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The Mannitol Repressor (MtlR) of *Escherichia coli*

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The mannitol operon of *Escherichia coli*, encoding the mannitol-specific enzyme II of the phosphotransferase system (MtlA) and mannitol phosphate dehydrogenase (MtlD), is here shown to contain a single additional downstream open reading frame which encodes the mannitol repressor (MtlR). MtlR contains 195 amino acids and has a calculated molecular weight of 21,990 and a calculated pI of 4.5. It is homologous to the product of an open reading frame (URF2D) upstream of the *E. coli gapB* gene but represents a novel type of transcriptional regulatory protein.

Studies in the late sixties and early seventies served to characterize genes encoding the catabolic enzymes concerned with hexitol catabolism in *Escherichia coli* and *Salmonella typhimurium* (3, 25, 50). Three hexitol catabolic operons were defined, and the gene orders were determined to be *mtlCAD*, *gutCAD*, and *gatCAD* for the mannitol, glucitol, and galactitol operons, respectively (23). The A genes encode enzymes II of the phosphoenolpyruvate:sugar phosphotransferase system (PTS), which phosphorylate the hexitols (3, 24); the D genes encode hexitol-phosphate specific, NAD-dependent dehydrogenases (28, 33, 53); and the C regions, recognized as a result of the isolation of *cis*-dominant regulatory mutations, were presumed to be in the operator-promoter (OP) regions of these three operons (23, 26).

The *mtlA* gene was the first gene encoding a PTS enzyme II to be sequenced (22), and later, the *mtlD* gene was sequenced (10, 18). The gene order proved to be *mtlOPAD*, as originally proposed (23). Sequencing of the glucitol operon revealed that it is complex, containing two or three transcriptional regulatory genes in addition to the transport and catabolic enzyme-encoding genes with the gene order *gutOPABDMRQ* (42, 46, 54–56). Although the *mtl* operon is 20-fold inducible, no transcriptional regulatory protein controlling its expression has yet been identified.

Some evidence suggests that, like regulation of *gut* operon expression, that of the *mtl* operon may be complex. Thus, *pts* mutants lacking either enzyme I or HPr of the PTS appear to express the *mtl* operon constitutively (41), and two classes of *mtlA* mutations affect *mtl* operon expression in opposing ways. *mtlA* mutants totally lacking enzyme II^{Mtl} activity are noninducible for *mtl* operon expression, whereas mutants lacking the phosphoenolpyruvate (PEP)-dependent sugar-phosphorylating activity of this enzyme but still possessing its mannitol-1-phosphate-dependent transphosphorylation activity synthesize the mannitol enzymes constitutively (27, 41). The operon is known to be subject to activation by the cyclic AMP-cyclic AMP receptor protein (CRP) complex, and mutations that specifically render the *mtl* operon independent of CRP frequently also render it constitutive (57). Rosenberg et al. (37) have shown that mannitol-1-phosphate accumulates when *E. coli* cells are grown in the absence of mannitol, even when the *mtlD* gene is defective. Novotny et al. (33) have identified high-molecular-weight mannitol-1-phosphate dehydrogenases that are apparently not encoded by the *mtlD* gene. These intriguing observations led to our current interest in the molecular details of the mechanism(s) by which *mtl* operon expression is regulated.

The DNA sequence upstream of the *mtlOP* region does not contain an open reading frame (ORF) that might function in *mtl* operon control (unpublished observations), and consequently, the downstream region was sequenced in a quest for a potentially relevant ORF(s). We found the initiation codon of a novel ORF encoding a protein of 195 amino acyl residues overlapping the termination codon of the *mtlD* gene. This ORF (ORF1) is homologous to one that precedes the *E. coli gapB* gene. We show that ORF1 is the terminal gene in the *mtl* operon, that it apparently encodes the mannitol repressor MtlR, and that it is probably expressed at low levels. We therefore designate this newly discovered gene *mtlR*.

MATERIALS AND METHODS

Materials. Restriction enzymes and the DNA polymerase I Klenow fragment were purchased from Bethesda Research Laboratories (Gaithersburg, Md.). Radioactively labeled chemicals were obtained from ICN Radiochemicals (Irvine, Calif.). Hydrobond membranes were purchased from Amersham (Arlington Heights, Ill.). Other chemicals were obtained from commercial sources and were of the highest purity available.

Bacterial strains and growth conditions. *E. coli* strains, vectors, plasmids, and bacteriophage are listed in Table 1. Cells were usually grown in Luria-Bertani (LB) broth. Strain LE392, used for propagation of phage λ, was grown in NZCYM medium as described by Maniatis et al. (29). For large-scale preparation of plasmid DNA, cells were grown in terrific broth (TB) (29). For some experiments, *E. coli* strains were also grown on YEG-Cl medium (19), minimal medium M63 (43), or Bacto MacConkey agar base plates supplemented to 0.5% with a carbon source.

DNA manipulations and sequencing. Standard DNA manipulations were carried out as described by Maniatis et al. (29). Electroporation was performed by the method of Dower et al. (12). Microscale preparation of λ DNA from plate lysates and large-scale preparation of λ DNA with infection at high multiplicity were performed according to Maniatis et al. (29). Plasmids were also prepared with the Qiagen kit (Qiagen Inc., Chatsworth, Calif.). Chromosomal DNA was prepared by the method described in Ramseier et al. (35). Southern blotting, hybridization, and hybrid detection were done by the protocol

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TABLE 1. *E. coli* strains, vectors, plasmids, and bacteriophage used in this study[a]

Strain, vector, or plasmid	Relevant characteristics	Reference or source
E. coli		
BL21(DE3)	*hsdS gal* (λ *cIts-857 indl Sam-7 nin-5 lacUV5*-T7 gene *1*)	48
DH5α	*supE44 ΔlacU169* (φ80 *lacZ*ΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	16
HB101	F^- *hsdS20* (r_B^- m_B^-) *recA13 ara-14 proA2 lacY1 galK2 rspL20 xyl-15 mtl-1 supE44*	6
LE392	*supE44 supF58 hsdR514 galK2 galT22 metB1 trpR55 lacY1*	4
MC1061	*hsdR mcrB araD139* Δ(*araABC-leu*)*7679* Δ*lacX74 galU galK rpsL thi*	31
LJ2704	As MC1061 but *mtlR*::Kan^r	This study
Vectors		
pBluescript SK+/KS+	Amp^r *lacZ'*	Stratagene, La Jolla, Calif.
pCRII TM	Amp^r Kan^r	Invitrogen, San Diego, Calif.
pET-19b	Amp^r T7 promoter	Novagen, Madison, Wis.
pKSS	Amp^r *pheS**	19
pRS415	Amp^r	47
pUC-4-KIXX	Amp^r Kan^r (Kan^r from Tn*5*)	Pharmacia, Uppsala, Sweden
Plasmids		
pCR1	pCRII TM bearing *mtlR*	This study
pER19	*mtlR* in pET-19b	This study
pRCLII	*Kpn*I fragment of pRKI in *Bam*HI of pKSS	This study
pRKI	Kan^r of pUC-4-KIXX in *Hin*dIII site of pRRII	This study
pML1	Ap^r *mtlA*::*lacZ*	This study
pRRI	Ap^r *mtlR* in pBluescript SK+	This study
pRRII	pRRI, Δ*Hin*dIII in multiple cloning site	This study

[a] The λ clones used (5D2 and 17G2) have been described by Kohara et al. (20).

outlined in the digoxigenin (DIG) labeling and detection kit from Boehringer (Mannheim, Germany). DNA fragments were isolated from agarose gels with the Qiaex gel extraction kit from Qiagen. PCRs were performed as described by the manufacturer (Perkin-Elmer Cetus, Norwalk, Conn.) with the Hybaid thermocycler. DNA fragments were generated by one cycle of 7 min at 95°C and 5 min at 55°C; 30 cycles of 3 min at 72°C, 1 min at 94°C, and 2 min at 55°C; and 1 cycle of 10 min at 72°C. The final concentration of deoxynucleoside triphosphates (dNTPs) in the reaction was 0.2 mM each. For labeling with DIG-dUTP, the concentrations of dTTP and DIG-dUTP were 0.13 and 0.07 mM, respectively. DIG-dUTP-labeled fragments were purified according to the instructions provided in the Boehringer kit.

The primers Mtl1 (5'-ACTGCTCGGTACGCTGGAAT-3') and Mtl2 (5'-GGGTTTGTTCCATTGTTGCC-3') (synthesized by John Tomich, Manhattan, Kans.) were used to determine the region downstream of *mtlD*.

Cloning of the regulatory region of the *mtl* operon. Two primers, pmtlA1 (5'-GGGGAATTCTTTAGAGGTGATGA GTTGCTT-3'), carrying an *Eco*RI site, and pmtlA2 (5'-AAAGGATCCCGGATGACATAAAAACACCCC-3'), harboring a *Bam*HI site, were used in PCR to amplify a 374-bp DNA fragment. This DNA fragment was digested with *Eco*RI and *Bam*HI and then cloned into the corresponding sites of pRS415. This gave rise to a transcriptional *mtlA*::*lacZ* fusion plasmid, called pML1, which encompasses a DNA region of 346 bp upstream of the translational start site of *mtlA* (see Fig. 3A).

Cloning and sequencing of the region downstream of the *mtl* operon. In the λ library established by Kohara et al. (20), one of the λ clones, 5D2 or 17G2, was expected to contain the mannitol (*mtl*) operon. To identify the correct clone and to subclone the appropriate fragment, Southern blotting was carried out with a DNA fragment that maps at the end of the known sequence of the *mtl* operon that was amplified by PCR and used as a probe. A 4.3-kbp *Kpn*I DNA fragment was found to harbor the end of the known *mtl* operon sequence and about 3 kb of downstream DNA. Cloning of the *Kpn*I DNA fragment into pBluescript KS+ resulted in plasmid pRRI.

Double-stranded DNA sequencing of about 1 kb of downstream DNA was carried out by Lofstrand Labs Ltd. (Gaithersburg, Md.) by the dideoxy chain termination method (45).

Construction of a chromosomal *mtlR*::Kan^r insertion mutant. To construct a mutant of *mtlR*, the following procedure was used. The *Hin*dIII site of pBluescript SK+ was deleted by cutting this cloning vector with *Xho*I and *Xba*I and religating the Klenow-treated plasmid. The 1.6-kb kanamycin resistance (Kan^r) cassette was excised from pUC-4-KIXX by using *Hin*dIII and inserted into the *Hin*dIII site of pRRII to give pRKI. The 5.9-kb *Kpn*I DNA fragment of pRKI, which contains the disrupted *mtlR* gene, was removed and cloned into the *Bam*HI site of pKSS. This step required blunt ends, which were generated by using the Klenow fragment of DNA polymerase I. The resulting plasmid was called pRCLII.

In vivo recombination with a pKSS derivative. The vector pKSS contains a gene coding for a mutated phenylalanyl tRNA synthetase (*pheS**) with relaxed substrate specificity. Strains bearing this plasmid incorporate *p*-chlorophenylalanine (p-Cl-Phe) in addition to phenylalanine into proteins, rendering them nonfunctional and leading to cell death. By this means, the presence of *pheS** on the plasmid allows screening for cells that have lost plasmid pKSS or its derivative when grown on medium containing the phenylalanine analog.

Plasmid pRCLII was transformed into *E. coli* MC1061 (*recA*$^+$), and the resultant strain was cloned. An overnight culture (5 ml) was used to inoculate 100 ml of an LB culture containing ampicillin (100 μg/ml) and kanamycin (50 μg/ml). After a 50-h incubation with aeration, aliquots were removed, diluted 10^6-fold, and plated on YEG-Cl containing kanamycin (50 μg/ml) and p-Cl-Phe (100 μg/ml). Colonies that were able to grow usually contained the chromosomal Kan^r marker and lacked the cloning vector. Single colonies were tested for ampicillin sensitivity to ensure that only the kanamycin resistance gene had been integrated. Integration of the whole plasmid would result in a cell with one normal and one relaxed

phenylalanyl tRNA synthetase, which might be capable of growth on p-Cl-Phe. Clones that were ampicillin sensitive contained no β-lactamase and therefore did not possess a copy of the plasmid. All ampicillin-sensitive clones were subjected to Southern blot analysis.

Southern blot analysis of mutated MC1061. Correct integration of the Kan^r cassette into the chromosome was verified by Southern hybridization of three different digests of chromosomal DNA. DNA of putative mutants was digested with *Hpa*I, *Pst*I, and *Sma*I and probed with the 2.2-kb DIG-dUTP-labeled *Hpa*I DNA fragment of pRRI containing *mtlR* and flanking DNA. Labeling of this probe was done by the Boehringer procedure for random-primed labeling of DNA. After hybridization, all of the above digests showed the expected shift in size or exhibited additional bands compared with the correspondingly digested wild-type DNA. *Sma*I digestion did not result in the expected 3.9-kb band. The DNA fragment downstream of the *Pst*I site originated from the λ phage, as was shown by further restriction analyses (data not shown). Twelve mutant clones were identified and designated LJ2704.

Construction of an ORF1 (*mtlR*) overexpression plasmid. In order to overexpress *mtlR*, this gene was amplified by PCR. Two primers, MTLRI (5′-AAACATATGGTGGACCAG GCGCAGGAC-3′), carrying an *Nde*I site for *mtlR*, and MTL RII (5′-AAAGTCGACCAGGTAGAGGGGCAGTAAGCA-3′), harboring a *Sal*I site, were used to generate a 0.6-kb DNA fragment, which was subsequently cloned into pCRII TM. This gave rise to pCR1. Plasmid pCR1 was then cut with *Nde*I and *Sal*I, and the 0.6-kb DNA fragment encompassing *mtlR* was cloned into the corresponding restriction sites of the expression vector pET-19b, resulting in pER19.

Enzymatic assays. Mannitol 1-phosphate dehydrogenase was determined by the method of Saier et al. (43). The reaction mixtures contained 0.1 M Tris-hydrochloride (pH 9), 1 mM NAD^+, and enzyme in a total volume of 1 ml. The A_{340} was monitored at room temperature until no further increase was observed. Subsequently, mannitol 1-phosphate was added, and the linear increase in absorbance with time was quantitated.

Assays for proteins of the PTS were performed essentially as described previously (39, 40). Assay mixtures (100 μl, final volume) contained 50 mM potassium phosphate buffer (pH 7.4), 12.5 mM $MgCl_2$, 25 mM KF, 2.5 mM dithiothreitol, 75 μM [^{14}C]mannitol (4 μCi/mmol), and 5 mM PEP. Enzyme II^{Mtl} activity was determined by complementation in the presence of a high-speed supernatant fraction of strain MC1061 grown in the presence of glucose. Membrane protein, prepared by ultracentrifugation, was used for the assay. Each of three membrane preparations from independently grown cells was assayed in duplicate, and the results were averaged. Reactions were initiated by addition of enzyme II^{Mtl} and terminated after 20 min at 37°C by addition of 1 ml of ice-cold water. [^{14}C]mannitol 1-phosphate was separated from the free sugar by ion-exchange chromatography (Bio-Rad AG1-X2 anion-exchange resin [analytical grade, 50/100 mesh, chloride form]) and determined by liquid scintillation counting, as described previously (21). The protein content was determined as described by Bradford (7).

In vivo labeling with [^{35}S]methionine. In order to identify the gene product of *mtlR*, in vivo labeling experiments were carried out with the T7 expression system as described by Tabor and Richardson (49). *E. coli* BL21 harboring pER19 was grown at 37°C in LB to an OD_{600} of 0.5. Aliquots (4 ml) of a cell suspension were washed and resuspended in M63 containing all amino acids except for methionine and cysteine at 0.01%. After the cells were shaken for 30 min at 37°C, isopropyl-β-D-thiogalactoside (IPTG) was added to a final concentration of 0.5 mM in order to induce synthesis of the T7 RNA polymerase. The cultures were shaken for 20 min at 37°C, and rifampin was then added to a final concentration of 400 μg/ml. After 20 min of aeration at 37°C, 0.5-ml aliquots were removed, and [^{35}S]methionine (10 μCi) was added and incubated for 5 min at 30°C. To remove the remaining [^{35}S]methionine, the cells were centrifuged, and the pellet was resuspended in gel loading buffer (100 μl).

Computer analyses. DNA and deduced protein sequences were analyzed with the programs in the Genetics Computer Group, Inc., Version 7.3 package. Screening for homology was performed by the use of BLAST (2) and FASTA (34). To establish homology, the program RDF2 (34) with 500 random shuffles was applied. A score of 9 standard deviations (SD) is considered sufficient to establish homology (11, 30). To predict secondary structure, the programs of Chou and Fasman (9), Garnier et al. (14), and Salzberg and Cost (44) were used.

RESULTS AND DISCUSSION

Cloning and sequencing of ORF1 (*mtlR*). One of the two selected clones in the Kohara λ library (20) was thought to contain the mannitol (*mtl*) operon. A downstream DNA fragment of the *mtlD* gene served as a probe for Southern analysis of the two clones in order to determine which carried the *mtl* operon downstream region. A 4.3-kb *Kpn*I DNA fragment was found to harbor this region and was subsequently cloned into pBluescript SK+. Approximately 1 kb of the downstream region was sequenced.

Sequence analysis. Computer analyses revealed two ORFs in the newly determined sequence. The ATG start codon of ORF1 overlaps the TAA stop codon of *mtlD* by one nucleotide. Thus, these two sequences may be translationally coupled. ORF1 is preceded by a reasonable Shine-Dalgarno sequence (Fig. 1). ORF2, which occurs downstream of ORF1, was only partially sequenced. No other ORF encoding a protein longer than 50 amino acyl residues was found.

Several palindromes were detected within ORF1 (hereafter designated *mtlR*) which may function to regulate transcription or translation (see Fig. 1). Overlapping and following the TAA translation termination codon of ORF1 (*mtlR*) are four palindromes which may play a role in transcriptional termination. Analysis of the translated protein sequence of ORF2 failed to reveal homology with other protein sequences in the data base. The *mtlR* gene codes for a protein of 195 amino acids with a calculated molecular mass of 21,990 Da and a calculated isoelectric point (pI) of 4.5. It has the amino acid composition and hydropathy plot of a typical water-soluble protein but contains no tryptophan, only one cysteine, elevated amounts of acidic residues (aspartate and glutamate; 15%, versus 10% for bulk proteins), and decreased amounts of glycine (4.6%, versus 8.3% for bulk proteins) (38). Although it shows a high leucine content (14.9%, versus 9.9% for bulk proteins), it does not contain a stretch of hydrophobic residues of sufficient length and hydrophobicity to suggest membrane localization.

The sequence was analyzed for secondary structure with three programs (see Materials and Methods). The N-terminal 10 residues can be drawn in a strongly amphipathic α-helix that is terminated at residue 11 with a prolyl residue followed by a strongly hydrophilic segment. Residues 20 to 38 are predicted to form an α-helix which proves to be strongly amphipathic. Residues 40 to 47 may form a β-strand. Residues 47 to 65 form a putative α-helix which proved to be moderately amphipathic, with the few hydrophilic residues in this region localized to one side of the helix. After a putative β-turn, residues 70 to 80 may form a β-strand, while residues 80 to 85 are predicted to form

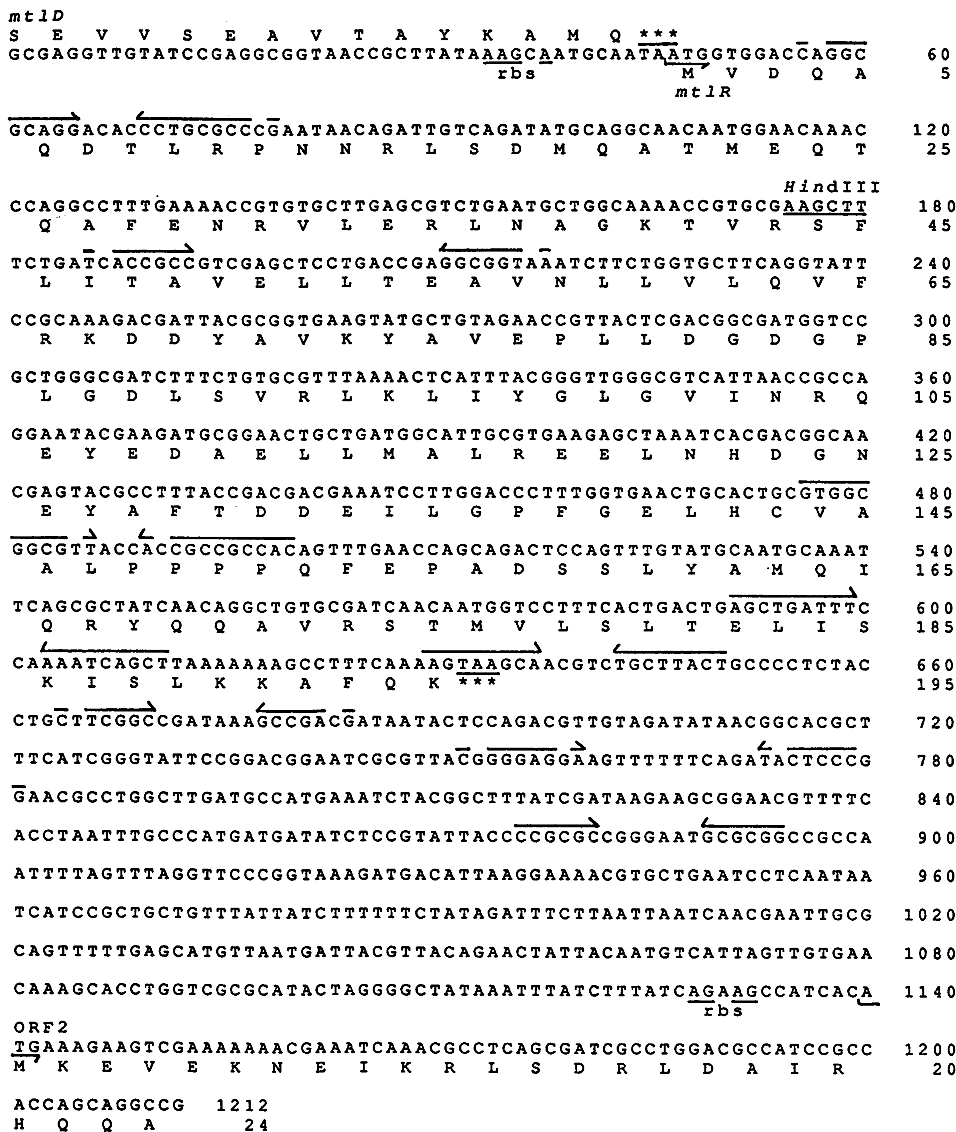

FIG. 1. Nucleotide sequence of the *mtlR* gene and flanking regions and deduced amino acid sequence of the encoded mannitol repressor MtlR. The putative ribosome-binding sites (rbs) for *mtlR* and the downstream unidentified ORF, ORF2, are underlined. Asterisks indicate the *mtlD* and *mtlR* gene termination codons (both TAA). Inverted repeats following *mtlR* may function in transcriptional termination for the *mtl* operon. Inverted repeats within *mtlR* that may have regulatory significance, possibly by decreasing the level of gene expression, are indicated by arrows. This sequence will appear in the EMBL/GenBank nucleotide sequence data base under accession number U03845.

a second β-turn followed by a β-strand (residues 88 to 98) with striking amphipathic character. Two α-helices (residues 105 to 120 and 125 to 132) are then predicted, both of which are rich in acidic but not basic residues. Finally, residues 162 through 178 probably form an extended β-strand. These analyses therefore suggest that MtlR contains about equal amounts of β and α structure in the sequence α β α β β α α β. No obvious helix-turn-helix motif was identified.

The three *mtl* operon genes were examined for frequency of rare-codon usage (data not shown). This frequency was greatly reduced for all three genes relative to untranslated DNA, particularly for *mtlA* and *mtlD*, but to a lesser extent for *mtlR*. The results clearly indicated that all three genes are expressed, *mtlA* and *mtlD* at a moderate level and *mtlR* at a low level.

mtlR proved to be homologous to an unidentified ORF, designated URF2A, that precedes the *gapB* gene, homologous to the glyceraldehyde 3-phosphate dehydrogenase gene (*gapA*) of *E. coli* (1). The deduced protein sequence of URF2A reported by Alefounder and Perham (1) is much smaller than that of *mtlR* (133 residues versus 195 residues, respectively). By examining the published nucleotide sequence of URF2A, it became evident that by the use of an earlier in-frame ATG with a better Shine-Dalgarno sequence than that selected by Alefounder and Perham, URF2A could be extended 66 bp at its 5′ end. This elongated ORF was thereafter called URF2D. After binary alignment of the deduced protein sequences of URF2D (155 amino acids) with the product of *mtlR*, it was apparent that the extended N-terminal sequence of URF2D was homologous to that of *mtlR*. Part of the binary alignment is shown in Fig. 2. The region shown in Fig. 2 exhibits 32% identity and 77% similarity over a stretch of 118 overlapping residues. To establish homology, the RDF2 program (34) with

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       30        40        50        60        70        80
MtlR   ENRVLERLNAGKTVRSFLITAVELLTEAVNLLVLQVF-RKDDYAVKYAVEPLLDGDGPLG
       :. :::.:.: ... ::. :: ..: .... ..  .:  .:.  :.:::.:::. .::::.
URF2D  EDDVLEQLDAQDNLFSFMKTAHTILLQGIRQFLPSLFVDNDEEIVEYAVKPLLAQSGPLD
          10        20        30        40        50        60


        90       100       110       120       130       140
MtlR   DLSVRLKLIYGLGVINRQEYEDAELLMALREELNHDGNEYAFTDDEILGPFGELHCVA
       :..: :.:::.:: ...  :.: . .  .... ::..... .:.:: . . ........
URF2D  DIDVALRLIYALGKMDKWLYADITHFSQFWHYLNEQDETPGFADDMTWDFISNVNSIT
          70        80        90       100       110       120
```

FIG. 2. Binary alignment of the MtlR sequence with that of URF2D of *E. coli*. Residue numbers in the two proteins are provided above and below the sequences, respectively. :, identical residues; ·, conservative substitutions. The depicted region exhibits 32% identity in a 118-amino-acid overlap with a single gap in the aligned sequence.

500 random shuffles was used. The score of 20 S.D. establishes that these two proteins have a common ancestry.

Of the 38 conserved residues shown in Fig. 2, 21 are hydrophobic (10 L, 4 V, 3 A, 3 F, and 1 I), four are structural (2 P and 2 G), five are semipolar hydroxy amino acids (3 Y, 1 S, and 1 T), and eight are strongly polar (5 D, 2 E, and 1 N). No basic residues are conserved. Most of the conservation was found between residues 72 and 100 in *mtlR*, a region which is predicted to be composed largely of β structure. Almost all of the conserved residues in this region are hydrophobic or of structural significance.

Expression and in vivo labeling of the *mtlR* gene product. In order to establish that *mtlR* is expressed, it was first cloned into pBluescript, and expression was attempted with the T7 expression system (49). No band of the expected molecular weight was observed. The gene was then cloned into the overexpression vector pET-19b (see Materials and Methods). The resulting plasmid, pER19, was transferred to strain BL21 bearing the T7 RNA polymerase under the control of the *lacUV5* promoter. Induction with IPTG resulted in expression of a single dominant band with an apparent molecular weight of 32,000 (data not shown). The calculated molecular mass of the gene product (22 kDa) differs from that seen in the T7 labeling experiment (32 kDa). This difference can be partly explained by the fact that *mtlR*, when cloned into pET-19b, acquired an additional 69 nucleotides at its 5′ end, which are transcribed and translated along with *mtlR*. These additional amino acyl residues (His tag) account for an increased molecular mass of 3 kDa. Furthermore, it is known that proteins with a His tag migrate like proteins with higher molecular weights (QIAexpress Manual from Qiagen, Inc.) (50a). Abnormal migration rates for some proteins on denaturing sodium dodecyl sulfate (SDS)-polyacrylamide gels is not uncommon (see e.g., reference 32).

Analysis of a chromosomal *mtlR*::Kan^r mutant. In order to determine the function of MtlR, the *mtlR* gene was inactivated by inserting a Kan^r cassette into the *Hin*dIII site of *mtlR* (shown in Fig. 1; see also Materials and Methods), and the introduced mutation was recombined into the chromosome, giving rise to strain LJ2704. This *Hin*dIII site marks the end of the previously sequenced region of the *mtl* operon (18). Wild-type (MC1061) and LJ2704 mutant cells were grown on MacConkey fermentation medium containing 0.5% mannitol or glucitol, but no difference in fermentation could be detected.

The effect of the *mtlR* mutation on *mtl* operon expression was examined by measuring the activities of the products of the two *mtl* operon structural genes for mannitol 1-phosphate dehydrogenase (MtlD) and the mannitol enzyme II (MtlA). The results are presented in Table 2. In the parental strain, MC1061, both activities were elevated about 10-fold by inclusion of mannitol in the growth medium. The *mtlR*::Kan^r mutation gave rise to a 20-fold increase in both MtlD and MtlA activities in the absence of sugar, and addition of 0.5% mannitol resulted in 2-fold repression, to the level of the induced parental strain (Table 2). Growth in the presence of 0.5% mannitol plus 0.5% glucose resulted in an approximately 50% drop in activity for both the parental and *mtlR* mutant strains (data not shown). It can therefore be concluded that loss of *mtlR* gene function gives rise to constitutive expression of the *mtl* operon and that this mutation does not abolish catabolite repression of *mtl* operon expression.

TABLE 2. Specific activities of the products of the mannitol operon in *E. coli* MC1061 (wild type) with and without plasmids pML1 and pRS415 and LJ2704 (*mtlR*::Kan^r) after growth in the presence or absence of mannitol

Strain	Sp act			
	Enzyme II^{Mtl} [a]		Mannitol 1-phosphate dehydrogenase[b]	
	Uninduced	Induced	Uninduced	Induced
MC1061	0.8 ± 0.1	7.5 ± 1.9	0.6 ± 0.1	8.7 ± 1.0
LJ2704	12.9 ± 0.9	5.7 ± 0.8	16.6 ± 4.2	10.9 ± 1.8
MC1061/pML1	3.1 ± 0.2	3.9 ± 0.5	3.4 ± 0.2	7.8 ± 0.6
MC1061/pRS415	0.8 ± 0.0	3.1 ± 0.4	0.4 ± 0.0	5.0 ± 0.3

[a] Specific activity values (reported as nanomoles of [^{14}C]mannitol phosphorylated per milligram of membrane protein per minute at 37°C) represent averages of three determinations with three independently grown pairs of bacterial cultures.

[b] Specific activity values (reported as millimoles of mannitol 1-phosphate oxidized to fructose 6-phosphate per milligram of soluble protein per minute) represent averages of three determinations with three independently grown cultures of bacteria.

Constitutive expression of both the *mtlA* and *mtlD* genes in strain LJ2704 strongly suggests that *mtlR* encodes the postulated but heretofore unidentified mannitol operon repressor. To provide further evidence that MtlR binds to the *mtl* operator-promoter region, *E. coli* MC1061 was transformed with plasmid pML1 (copy number, approximately 20) bearing the upstream region of the *mtl* operon with the mannitol structural genes inactivated by the presence of an *mtlA*::*lacZ* transcriptional fusion mutation (see Fig. 3A, Table 1, and Materials and Methods). As a control, the same strain was transformed with the parental plasmid pRS415, which lacks the *mtl* regulatory region. The data in Table 2 show the effects of these two plasmids on synthesis of the two mannitol operon gene products, MtlD and MtlA. As expected, transformation of MC1061 with plasmid pRS415 had no effect on the inducible expression of these two genes. By contrast, inclusion of plasmid pML1, bearing the *mtl* regulatory region, gave rise to nearly constitutive expression of the chromosomal *mtlA* and *mtlD* genes (Table 2). We interpret these results to suggest that the mannitol repressor protein MtlR is present in the bacterial cell in relatively low amounts, so that a moderately high copy number of the *mtlOP* region effectively titrates out the repressor, rendering it no longer available for binding to the chromosomal *mtlOP* region. Figure 3B shows the putative operator sequence of the *mtl* operon, which reveals two palindromes which might serve as the putative MtlR binding site.

Transcriptional regulation of the *mtl* operon. The results presented above establish that the mannitol operon consists of

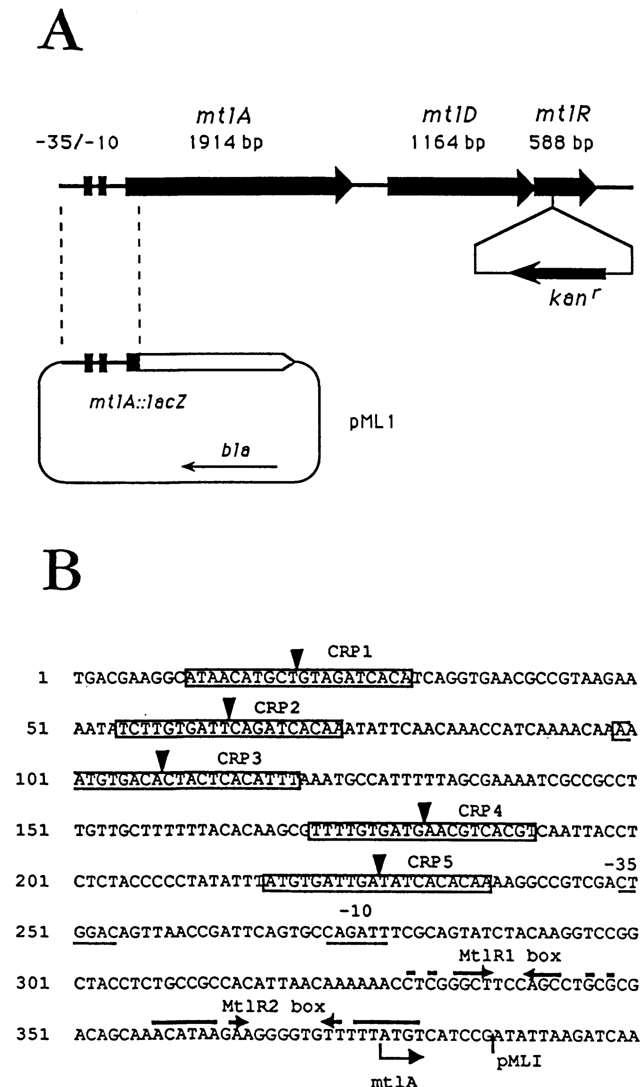

FIG. 3. Schematic depiction of the *mtl* operon of *E. coli*. (A) The operon consists of the operator-promoter region (shown in detail in panel B) followed by the three structural genes *mtlA*, *mtlD*, and *mtlR*. *mtlA* and *mtlD* are separated by an intercistronic region of 227 bp in which the repetitive extragenic palindromic structure is found (19, 22). The *mtlR* gene overlaps the *mtlD* gene by 1 bp, suggestive of translational coupling. Several stem-loop structures (see Fig. 1) that may serve as transcriptional terminators follow *mtlR*. The operator-promoter region of the *mtl* operon fused to *lacZ* in plasmid pML1 is shown below the operon. (B) Sequence of the operator-promoter region of the *mtl* operon. The five putative CRP consensus sequences are boxed. The −35 and −10 regions of the promoter are indicated, as are the two palindromes (MtlR1 and MtlR2 boxes) that might serve as binding sequences for MtlR. ↳ *mtlA*, translational start site of *mtlA*. The point of fusion with *lacZ* in pML1 is indicated (pML1).

three structural genes (*mtlADR*) following the operator-promoter region, to which MtlR and CRP presumably bind. The *mtlOP* region was previously shown to contain five well-conserved consensus sequences for CRP binding, each in proximity to the others and all upstream of the *mtlA* translational start site (Fig. 3B) (10, 18). Although several catabolite-repressible operons are known to possess more than one CRP-binding site (5, 15, 17, 36, 52), five is the most such binding sites yet to be reported for any operon. Since many mtl^+, operon-specific, CRP-independent mutants were found to express the *mtl* operon constitutively (57), CRP and MtlR may bind to overlapping sites and function antagonistically. This possibility is supported by the fact that *crp* mutants of *E. coli* and *S. typhimurium* are noninducible (unpublished results). By contrast, for the fructose (*fru*) operon, *crp* mutants exhibit depressed *fru* expression but retain inducibility (13). It should be noted that CRP-independent mutations in the *lac* operon do not give rise to *lac* operon constitutivity (57).

Three ambiguous observations must be explained in order to understand transcriptional regulation of the *mtl* operon. (i) The *mtl* operator-promoter region has been shown to bind the pleiotropic transcriptional regulator FruR (8, 34a, 35, 51), but its role in transcriptional regulation is not yet defined. It is interesting that a FruR-binding consensus sequence has been identified upstream of the *gapB* gene, near the region where the MtlR homolog was found (34a). (ii) Mannitol operon expression is induced in the absence of exogenous mannitol in *pts* mutants lacking either enzyme I or HPr of the PTS as cells approach high cell density, but it is not induced when cells are grown continuously at low cell density (37a, 41). (iii) Enzyme II^{Mtl} (*mtlA*) mutations can render *mtl* operon expression either noninducible (as observed for complete-loss-of-function mutants) or constitutive (as observed for mutants which lack PEP-dependent mannitol phosphorylation activity but retain mannitol-1-phosphate transphosphorylation activity [41]).

Mannitol-1-phosphate accumulation, resulting from fructose-6-phosphate reduction (37) in the absence of exogenous mannitol, may provide an explanation for some of these observations, assuming that free cytoplasmic mannitol and not mannitol-1-phosphate is the inducer. Displacement of MtlR from the DNA may require the competitive participation of the cyclic AMP-CRP complex. Cytoplasmic mannitol accumulation from mannitol-1-phosphate may require the sugar phosphate binding site (and thus transphosphorylation activity) of enzyme II^{Mtl} if this enzyme is to catalyze mannitol-1-phosphate hydrolysis. This possibility would explain why *mtlA* mutants that retain transphosphorylation activity appear to express the *mtl* operon constitutively, while *mtlA* null mutants are noninducible. A soluble mannitol-1-phosphate phosphatase appears to be lacking in enteric bacteria (37a). In the presence of a functioning PTS (enzyme I plus HPr plus enzyme II^{Mtl}), free mannitol generated internally and released into the medium would be taken up and rephosphorylated. Consequently, loss of enzyme I or HPr prevents inducer destruction and leads to high-level expression of the *mtl* operon as cells approach the stationary phase of growth. This working hypothesis should allow the design of new experiments to reveal the detailed mechanism by which mannitol operon expression is regulated.

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