sequencing reactions. Data were analyzed with the MELBDBSYS suite of computer programs developed by A. Kyne from the original DB system of Staden (62, 63).

Primer extension. A synthetic 22-base oligonucleotide primer was labeled at its 5′ end with [γ-^{32}P]ATP by using T4 polynucleotide kinase, followed by passage through a 1-ml

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TABLE 1. *E. coli* K-12 strains and plasmids used in this study

Strain or plasmid	Relevant characteristics[a]	Source or reference
Strains		
JP2116	*his-1 metB1 aroP mtr-24*	D. Tribe
JP3561	*thr-1 leu-1 thi-1 lacZΔM15*	37
JP4822	JP3561 *tyrR366*	71
JP7216	JP2116 *tyrR366*	P1 *kc* transduction
JP7217	JP3561(pMU1065)	By transformation
JP7218	JP3561 *trpR363*	P1 *kc* transduction
JP7219	JP4822 *trpR363*	P1 *kc* transduction
JP7220	JP3561(pRPG16)	By transformation
Plasmids		
pB13-1	Ap^r Tc^r, 12.9-kb *Hin*dIII fragment containing *mtr* gene in pBR322	52
pBR322	Ap^r Tc^r, ColE1 derivative	10
pMU525	Tp^r, *lac'ZYA'* low-copy-number gene fusion vector, IncW replicon	53
pMU577	Tp^r, *galK'-lac'ZYA'*, low-copy-number transcriptional fusion vector; derivative of pMU575 (71) containing modified polylinker, IncW replicon	This laboratory
pMU1065	Km^r, 1.77-kb *Eco*RV-*Pvu*II fragment containing *tyrR* gene (20) in pACYC177 (16)	E. Cornish
pMU3175	Ap^r, 2.6-kb *Eco*RI-*Kpn*I fragment containing *mtr* gene in pBR322	This study
pMU3185	Tp^r, 383-bp *mtr* fragment in pMU525, *mtr-lacZ* translational fusion	This study
pMU3186 to pMU3188	Derivatives of pMU3185 constructed by site-directed mutagenesis (see Fig. 4)	This study
pMU3190	Tp^r, 189-bp *mtr* fragment in pMU577, *mtr-lacZ* transcriptional fusion	This study
pMU3191 to pMU3207	Derivatives of pMU3190 constructed by site-directed mutagenesis (see Fig. 4)	This study
pRPG16	Km^r, 1.3-kb *Bam*HI fragment containing *trpR* gene in pACYC177 (16)	39

[a] The genetic nomenclature is that described by Bachmann (5). Allele numbers are indicated where known.

Sephadex G-50 spun column (56). This probe was used to determine the 5′ end of in vivo transcripts by the primer extension technique described by Hudson and Davidson (36).

Plasmid construction. The plasmid pMU3190 contains the regulatory region of the *mtr* gene transcriptionally fused to the *lacZ* gene. Construction of this vector was facilitated by making use of an M13mp18 derivative, which had been prepared by exonuclease III digestion as part of the sequencing strategy and which contained a DNA fragment from position 26 to the *Kpn*I restriction site (see Fig. 2 and 3). This vector was digested with *Hin*dIII, which cuts in the M13mp18 polylinker, and *Bgl*I, which cuts at position 214 in the *mtr* DNA. A fragment containing 189 bp of *mtr* DNA (nucleotides 26 to 214) and some M13mp18 polylinker DNA was isolated, and the ends were made blunt by treatment with T4 DNA polymerase. This fragment was cloned into the *Sma*I site of the low-copy-number, promoter-cloning vector, pMU577. This vector is identical to the previously described pMU575 (71), except for the addition of *Eco*RI and *Bgl*II restriction sites in the polylinker region. The entire *mtr* fragment and its junctions with the vector were sequenced. The resultant construct placed the expression of the *lacZ* gene under the control of transcriptional events taking place at the *mtr* promoter.

The plasmid pMU3185 contains the regulatory region and part of the coding region of the *mtr* gene translationally fused to the *lacZ* gene. A 383-bp DNA fragment extending from positions 14 to 396 was amplified by the polymerase chain reaction (56) and cloned into M13mp18, where its sequence was verified. The fragment was then cloned into the low-copy-number, translational-fusion vector pMU525 (53), such that codon 60 of the *mtr* gene was joined in phase to codon 8 of the *lacZ* structural gene. Expression of β-galactosidase from this construct is placed under the control of transcription and translation initiation signals present in the *mtr* gene.

Site-directed mutagenesis. Site-directed mutagenesis with synthetic oligonucleotides was performed on M13mp18 or M13mp19 derivatives containing the *mtr* DNA fragment from pMU3185 or pMU3190, by the method of Taylor et al. (67), with a commercial kit (Amersham). Following the isolation of desired mutations, the entire *mtr* fragment was sequenced to ensure that only the desired change was present. The *mtr* fragments were then cloned into pMU525 or pMU577, the resultant constructs were transformed into relevant *lacZ* strains, and β-galactosidase assays were performed.

Assay of β-galactosidase activity. Cultures were grown in half-strength buffer 56, containing 0.2% glucose and required growth factors, at 37°C in a rotary water bath to the mid-exponential phase. β-Galactosidase activity was assayed as described by Miller (44). Each assay was performed in duplicate on at least four separate occasions. The extent of repression is presented as the ratio of β-galactosidase activity obtained in a *tyrR trpR* strain to that in a $trpR^+$ strain. The extent of activation is presented as the ratio of β-galactosidase activity obtained in a $tyrR^+$ strain to that in a *tyrR trpR* strain.

Transport assays. The uptake of [^{14}C]tryptophan was assayed essentially as described by Wookey et al. (70).

Nucleotide sequence accession number. The nucleotide sequence data reported in this communication have been submitted to the GenBank data base and assigned the accession number M58338.

RESULTS

Cloning of the *mtr* gene. The *infB* gene (69 min), which codes for translation initiation factor IF2, was isolated on a 40-kb DNA fragment by Plumbridge et al. (51). Maxicell analysis indicated that this fragment also contained a number of other genes (52). Both the *infB* and *mtr* genes are known

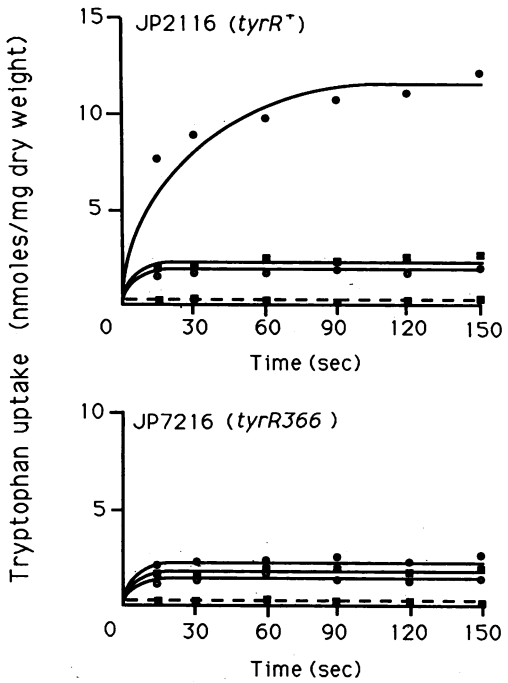

FIG. 1. Uptake of tryptophan by isogenic *tyrR*$^+$ and *tyrR* strains in the presence (——) or absence (– – –) of plasmid pB13-1. Strains were grown in minimal medium (■), minimal medium containing 1 mM phenylalanine (●), or minimal medium containing 1 mM phenylalanine and 1 mM tryptophan (○). The uptake of tryptophan in strains not harboring the plasmid pB13-1 grown in the presence of phenylalanine or tryptophan was not significantly different from that in strains grown solely in minimal medium. Strains were grown in 0.2% glucose as the carbon source, under which conditions expression of the *tnaB* gene is minimal as a result of catabolite repression.

to be closely linked to *argG* (35, 51), and it was therefore possible that *mtr* was present on this fragment. A 12.9-kb *Hin*dIII fragment known to harbor a number of genes, including *rpsO* and *pnp*, was subcloned into the vector pBR322 to produce plasmid pB13-1 (52). We obtained plasmid pB13-1 and found that its presence in *mtr* strains eliminated their resistance to 5-methyltryptophan. The plasmid was introduced into strains JP2116 and JP7216, which are isogenic *tyrR*$^+$ and *tyrR* strains that are unable to actively transport tryptophan (*aroP mtr*). These strains were grown in the presence or absence of phenylalanine and tryptophan and assayed for tryptophan transport (Fig. 1). The results confirmed that pB13-1 contained a gene which codes for a tryptophan permease whose synthesis is regulated in the same manner as that expected of the *mtr* gene (68).

A restriction map of the 12.9-kb *Hin*dIII fragment was produced (Fig. 2). Further subcloning indicated that the 2.6-kb *Eco*RI-*Kpn*I fragment was the smallest DNA fragment which showed the same regulated expression of *mtr* (data not shown). Subcloning of this fragment indicated that the gene spanned both the *Bgl*I and *Bgl*II restriction sites. Comparison with the *E. coli* physical map of Kohara et al. (41) revealed that the 12.9-kb *Hin*dIII fragment was homologous to a region near 69 min on the chromosome. The *mtr* gene is located between positions 3370 and 3375 kb and is expected to be present on lambda Miniset clone 518 (41).

The nucleotide sequence of both strands of the 2.6-kb *Eco*RI-*Kpn*I fragment was determined. While this communication was being completed, we became aware that Heatwole and Somerville had also sequenced the *mtr* gene (33). The sequences are in agreement except at the following positions: CG rather than GC at positions 68 and 69 (Fig. 3) (positions 324 and 325 in reference 33); A rather than T at position 57 (33); and the addition of C, G, and C residues between positions 1759 and 1760, 1763 and 1764, and 1765 and 1766 (33), respectively. We therefore present only the

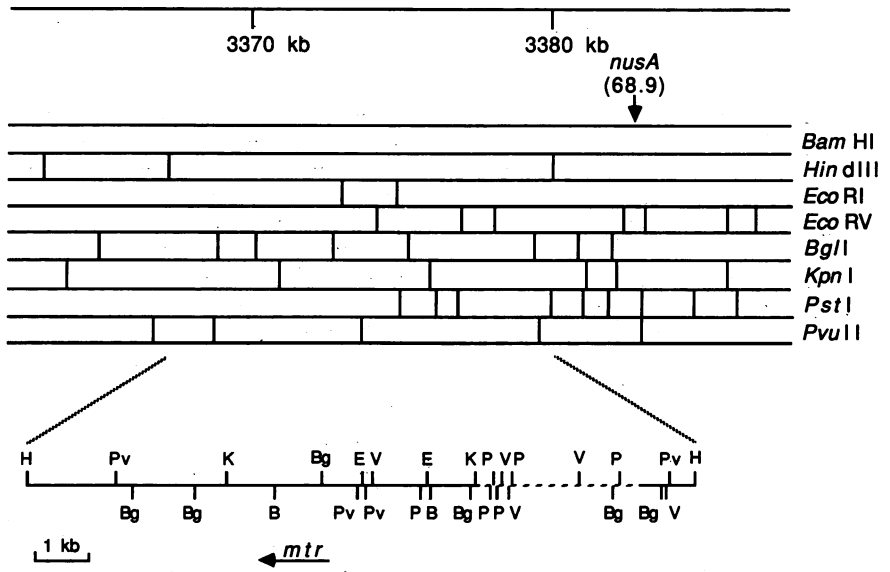

FIG. 2. Location of the *mtr* gene on the physical map of the *E. coli* chromosome. The 12.9-kb *Hin*dIII fragment of pB13-1 was mapped with all the restriction enzymes used in the construction of the *E. coli* physical map of Kohara *et al.* (41), with the addition of *Bgl*II. A section of the physical map from positions ca. 3360 to 3390 kb, exhibiting a similar restriction pattern, is redrawn above the restriction enzyme map of the 12.9-kb *Hin*dIII fragment. The nucleotide sequence of the segment represented by the broken line has been previously determined (54, 66). Abbreviations: B, *Bgl*II; Bg, *Bgl*I; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; P, *Pst*I; Pv, *Pvu*II; V, *Eco*RV.

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GAAGGCCGCGCTCCGCGTCGTGATGATTCTACCGGTCGTCGTCGTTTCGGTGGTGATGCG   60
TAATCATCGCTGAACAGCGAACACAATCTGTAAAATAATATATACAGCCCCGATTTTTAC  120
                          TYR R box
CATCGGGGCTTTTTTTCTGTCTTTTGTACTCGTGTACTGGTACAGTGCAATGCATAACAA  180
          -35  Trp-repressor  binding  site  -10        **
                                                M  A  T  L  T  T  T  Q     8
CGCAGTCGCACTATTTTTCACTGGAGAGAAGCCCTCATGGCAACACTAACCACCACCCAA  240
                          SD      BglI
 T  S  P  S  L  L  G  G  V  V  I  I  G  G  T  I  I  G  A  G    28
ACGTCACCGTCGCTGCTTGGCGGCGTGGTGATTATCGGCGGCACCATTATTGGCGCAGGG  300

 M  F  S  L  P  V  V  M  S  G  A  W  F  F  W  S  M  A  A  L    48
ATGTTTTCTCTGCCAGTGGTCATGTCCGGGGCGTGGTTTTTCTGGTCAATGGCGGCGCTG  360

 I  F  T  W  F  C  M  L  H  S  G  L  M  I  L  E  A  N  L  N    68
ATCTTTACCTGGTTCTGTATGCTGCATTCCGGCTTGATGATTCTGGAAGCTAACCTGAAT  420
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FIG. 3. Nucleotide sequence of the antisense strand of the *mtr* regulatory region. A TYR R box and Trp repressor-binding site are indicated in bold type. The −35 and −10 regions of a σ^{70}-dependent promoter are underlined, and a putative σ^{54}-dependent promoter sequence is overlined. The transcription initiation site is marked by a double asterisk, and a putative Shine-Dalgarno (SD) sequence is indicated by a double underline. The *Bgl*I restriction endonuclease site used in the construction of the *mtr-lacZ* transcriptional fusion is underlined.

sequence relevant to the work detailed in this communication, namely the regulatory region of the *mtr* gene and some sequence of the structural region (Fig. 3).

Computer analysis revealed a large open reading frame beginning with an ATG codon at position 217 and extending for 1,245 bp. This open reading frame is preceded by a possible Shine-Dalgarno sequence (60) and codes for a protein consisting of 414 amino acid residues. The protein has a calculated molecular weight of 44,332 and is highly hydrophobic, consistent with its presumed location spanning the cytoplasmic membrane. No other substantial open reading frames were present in the sequenced fragment.

To verify the start point of translation of the *mtr* gene product, we constructed an *mtr-lacZ* translational fusion as described in Materials and Methods. The gene fusion codes for a hybrid protein whose transcription and translation are dependent on signals present in the *mtr* DNA fragment. The ATG codon at positions 217 to 219 is the first such codon present in the *mtr* mRNA and is preceded by a possible Shine-Dalgarno sequence. No other ATG or GTG triplet in the 5′ end of the mRNA is preceded by such a sequence. The A residue of the ATG codon at position 217 was therefore changed to G and C residues by site-directed mutagenesis (see Materials and Methods). A termination codon was also inserted between the putative initiation codon and the next ATG codon at positions 361 to 363 by converting the TCA (Ser) codon at positions 244 to 246 to a TAA (stop) codon. The plasmids harboring these mutations were designated pMU3186, pMU3187, and pMU3188, respectively (Fig. 4). β-Galactosidase assays indicated that a GTG codon at positions 217 to 219 resulted in approximately a 75% reduction in expression compared with that of the wild-type sequence, whereas a CTG codon at this position led to a 90% reduction in expression (Table 2). The presence of a termination codon at positions 244 to 246 virtually abolished expression. These findings should indicate that translation of the *mtr* gene commences at the ATG codon at positions 217 to 219, but direct evidence of this by determination of the sequence of the amino terminus of the *mtr* gene product has not yet been obtained.

A detailed analysis of the *mtr* gene product will be presented in another communication (58).

Regulation of *mtr* gene expression. Inspection of the sequence upstream of the start point of translation revealed possible promoter-operator sites (Fig. 3). A DNA fragment extending from positions 26 to 214 was cloned upstream of

TABLE 2. β-Galactosidase assays of strain JP3561 containing *mtr-lacZ* translational fusion vectors

Plasmid	Mutation	β-Galactosidase sp act[a]
pMU3185	Wild type	33.0
pMU3186	ATG→GTG at 217	7.7
pMU3187	ATG→CTG at 217	3.3
pMU3188	TCA→TAA at 245	<1.0

[a] The units of β-galactosidase specific activity are those defined by Miller (44).

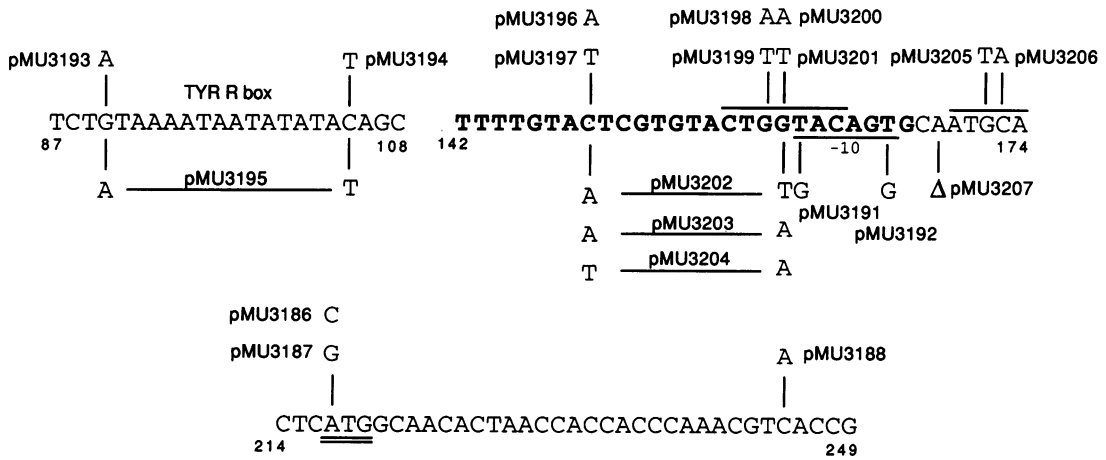

FIG. 4. Mutations in *mtr* introduced by site-directed mutagenesis. The mutations constructed in the regulatory and structural regions of the *mtr* gene are indicated above or below the sequence, and the respective plasmid numbers are shown. Double mutations are indicated by horizontal bars. The numbering of the sequences is taken from Fig. 3. The TYR R box is marked, and the Trp repressor-binding site is shown in bold type. The −10 promoter region is underlined, and the −24 and −12 regions of a putative σ^{54}-dependent promoter are overlined. The putative initiation codon is marked by a double underline.

TABLE 3. β-Galactosidase assays of strains containing *mtr-lacZ* transcriptional fusion vector

Strain	Description	β-Galactosidase sp act of strains grown in[a]:				
		MM	Phe	Tyr	Trp	Phe + Trp
JP3561	*tyrR*$^+$ *trpR*$^+$	894	5,009	1,813	332	540
JP4822	*tyrR366 trpR*$^+$	477	529	463	221	235
JP7218	*tyrR*$^+$ *trpR363*	2,517	5,434	7,217	4,389	4,235
JP7219	*tyrR366 trpR363*	1,853	1,890	2,053	1,780	1,674
JP7217	Multicopy *tyrR*$^+$, *trpR*$^+$	1,314	5,493	5,555	345	NT[b]
JP7220	*tyrR*$^+$, multicopy *trpR*$^+$	117	721	394	12	NT

[a] MM, minimal medium; Phe, minimal medium containing 1 mM phenylalanine; Tyr, minimal medium containing 1 mM tyrosine; Trp, minimal medium containing 1 mM tryptophan; Phe + Trp, minimal medium containing 1 mM phenylalanine and 1 mM tryptophan. The units of β-galactosidase specific activity are those defined by Miller (44).

[b] NT, not tested.

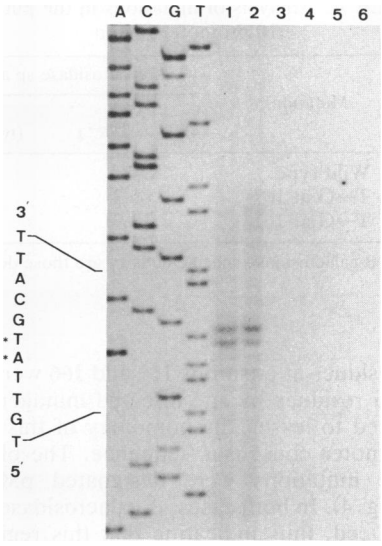

FIG. 5. Determination of the transcription initiation site of *mtr* by primer extension analysis. The 22-base oligonucleotide primer was hybridized, as follows: to RNA from JP2116(pMU3175) grown in minimal medium containing 1 mM phenylalanine (lanes 1 and 5); to RNA from JP2116(pMU3175) grown in minimal medium containing 1 mM tryptophan (lane 2); to RNA from JP2116 (lane 3); to RNA from JP2116(pBR322) (lane 4); and in the absence of RNA (lane 6). It was then extended with reverse transcriptase. No reverse transcriptase was added to the sample in lane 5. Reference sequencing reactions (lanes A, C, G, and T) were initiated by the same primer. The sequence of the coding strand in the region of transcription initiation is shown, and the start points are marked by asterisks.

the *lacZ* structural gene situated on the low-copy-number, promoter-cloning vector, pMU577, as described in Materials and Methods. The resulting construct, pMU3190, was used to further investigate the regulation of *mtr* expression.

The plasmid was initially transformed into three strains: JP3561, which is haploid *tyrR*$^+$; JP4822, which is a *tyrR366* derivative; and JP7217, which is a derivative of JP3561 that contains the multicopy *tyrR*$^+$ plasmid pMU1065. Strains were grown in minimal medium in the presence or absence of the various aromatic amino acids and assayed for β-galactosidase (Table 3). Increased gene expression was clearly evident when cells were grown in the presence of phenylalanine, as had previously been shown by transport assays (68). Cells grown in the presence of tyrosine also exhibited higher β-galactosidase levels. The addition of tryptophan not only abolished phenylalanine-mediated activation but also led to a level of expression lower than that seen in cells grown only in minimal medium. The presence of multiple copies of *tyrR* produced a marked increase in β-galactosidase levels in cells grown in the presence of tyrosine, but did not significantly alter expression under other growth conditions. Although phenylalanine- and tyrosine-mediated induction was absent in a *tyrR366* background, tryptophan-mediated repression was still apparent, and to a slightly greater degree.

The plasmid pMU3190 was transformed into strains JP7218 and JP7219, which are *trpR363* derivatives of strains JP3561 and JP4822, respectively, and into JP7220, which is a derivative of JP3561 that contains the multicopy *trpR*$^+$ plasmid pRPG16. Without functional Trp repressor, tryptophan-mediated repression was abolished (Table 3). The overall level of β-galactosidase produced was increased under all growth conditions. The β-galactosidase levels in strain JP7219 provided a measure of the promoter activity unregulated by TyrR protein or Trp repressor. The presence of functional Trp repressor resulted in the repression of *mtr* expression, and this repression was greater on the addition of tryptophan. Increasing the levels of Trp repressor further accentuated repression. In the strain possessing functional TyrR protein but no Trp repressor (strain JP7218), growth in the presence of any of the three aromatic amino acids caused an enhancement of *mtr* expression. The presence of tyrosine as the only aromatic amino acid supplement resulted in a greater level of induction than that observed with phenylalanine as the sole supplement. These findings indicated that the TyrR protein was involved in the activation of *mtr* expression but not in repression, as previously believed (68). Repression of *mtr* expression is brought about by the Trp repressor.

Examination of the sequence of the regulatory region revealed sequences homologous to previously described TyrR protein and Trp repressor-binding sites (7, 40, 61). A putative TYR R box was identified at positions 87 to 108, and a sequence resembling an operator for the Trp repressor was located at positions 142 to 167 (Fig. 3).

Determination of the transcription initiation site and identification of the *mtr* promoter. To accurately localize the *mtr* promoter, we performed primer extension analysis to map the 5′ end of *mtr* mRNA. A 22-base, single-stranded, synthetic oligonucleotide primer, possessing the same sequence as the sense strand from positions 215 to 236, was labeled with ^{32}P as described in Materials and Methods. The primer was hybridized to RNA isolated from a strain containing plasmid pMU3175 (a pBR322 derivative containing the 2.6-kb *Eco*RI-*Kpn*I fragment), grown in minimal medium supplemented with either phenylalanine or tryptophan. The primer was extended with reverse transcriptase, and the products of extension were electrophoresed next to the sequencing reactions of an *mtr* clone primed by the same 22-base oligonucleotide. The results of the primer extension analysis (Fig. 5) demonstrated that the start point of transcription is located at nucleotides 174 to 175 (Fig. 3).

Examination of the nucleotide sequence upstream of the start point of transcription revealed sequences somewhat homologous to the canonical *E. coli* −10 and −35 promoter sequences (32) (Fig. 3). To ascertain whether these particular sequences were in fact involved in transcription initia-

TABLE 4. Analysis of mutations in the putative −10 promoter region

Plasmid	Mutation	β-Galactosidase sp act in strain[a]: JP3561 (*tyrR*$^+$ *trpR*$^+$)	JP7219 (*tyrR366 trpR363*)
pMU3190	Wild type	894	1,853
pMU3191	T→G at 167	5.3	4.7
pMU3192	T→G at 161	13.7	9.2

[a] The units of β-galactosidase specific activity are those defined by Miller (44).

tion, the T residues at positions 161 and 166 were separately changed to G residues by site-directed mutagenesis. These changes served to lessen the homology of this region with the −10 promoter consensus sequence. The plasmids harboring these mutations were designated pMU3191 and pMU3192 (Fig. 4). In both cases, β-galactosidase levels were severely reduced, thus indicating that this region of DNA plays an important role in promoter function (Table 4).

Analysis of operator sites. The semipalindromic sequence extending from positions 87 to 108 exhibited homology to the consensus TYR R box sequence, N_2TGTAAAN$_6$TTTA CAN$_2$ (7, 22). To ascertain whether this region functioned as a TYR R box, we altered a number of bases in this sequence by site-directed mutagenesis. The symmetrical G · C base pairs are conserved in every TYR R box identified, and alteration of these residues in other TYR R boxes invariably results in loss of function, causing derepression and, in the case of *tyrP*, reduced activation (7, 18, 19, 22, 28, 37, 71). We separately changed the G residue at position 90 to an A and the C residue at position 105 to a T and constructed a double mutant containing both alterations (Fig. 4). As a result of the mutations, the TyrR-mediated activation was severely reduced or abolished (Table 5). When the effects of repression were absent (strain JP7218), the level of β-galactosidase was similar to that produced by an unregulated promoter (strain JP7219) and ratios approached unity. These results indicated that the region from positions 87 to 108 was involved in TyrR-mediated activation of *mtr* expression.

The sequence encompassing positions 142 to 167 exhibited homology to the three known Trp repressor-binding sites (8, 30, 61, 72, 73). To investigate whether this region was involved in the regulation of *mtr* expression mediated by the Trp repressor, we constructed a number of mutations by site-directed mutagenesis. The effect of these mutations was examined by measuring β-galactosidase levels in *trpR*$^+$ and *trpR mtr-lacZ* fusion strains grown in the presence of tryptophan. The mutations are described in Fig. 4, and the assay results are shown in Table 6. Some of the strains containing these mutant alleles exhibited significant differences in unregulated promoter activity from that of the wild type. Comparison in these cases is facilitated by presenting the degree of repression as the ratio of activity conferred on a *tyrR trpR* strain to that on *trpR*$^+$ strains.

It can be seen that a single-base change of C to T at position 149 has no effect on repression. A change of C to A at the same position, however, almost abolishes repression in a *tyrR*$^+$ *trpR*$^+$ strain, but has a less drastic effect in a *tyrR* derivative. A single-base change of G to A at position 159 reduces promoter activity, but causes a slight increase in the degree of repression in the absence of the stimulatory affects of TyrR. A change of G to T at the same position leads to greater reduction in promoter activity (to about one-ninth that of the wild-type level) and largely abolishes repression. Changing G to T at position 160 causes a slight reduction in promoter strength and in repression, whereas changing G to A at the same position reduces both promoter strength and repression by about half. A quite significant decrease in the level of repression is seen with the double mutations present in plasmid pMU3202 and pMU3203, and repression is reduced to one-third of wild-type levels by the double mutation present in plasmid pMU3204.

The results observed in the *tyrR*$^+$ *trpR*$^+$ strains and *tyrR366 trpR*$^+$ strains are not always the same, indicating some dynamic interaction between the binding of these two proteins and RNA polymerase.

Analysis of a putative σ^{54}-dependent promoter. As indicated above, some mutations in the Trp repressor-binding site had a significant effect on promoter strength, particularly the changes at positions 159 and 160 (Table 6). It has been found that *E. coli* promoter spacer regions contain nonrandom sequences and that the structure of spacer DNA may modulate promoter recognition (3, 4, 9). The bases at positions 159 and 160 may play some role in the function of this promoter. Alternatively, these changes may have altered the sequence of a promoter distinct from that identified previously.

The TyrR protein is related to a family of regulatory proteins, including NtrC, NifA, and DctD, all of which are able to bind ATP (24, 25, 27, 55, 65). With the exception of TyrR, these proteins have been found to activate transcription in conjunction with σ^{54}-RNA polymerase from characteristic σ^{54}-dependent promoters (24, 31, 42). All the other genes of the TyrR regulon are known to contain σ^{70}-dependent promoters, but a sequence exhibiting homology to a σ^{54}-dependent promoter is present in the regulatory region of the *mtr* gene (Fig. 3). The consensus sequence for σ^{54}-dependent promoters is CTGGCACN$_5$TTGCA (23, 46). All σ^{54}-dependent promoters so far identified are characterized by a GG doublet around position −24 and a GC doublet at

TABLE 5. Analysis of mutations in the TYR R box of *mtr*

Plasmid	Mutation(s)	β-Galactosidase sp act in strain[a]: JP7219 (*tyrR366 trpR363*)	JP3561 (*tyrR*$^+$ *trpR*$^+$)[b] Phe	Tyr	Trp	JP7218 (*tyrR*$^+$ *trpR363*)[b] Phe	Tyr	Trp
pMU3190	Wild type	1,853	5,009 (2.7)	1,813 (1.0)	332 (0.2)	5,434 (2.9)	7,217 (3.9)	4,389 (2.4)
pMU3193	G→A at 90	2,109	1,180 (0.6)	515 (0.2)	229 (0.1)	1,915 (0.9)	2,171 (1.0)	1,924 (0.9)
pMU3194	C→T at 105	2,169	1,435 (0.7)	481 (0.2)	241 (0.1)	1,868 (0.9)	1,904 (0.9)	1,892 (0.9)
pMU3195	G→A at 90; C→T at 105	2,198	1,239 (0.6)	532 (0.2)	274 (0.1)	1,949 (0.9)	1,938 (0.9)	1,796 (0.8)

[a] Abbreviations are defined in Table 3, footnote *a*. The units of β-galactosidase specific activity are those defined by Miller (44).
[b] Values in parentheses represent the extent of activation as the ratio of β-galactosidase activity obtained in the *tyrR*$^+$ strains to that in strain JP7219.

TABLE 6. Analysis of mutations in the Trp repressor-binding site

Plasmid	Mutation(s)	β-Galactosidase sp act in strain[a]:		
		JP7219 (*tyrR366 trpR363*)	JP3561 (*tyrR*$^+$ *trpR*$^+$)[b]	JP4822 (*tyrR366 trpR*$^+$)[b]
pMU3198	G→A at 159	886	197 (4.5)	87 (10.2)
pMU3190	Wild type	1,853	332 (5.6)	221 (8.4)
pMU3197	C→T at 149	1,949	303 (6.4)	247 (7.9)
pMU3201	G→T at 160	1,328	272 (4.9)	209 (6.4)
pMU3196	C→A at 149	1,760	1,095 (1.6)	349 (5.0)
pMU3204	C→T at 149; G→A at 160	1,742	813 (2.1)	351 (5.0)
pMU3200	G→A at 160	863	260 (3.3)	267 (3.2)
pMU3202	C→A at 149; G→T at 160	1,510	1,505 (1.0)	534 (2.8)
pMU3199	G→T at 159	206	259 (0.8)	112 (1.8)
pMU3203	C→A at 149; G→A at 160	1,725	2,944 (0.6)	937 (1.8)

[a] Strains were grown in minimal medium, with the addition of 1 mM tryptophan in the growth medium of *trpR*$^+$ strains. The units of β-galactosidase specific activity are those defined by Miller (44).

[b] Values in parentheses represent the extent of repression as the ratio of β-galactosidase activity obtained in strain JP7219 to that in the *trpR*$^+$ strains. Values are presented in decreasing order of repression observed in strain JP4822.

position −12 relative to the start point of transcription, which are invariably separated by 10 nucleotides. Alteration of these bases or the spacing between them severely reduces promoter efficiency (12, 13, 47). The sequence between positions 157 and 174 in *mtr* shows striking homology to the consensus sequence, except that 11 rather than 10 bp is present between the conserved GG and GC motifs.

The mutations introduced at positions 159 and 160 alter the bases that make up the GG doublet and could account for the decrease in promoter strength. We therefore mutated the GC doublet at positions 172 to 173 to ascertain whether this would affect promoter strength or TyrR-mediated activation (Fig. 4). The base pair at position 169 was deleted to reduce the spacing between the two doublets to 10 bp. β-Galactosidase assays indicated that neither alterations to the GC motif nor alterations to the spacing between the two halves of the putative promoter significantly affected promoter function or activation by the TyrR protein (Table 7). Changes to the GG motif affect promoter strength, but TyrR is still able to cause some activation of expression. The GC doublet lies outside the σ^{70}-dependent promoter, unlike the GG doublet, which lies in the spacer region.

It was also found that *mtr* expression was not affected when a mutation in the *ntrA* (*rpoN*) gene, which codes for functional σ^{54}, was introduced into strain JP3561 (data not shown). Primer extension analysis and mutagenesis of the putative −10 promoter region indicated that transcription is taking place only via a σ^{70}-dependent promoter. No transcript could be found to originate at the position expected if the putative σ^{54}-dependent promoter was functional. It would appear that the putative σ^{54}-dependent promoter sequence is nonfunctional.

TABLE 7. Analysis of mutations in putative σ^{54}-dependent promoter sequence

Plasmid	Mutation	β-Galactosidase sp act in strain[a]:			
		JP7219 (*tyrR366 trpR363*)	JP7218 (*tyrR*$^+$ *trpR363*)		
			Phe	Tyr	Trp
pMU3190	Wild type	1,853	5,434	7,217	4,389
pMU3198	G→A at 159	886	5,586	7,893	4,200
pMU3199	G→T at 159	206	2,957	4,079	1,696
pMU3200	G→A at 160	863	5,301	7,391	4,587
pMU3201	G→T at 160	1,328	5,234	8,262	4,982
pMU3205	G→T at 172	2,025	5,251	7,073	4,144
pMU3206	C→A at 173	1,844	4,999	6,738	3,771
pMU3207	ΔA at 169	1,924	4,814	5,846	4,403

[a] Abbreviations are defined in Table 3, footnote *a*. The units of β-galactosidase specific activity are those defined by Miller (44).

DISCUSSION

In this study, the *mtr* gene, encoding a tryptophan-specific permease, was cloned and sequenced. Comparison of restriction enzyme sites in *mtr* and surrounding DNA positioned the gene between positions 3370 and 3375 kb on the *E. coli* physical map of Kohara et al. (41), and it is transcribed in an anticlockwise direction on the *E. coli* chromosome.

The gene encodes a protein consisting of 414 amino acids. Translation was shown to commence at the first ATG codon present in the mRNA, situated at positions 217 to 219 (Fig. 3). This codon is preceded by a possible Shine-Dalgarno sequence, but this sequence does not exhibit strong homology to the 3′ end of 16S rRNA (60). This may explain the low level of expression produced by the translational fusion vector as compared with the transcriptional fusion vector. It is also possible that the 60 amino acids encoded by the *mtr* gene present at the N terminus of the fusion protein cause the β-galactosidase to be partially buried in the membrane, thus diminishing its activity. Current models of the secondary structure of the transport protein indicate that the junction between the Mtr protein and the β-galactosidase would be slightly buried on the inner side of the cytoplasmic membrane. A detailed discussion of the possible structure of the *mtr* gene product, and comparison with other transport proteins, will be presented in another communication (58).

Notwithstanding the relationship of the TyrR protein to a number of regulatory proteins known to activate transcription in association with σ^{54}-containing RNA polymerase (65) and the existence of a sequence upstream of the transcription start point with many similarities to σ^{54}-dependent promoters (42), *mtr* is transcribed from a σ^{70}-dependent promoter. Support for this conclusion comes from the accurate mapping of the transcription initiation site, site-directed mutagenesis affecting each of the putative σ^{54}- and σ^{70}-dependent promoter sequences, and the introduction of a mutant form of the σ^{54} gene, *ntrA*.

By studying the expression of β-galactosidase in strains containing an *mtr-lacZ* transcriptional fusion vector, it was

found that TyrR protein is involved only in activation of *mtr* expression. The abolition of activation in the presence of tryptophan, originally observed in transport assays (68), is not mediated by the TyrR protein, but rather by the Trp repressor. In *mtr-lacZ* fusion strains the level of β-galactosidase in cells grown in the presence of tryptophan is repressed approximately twofold below levels obtained in minimal medium. The degree of tryptophan-mediated repression observed in transport assays is, however, much lower than this. The difference may be due to the different genetic backgrounds of the strains used in these two assays. The strains used for the β-galactosidase assays contain a functional *aroP* gene, which encodes the general aromatic amino acid transport system, whereas the strains used in the transport assays do not. Alternatively, since the strains used in the transport assays are *tnaB*$^+$, the higher-than-expected level of tryptophan transport in cells grown in the presence of tryptophan may reflect some escape from catabolite repression (15, 64), allowing some expression of *tnaB*. This may not be revealed in assays of an *aroP mtr* control strain, as in this case intracellular tryptophan levels would be expected to remain low.

An analysis of the nucleotide sequence upstream of the transcription start point reveals a sequence with close homology to the Trp repressor-binding sites present in *aroH*, *trpR*, and the *trp* operon (30, 61, 72, 73), extending from positions 142 to 167. A TYR R box is evident some 28 bp upstream of the −35 promoter region. In a recent study of the activation of expression of the *tyrP* gene, it was shown that a TYR R box at the same position provides the greatest TyrR-mediated activation of this gene (1). It is hypothesized that there may be some cooperativity in the binding of TyrR protein and RNA polymerase, as is thought to occur in the NifA-mediated activation of *nifH* in *Klebsiella pneumoniae* (14). This could account for the restoration of promoter activity in promoter-down mutations in the presence of TyrR, as is the case with the mutations at position 159 (Table 7). The sequence recognized by the Trp repressor, on the other hand, is found in a position where repressor binding would be expected to inhibit the binding of RNA polymerase to the promoter and hence cause repression. When endogenous tryptophan levels are high, repression is completely dominant over activation. However, under conditions where endogenous levels of tryptophan are lower, as in cells grown in minimal medium, or in a situation in which the levels are high but the affinity of the Trp repressor for its target has been weakened by mutation, repression and activation can be seen to be involved in some sort of dynamic interaction, in which the overall level of repression is affected by the presence or absence of the activation system.

In a *trpR* background, any of the aromatic amino acids are able to cause activation of *mtr* expression in a TyrR-mediated manner. The greatest level of activation is seen in the presence of tyrosine, whereas tryptophan is the least effective of the three, although still able to induce a twofold increase in expression. In contrast, in *trpR*$^+$ strains, in which tryptophan represses *mtr* expression, the level of tyrosine-mediated activation is greatly diminished, whereas phenylalanine-mediated induction remains largely unchanged. The mechanism responsible for these effects requires further investigation.

Analysis of many different TYR R boxes and various mutants thereof (7, 18, 19, 22, 28, 37, 71) has established the importance of the consensus sequence N_2TGTAAAN_6TTT ACAN_2 in determining the strength of interaction with the TyrR protein. The TYR R box identified in *mtr* matches the consensus sequence at 11 of 12 positions. It has also recently been shown that TYR R boxes with the strongest affinity for TyrR protein contain a large number of symmetrical base pairs and have a central region of 6 bp which is A+T rich (50). This is also the case with this TYR R box controlling *mtr* expression. Site-directed mutagenesis of this box confirmed its role in activation of *mtr* expression.

Currently, two major models have been proposed for the structure of the operators recognized by the Trp repressor. One model holds that a single Trp repressor dimer recognizes an imperfect palindromic sequence of approximately 24 bp, containing a single central axis of symmetry (8, 40, 72). A recent paper, however, suggests that the operator is actually composed of two Trp repressor-binding sites whose axes of symmetry are located 4 bp either side of the central axis of symmetry and that two Trp repressor dimers bind to one operator (61). The sequence of the putative binding site of the Trp repressor in *mtr* is compared with both these models in Fig. 6. The sequence can be seen to conform to both models, exhibiting a similar degree of homology to each. Mutational changes which affect the repression of *mtr* (Table 6) affect bases which can be seen to play an important role in operator function according to either operator model (Fig. 4 and 6). The effects of these mutations confirm the role of this region in the tryptophan-mediated repression of *mtr*, but do not help to distinguish between the two models.

Interestingly, the only mutation which brought the Trp repressor-binding site in *mtr* closer to consensus according to either model (G to A at position 159) produced a slight increase in the degree of repression in the absence of TyrR-mediated activation. Previous studies have indicated that a G residue at this position in other Trp repressor-binding sites does not significantly affect operator function (6). A possible explanation for the presence of a G rather than an A residue at this position in *mtr* may be the positional constraints imposed by promoter functions performed by the same region.

It is interesting to compare the regulation of *mtr* with that of *tyrP*. The expression of the *tyrP* gene, which codes for the tyrosine-specific transport system, is enhanced when the growth medium is supplemented with phenylalanine, as is the case with *mtr* (38, 68). Expression of each gene is repressed in the presence of the particular amino acid that it is responsible for transporting. That is, *tyrP* expression is repressed by tyrosine and *mtr* expression is repressed by tryptophan. In cells growing in minimal medium, however, there is considerable expression of both *tyrP* and *mtr*, and subsequent repression of expression of these genes occurs only when enough of each amino acid has been transported into the cell to significantly increase intracellular pools. For *mtr*, a large intracellular pool of either phenylalanine or tyrosine is able to activate increased synthesis of the tryptophan-specific transport system. For *tyrP*, the TyrR protein is used both to activate and repress gene expression. Repression, which is tyrosine mediated, requires the cooperative binding of two TyrR molecules to adjacent TYR R boxes, whereas activation, which is phenylalanine mediated, involves TyrR protein binding to only one of these boxes (2). In a manner which is analogous to what has been found for *mtr*, if the promoter-proximal TYR R box is made nonfunctional so that only the upstream box is able to bind TyrR protein, tyrosine-mediated activation of *tyrP* is also observed (2). Although some repression of the *aroP* gene by TyrR protein is also mediated by tryptophan, it is less effective than either tyrosine- or phenylalanine-mediated repression of this gene (18). Furthermore, the use of TyrR

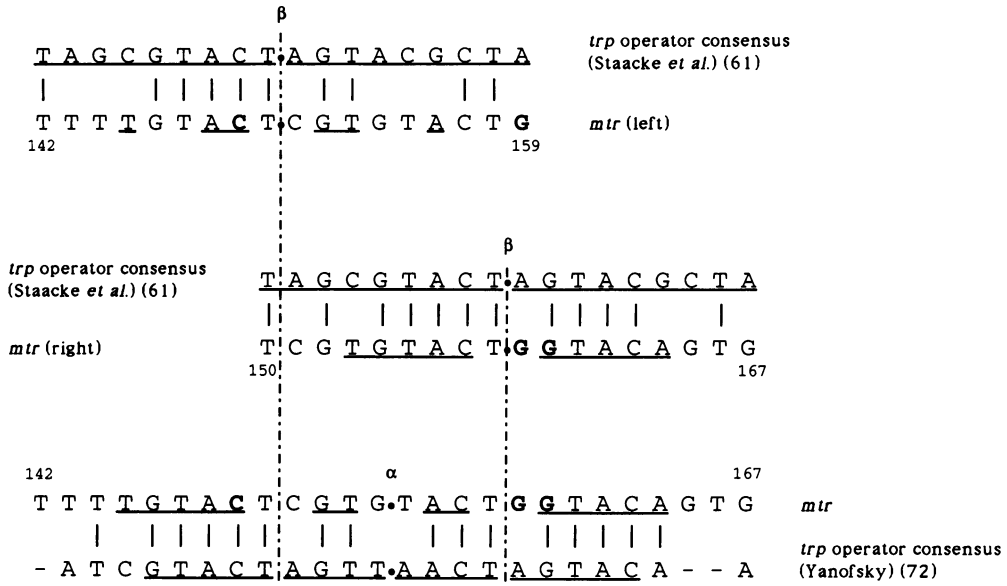

FIG. 6. Comparison of the Trp repressor-binding site present in the *mtr* regulatory region with the consensus Trp repressor-binding site sequences of Staacke et al. (61) and Yanofsky and Crawford (72). The central axis of symmetry of the "Yanofsky operator" is marked by an α. The axis of symmetry of the "Müller-Hill operator" is marked by a β. Identical bases occurring in the same position in both the *mtr* and consensus sequences are indicated by vertical bars. Regions of symmetry surrounding the α and β axes of symmetry are underlined. Positions subjected to site-directed mutagenesis are shown in bold type.

protein for both activation and repression, as for *tyrP*, places certain constraints on the positioning of the TYR R boxes, such that the position which provides best repression is suboptimal for activation (1). For *mtr* the use of TyrR protein for activation and the Trp repressor for repression avoids these constraints, and the positions of the binding sites of the two regulatory proteins can be optimized.

During the preparation of this communication we became aware that Heatwole and Somerville had independently cloned and sequenced the *mtr* gene (33). They point out the existence of a second potential TYR R box at positions 57 to 78. Although we have not mutated this box to determine its function, its position 8 bp upstream of the TYR R box discussed in this paper would, by analogy with *tyrP* (1), just allow cooperative tyrosine-mediated interaction between molecules of TyrR protein binding to each box, such as has been shown for tyrosine-mediated repression of *tyrP* expression (2). For *mtr*, however, the position of the double boxes is such that cooperative binding would be expected to activate rather than repress expression.

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