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Multiple Phosphorylation Events Regulate the Activity of the Mannitol Transcriptional Regulator MtlR of the Bacillus stearothermophilus Phosphoenolpyruvate-dependent Mannitol Phosphotransferase System*

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Phosphotransferase system; IICB mtl, mannitol permease; IIA mtl, enzyme I of the PTS; PRD, PTS regulation domain; P-enolpyruvate, enzyme IIA of the mannitol PTS; HPr, histidine phosphocarrier protein; phosphorylated by HPr, whereas a third IIA-like domain

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D-Mannitol is taken up by Bacillus stearothermophilus and phosphorylated via a phosphoenolpyruvate-dependent phosphotransferase system (PTS). Transcription of the genes involved in mannitol uptake in this bacterium is regulated by the transcriptional regulator MtlR, a DNA-binding protein whose affinity for DNA is controlled by phosphorylation by the PTS proteins HPr and IICBmtl. The mutational and biochemical studies presented in this report reveal that two domains of MtlR, PTS regulation domain (PRD-I) and PRD-II, are phosphorylated by HPr, whereas a third IIA-like domain is phosphorylated by IICBmtl. An involvement of PRD-I and the IIA-like domain in a decrease in affinity of MtlR for DNA and of PRD-II in an increase in affinity is demonstrated by DNA footprint experiments using MtlR mutants. Since both PRD-I and PRD-II are phosphorylated by HPr, PRD-I needs to be dephosphorylated by IICBmtl and mannitol to obtain maximal affinity for DNA. This implies that a phosphoryl group can be transferred from HPr to IICBmtl via MtlR. Indeed, this transfer could be demonstrated by the phosphoenolpyruvate-dependent formation of [3H]mannitol phosphate in the absence of IIAmtl. Phosphoryl transfer experiments using MtlR mutants revealed that PRD-I and PRD-II are dephosphorylated via the IIA-like domain. Complementation experiments using two mutants with no or low phosphoryl transfer activity showed that phosphoryl transfer between MtlR molecules is possible, indicating that MtlR-MtlR interactions take place. Phosphorylation of the same site by HPr and dephosphorylation by IICBmtl have not been described before; they could also play a role in other PRD-containing proteins.

Many bacteria transport d-mannitol and other carbohydrates via a phosphoenolpyruvate-dependent phosphotransferase system (PTS) (1–3). Recently, the mannitol operon of Bacillus stearothermophilus was cloned (4) and shown to consist of four genes, mtlA, mtlR, mtlF, and mtlD, coding for the mannitol transporter IICBmtl, the transcriptional regulator MtlR, the phosphotransferase IIAmtl, and mannitol-1-phosphate dehydrogenase, respectively. Analysis of the mannitol promoter revealed a catabolite response element overlapping the mannitol promoter, indicating that this operon is sensitive to catabolite repression. When favorable catabolites like glucose are utilized, HPr is phosphorylated by a kinase on a specific serine (5) that forms a complex with the CcpA repressor. Binding of this complex to catabolite response element sites located in or near the promoter regions of catabolic operons will prevent expression of these operons (6). In addition to catabolite repression, the expression of the mannitol operon is probably also regulated by the mannitol regulator MtlR (7).

Domains in this protein show similarity to domains of two types of transcriptional regulators: DNA-binding proteins and anti-terminators. A helix-turn-helix motif is situated at the N terminus that is similar to those of DNA-binding transcriptional regulators of the DeoR family. The center of the protein sequence contains two domains resembling the PTS regulation domains (PRD-I and PRD-II) of the anti-terminators LicT, SacY, and BglI (7, 8). Anti-terminators are RNA-binding proteins that prevent premature termination of transcription at a terminator located between the promoter and the functional genes. Combinations of DNA-binding helix-turn-helix motifs and PRDs have been found in other proteins such as LevR and LicR. The activity of most of these proteins can be regulated by phosphorylation of PRD-I and/or PRD-II by the PTS components HPr and/or IIB. Based on these similarities, it was assumed that MtlR is a DNA-binding protein whose activity is regulated by the PTS (8).

In this paper, we link phosphorylation of the individual domains of MtlR by HPr and IICBmtl to the regulation of this protein. The residues involved in HPr- and IICBmtl-dependent phosphorylation are mapped and correlated with either an increase or decrease in the affinity of MtlR for DNA. In addition to PRD-I and PRD-II, a third phosphorylation domain is presented that is involved in the phosphorylation and dephosphorylation of MtlR by IICBmtl.

EXPERIMENTAL PROCEDURES

Materials—Restriction enzymes, T4 DNA polymerase, nucleotides, oligonucleotide kinase, pyruvate kinase, and isopropyl-1-thio-β-D-galactopyranoside were purchased from Roche Molecular Biochemicals. [γ-32P]ATP (3000 Ci/mmol) and [3H]mannitol (15–30 Ci/mmol) were obtained from Amersham Pharmacia Biotech and ICN, respectively. RNase-free RNase I DNase I was obtained with the Promega Core footprinting system, and Ni2+-nitrilotriacetic acid-agarose was from Qiagen Inc. Primers were synthesized by Eurosequence B. V. Groningen. P-enolpyruvate and yeast tRNA were purchased from Sigma. Site-directed mutagenesis was performed with the QuickChange kit from Stratagene. α-Chymotrypsin (50.5 units/mg) was obtained from Worth-
ingston. Anti-His tag and anti-mouse antibodies were purchased from Amersham Pharmacia Biotech and Sigma, respectively.

**Purification of B. stearothermophilus EI, HPr, IIA_{mtl}, IIC_{mtl}, and MtlR—**MtlR and mutants of MtlR were overexpressed in *Escherichia coli* BL21(DE3) (9); *B. stearothermophilus* EI and HPr were expressed in *E. coli* ZSCL12LHAC (10); and *B. stearothermophilus* IIA_{mtl} was expressed in *E. coli* JM101 (11). These proteins were purified as described by Henstra et al. (7). The *B. stearothermophilus* mannitol transporter IIC_{mtl} was expressed in the mannitol deletion *E. coli* strain LGS322 (12) and purified as described by Henstra et al. (4). P90 protein activities were measured as mannitol phosphorylation activity as described by Robillard and Blaauw (13).

General Methods—DNA was isolated from agarose gels using the gel extraction kit from QIAGEN Inc. Protein concentrations were determined according to Bradford (14). General DNA manipulations were performed as described by Sambrook et al. (15). Sequence data base searches were performed using the program BLAST at NCBI (16).

**MtlR Mutants**—The mutants of MtlR that were made following the QuickChange kit protocol of Stratagene are listed in Table I. Two complementary primers containing the mutation were created and used in a PCR amplifying 25 ng of the MtlR expression plasmid pETMtlRhis. The sequences of one strand of each of the complementary primers are listed in Table II. The PCR mixture was first incubated for 10 min at 94 °C, followed by 18 cycles of 1-min denaturation at 94 °C, 1-min annealing at 52 °C, and 16-min elongation at 68 °C. Methylated template DNA was digested by DpnI, and the remaining PCR product was precipitated and re-dissolved in 2 μl of triple-distilled water. After transformation to *E. coli* XLI-Blue, the plasmid was isolated and checked for the mutation by restriction analysis. After checking the entire MtlR sequence of a mutant, the plasmid was transformed to the T7 expression strain BL21(DE3). Double mutants of MtlR were created in a second round using one of the single mutants as template exactly as described above.

**DNA Footprinting**—DNA footprinting was performed essentially as described by Henstra et al. (7). A single-end 32P-labeled DNA probe of the mannitol promoter region was synthesized in a PCR in which one of the primers was labeled. 28.5 pmol of the forward primer sah1 (5'-AGG TGA ATT GTT AAA G-3') labeled with 100 μM dNTPs, 2.5 units of Taq DNA polymerase, 57 pmol of universal reverse primer (5'-CGAAGATTTGGTCGTT-3'), priming at position 9-10 was labeled with 100 μCi of [γ-32P]ATP (3000 Ci/mmol) by T4 polynucleotide kinase as recommended by Roche Molecular Biochemicals. The labeled primer was purified by chloroform/phenol and chloroform extractions and precipitated by ethanol precipitation. 19 pmol of the labeled forward primer was built into a 473-base pair probe by PCR in a mixture containing 10 mM Tris, 1.5 mM MgCl$_2$, 50 mM KCl, 200 μM dNTPs, 2.5 units of Taq DNA polymerase, 57 pmol of universal reverse primer (5'-CACGAGAAA-CAGCTATGACC-3'), and 1 ng of template DNA. The 5′K-derived subclone pSKCH550, containing the area of the mannitol promoter from C1aI (position −354) to HindIII (position +212), was used as template DNA. After 30 cycles of 1-min denaturation at 94 °C, 1-min annealing at 55 °C, and 1-min elongation at 72 °C, the 473-base pair PCR product was separated by electrophoresis on a 0.8% agarose gel and isolated from the gel with the QIAGEN gel extraction kit.

**RESULTS**

Two domains, PRD-I and PRD-II, are expected to contain the phosphorylation sites based on the homology of MtlR to anti-terminators such as SacY and BglG and the DNA-binding regulators LieR and LevR. Alignment of these domains reveals two conserved histidines in each PRD (Fig. 1, B and C) that could be the phosphorylation sites involved in the regulation of MtlR. To demonstrate their involvement, mutants were made in which one or two of the conserved histidines were replaced by alanine. Wild-type MtlR and mutant MtlR were expressed in *E. coli* BL21(DE3) and purified by Ni$_2$-nitrilotriacetic acidagarose chromatography. The expression levels, yield, and purity of all the mutants are comparable to those of the wild-type protein (data not shown). The purified proteins were used in [γ-32P]P-enolpyruvate-dependent phosphorylation and footprint experiments to examine the effects of the mutation on HPr- and IIC_{mtl}-dependent phosphorylation and the binding properties of the protein for the mannitol operator.

**Table I**

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Mutant</th>
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<tbody>
<tr>
<td>pETMtlRhis</td>
<td>Wild-type His-tagged MtlR</td>
</tr>
<tr>
<td>pETMtlR-AHHH</td>
<td>H236A</td>
</tr>
<tr>
<td>pETMtlR-RAHH</td>
<td>H295A</td>
</tr>
<tr>
<td>pETMtlR-HAH</td>
<td>H348A</td>
</tr>
<tr>
<td>pETMtlR-HMAH</td>
<td>H405A</td>
</tr>
<tr>
<td>pETMtlR-HAAH</td>
<td>H236A/H95A</td>
</tr>
<tr>
<td>pETMtlR-HAAH</td>
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<tr>
<td>pETMtlR-HAH-H598A</td>
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**Table II**

<table>
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<tr>
<th>Mutation</th>
<th>Primer</th>
<th>Restriction site</th>
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<tr>
<td>H236A</td>
<td>3'-CCGATATTTTGGTCGTT</td>
<td>NheI</td>
</tr>
<tr>
<td>H295A</td>
<td>3'-CCGAGGATTTGCGATAATC-5'</td>
<td>NcoI</td>
</tr>
<tr>
<td>H348A</td>
<td>3'-CCTTTGCGCCGATCTAATCC-5'</td>
<td>DraI</td>
</tr>
<tr>
<td>H405A</td>
<td>3'-CCGTTTGGCGCATCTAATT-5'</td>
<td>PstI</td>
</tr>
<tr>
<td>H598A</td>
<td>3'-CCGAGGAAGTTCGCTAGTTTAGTACGTTCTAATCAGGCTCGAG-5'</td>
<td>NheI</td>
</tr>
</tbody>
</table>
A careful analysis of the MtlR sequence was performed to identify additional putative phosphorylation sites. A sequence with low identity to IIA proteins of the fructose family, including H598A of B. steaothermophilus, was found at the C-terminus of MtlR. IIA proteins or domains are responsible for the transfer of the phosphoryl group from HPr to the B domain of the transporter. The new putative phosphorylation site is His-598 since it aligns with the active-site phosphohistidines of the IIA proteins. To test whether His-598 is involved in PTS-dependent phosphorylation, H598A mutants were made, and $^{32}$P-enolpyruvate-dependent phosphorylation was performed as described for the PRD mutants. Based on these experiments (Fig. 2B), HPr-dependent phosphorylation was not affected by the H598A, H236A/H598A, or H295A/H598A mutation. On the other hand, the IICB$^{\text{mut}}$-dependent phosphorylation levels of all H598A mutants were affected; they did not exceed the HPr background phosphorylation. These data indicate that the H598A mutation strongly reduces or completely inhibits IICB$^{\text{mut}}$-dependent phosphorylation.

The phosphorylation experiments described above do not exclude HPr-dependent phosphorylation of PRD-I. We must consider whether mutations in PRD-II have reduced the efficiency of phosphorylation of PRD-I by HPr. Initial protease digestion experiments showed that MtlR can be primarily cut at sites in between PRD-I and PRD-II (data not shown). This implies that the phosphorylation of PRD-I by HPr or IICB$^{\text{mut}}$ could be resolved using limited proteolysis of MtlR. This was done by phosphorylation of wild-type MtlR by HPr or IICB$^{\text{mut}}$ using $^{32}$P-enolpyruvate, followed by partial digestion of the phosphorylated protein by a-chymotrypsin. Phosphorylated fragments were separated by SDS-polycrylamide gel electrophoresis and transferred to nitrocellulose. The phosphorylated fragments were visualized using a PhosphorImager (Fig. 3A). Fragments containing the N-terminal His tag were colored using His-tag-specific antibodies (Fig. 3B). The mass of each fragment was determined using a partial CNBr digest of MtlR (eco, E.coli). HTH, helix-turn-helix.
Additional cleavage products containing the IICB<sup>mtl</sup> phospho-ol MtlR that could not be removed during purification (7). Protein (Fig. 3), are probably phosphorylated degradation products and IICB<sub>mtl</sub>.

Replacement of histidine by alanine is indicated by <sup>A</sup> lane 10. Since mutations affecting PTS-de-

 affinity of MtlR for DNA is dependent on its phosphorylation state (7). Since mutations affecting PTS-de-

 the N-terminal His-tagged peptides is shown in <sup>A</sup> lane 1. The positions (pos.) of these residues are indicated to the left. The control experiment without MtlR is presented in lane 10. In the <sup>A</sup> upper panels, phosphorylation was carried out with 8 μM [γ<sup>32</sup>P]<sub>P</sub>-enolpyruvate, 0.04 mg/ml EI, and 5 μM HPr. In the middle panels, phosphorylation was carried out with 8 μM [γ<sup>32</sup>P]<sub>P</sub>-enolpyruvate, 0.04 mg/ml EI, 0.5 μM HPr, 0.4 μM IIA<sup>mtl</sup>, and 0.02 μM IICB<sup>mtl</sup>. In the <sup>A</sup> lower panels, a reaction without IICB<sup>mtl</sup> was performed for each mutant to determine the background of HPr-dependent phosphorylation (HPr Backgr.) in the IICB<sup>mtl</sup> phosphorylation experiment. The mixtures were incubated for 5 min at 30 °C, and the reactions were then started by the addition of 0.09 mg/ml MtlR or MtlR mutant. After 20 min at 30 °C, the reactions were stopped with 0.4 volume of denaturation buffer.

**Fig. 2. Phosphorylation of MtlR and MtlR mutants by HPr and IICB<sup>mtl</sup>.** Shown is the phosphorylation of MtlR and MtlR mutants with single or double mutations of putative histidine phosphorylation sites replaced by alanine in PRD-I and PRD-II (A) and the IIA-like domain (B). Replacement of histidine by alanine is indicated by A above the lanes. The positions (pos.) of these residues are indicated to the left. The control experiment without MtlR is presented in lane 10. In the upper panels, phosphorylation was carried out with 8 μM [γ<sup>32</sup>P]<sub>P</sub>-enolpyruvate, 0.04 mg/ml EI, and 5 μM HPr. In the middle panels, phosphorylation was carried out with 8 μM [γ<sup>32</sup>P]<sub>P</sub>-enolpyruvate, 0.04 mg/ml EI, 0.5 μM HPr, 0.4 μM IIA<sup>mtl</sup>, and 0.02 μM IICB<sup>mtl</sup>. In the lower panels, a reaction without IICB<sup>mtl</sup> was performed for each mutant to determine the background of HPr-dependent phosphorylation (HPr Backgr.) in the IICB<sup>mtl</sup> phosphorylation experiment. The mixtures were incubated for 5 min at 30 °C, and the reactions were then started by the addition of 0.09 mg/ml MtlR or MtlR mutant. After 20 min at 30 °C, the reactions were stopped with 0.4 volume of denaturation buffer.

**Fig. 3. Limited proteolysis of MtlR phosphorylated by HPr and IICB<sup>mtl</sup>.** MtlR was phosphorylated by HPr (lanes 1 and 2) and IICB<sup>mtl</sup> (lanes 3 and 4) exactly as described for Fig. 2A. Part of the phosphorylated MtlR protein was digested by α-chymotrypsin for 2 min. Both uncleaved MtlR (lanes 1 and 3) and cleaved MtlR (lanes 2 and 4) were analyzed by SDS-polyacrylamide gel electrophoresis as described under “Experimental Procedures.” The autoradiogram presenting phosphorylated peptides is shown in A, and the Western blot showing only the N-terminal His-tagged peptides is shown in B. Band I is uncleaved MtlR, and Bands II and III are cleavage products of interest. His-tagged MtlR, partly cleaved by CNBr, was used as a reference. The positions (Pos) of the N-terminal His-tagged peptides in the CNBr digest are indicated to the right. For each CNBr cleavage fragment, the cleavage location in the sequence and the mass of the peptide are given.

Phosphorylation to approximately the same extent. When the protein was phosphorylated by IICB<sup>mtl</sup>, only Band II, containing PRD-II and the IIA-like domain, was phosphorylated. This excludes a possible phosphorylation of PRD-I by IICB<sup>mtl</sup>.

The weak bands, visible in lanes containing the uncut protein (Fig. 3), are probably phosphorylated degradation products of MtlR that could not be removed during purification (7). Additional cleavage products containing the IICB<sup>mtl</sup> phosphorylation site His-598 could explain the additional labeled polypeptides such as fragment IV present only in digests of MtlR phosphorylated by IICB<sup>mtl</sup> (Fig. 3A, lane 4).

**Phosphorylation and the Affinity of MtlR Mutants for DNA Binding—**The affinity of MtlR for DNA is dependent on its phosphorylation state (7). Since mutations affecting PTS-de-

pendent phosphorylation would also affect the response of the protein to different phosphorylation conditions, we performed quantitative DNA footprint experiments using several mutants. Four different phosphorylation conditions per mutant were examined: 1) dephosphorylation of MtlR in the presence of EI, HPr, IIA<sup>mtl</sup>, IICB<sup>mtl</sup>, and mannnitol (Fig. 4, □); 2) phosphorylation of MtlR by HPr in the presence of P-enolpyruvate, EI, and HPr (○); 3) phosphorylation of MtlR by HPr and IICB<sup>mtl</sup> in the presence of P-enolpyruvate, EI, HPr, IIA<sup>mtl</sup>, and IICB<sup>mtl</sup> (△); and 4) phosphorylation of MtlR by HPr in the presence of P-enolpyruvate, EI, and HPr and simultaneous dephosphorylation by IICB<sup>mtl</sup> and mannnitol (◇). Since MtlR was purified in a phosphorylated form, MtlR and its mutants were first dephosphorylated by incubation at pH 6.5 for 2 h at 30 °C. After incubation of MtlR and the mutant proteins under the different phosphorylation conditions, the proteins were diluted to various concentrations using the same phosphorylation mixtures to maintain identical phosphorylation conditions during the DNA binding experiments for all dilutions. Binding of MtlR to DNA was followed by measuring the intensity of the footprint located at positions −46 to −41 (7). The level of protection of this region at each protein concentration was calculated from the decrease in intensity of this area compared with that of the unprotected DNA. These values are plotted against the logarithm of the protein concentration for each phosphorylation condition and each mutant in Fig. 4. The midpoint of the sigmoidal curves represents the concentration of MtlR that gives a protection level of 50% and is a measure of the affinity of the protein for DNA. Wild-type MtlR (Fig. 4A) behaved under the four phosphorylation conditions as observed previously (7). Phosphorylation by HPr resulted in a small increase, whereas phosphorylation by HPr and IICB<sup>mtl</sup> resulted in a decrease in binding affinity compared with the non-phosphorylated protein. Maximal stimulation was observed with the combination of phosphorylation of MtlR by HPr and dephosphorylation via IICB<sup>mtl</sup> and mannnitol.

When one of the conserved histidines in PRD-I was replaced (Fig. 4, B and C), the affinity of these mutants, when phosphorylated by HPr (○) alone or by HPr and IICB<sup>mtl</sup> (◇), increased 4–5-fold compared with the affinity of wild-type MtlR under
the B. stearothermophilus Mannitol Regulator MtlR

FIG. 4. DNA binding of MtlR and MtlR mutants exposed to different phosphorylation conditions. MtlR and MtlR mutants were dephosphorylated or phosphorylated by adding different components of the PTS, including P-enolpyruvate and mannitol, as described under "Results." After incubation, the samples were diluted to different concentrations of MtlR, and the binding to DNA was determined by DNA footprinting as described under "Experimental Procedures." For each phosphorylation condition, the relative protection is plotted against the logarithm of the concentration of MtlR or MtlR mutant. The relative protection is a measure of the number of DNA molecules bound against the logarithm of the concentration of MtlR or MtlR mutant. The four phosphorylation conditions and the components present in each of these conditions are presented at the top. The concentrations of these components, when added, were as follows: 5 mM P-enolpyruvate, 0.04 mg/ml EI, 9 mM P-enolpyruvate, formation of mannitol phosphate to a certain level depended phosphorylation of mannitol was not observed if MtlR were already phosphorylated. The P-enolpyruvate-independent phosphorylation of mannitol phosphate was observed (\( \Delta \)) when the difference between the reaction with and without P-enolpyruvate. The slope of the P-enolpyruvate-dependent reaction was determined by linear regression and is a measure for the phosphoryl transfer rate from P-enolpyruvate to \([^{3}H]\)mannitol via the PTS.

FIG. 5. \([^{3}H]\)Mannitol phosphorylation catalyzed by MtlR and components of the PTS. The formation of \([^{3}H]\)mannitol phosphate by 0.04 mg/ml EI, 1.5 mM HPr, 0.05 mg/ml MtlR, and 0.02 mM IICB\( ^{\text{mtl}} \) was followed in the presence (○) and absence (□) of P-enolpyruvate (PEP). The P-enolpyruvate-dependent component of the \([^{3}H]\)mannitol phosphorilation (△) was calculated from the difference between the reaction with and without P-enolpyruvate. The slope of the P-enolpyruvate-dependent reaction was determined by linear regression and is a measure for the phosphoryl transfer rate from P-enolpyruvate to \([^{3}H]\)mannitol via the PTS.

If one or both conserved histidines of PRD-II was replaced by alanine (Fig. 4, E–G), the positive effects of phosphorylation by HPr disappeared (△), Phosphorylation by HPr alone (○) resulted even in a decrease in affinity for the DNA compared with the non-phosphorylated protein (□). The negative effects of phosphorylation by IICB\( ^{\text{mtl}} \) appeared to be unaffected in these mutants (△). The observed negative effect of phosphorylation by HPr (○) on these mutants disappeared if the protein was simultaneously dephosphorylated by IICB\( ^{\text{mtl}} \) and mannitol (△). However, under these conditions, the affinity of the protein did not exceed that of the non-phosphorylated protein (compare △ and □). Replacement of His-598 by alanine had a dramatic effect on the regulation of MtlR by the PTS. The H598A mutant had a low affinity under all phosphorylation conditions, comparable to that of wild-type MtlR phosphorylated by HPr and IICB\( ^{\text{mtl}} \).

MtlR-dependent Phosphoryl Transfer from HPr to IICB\( ^{\text{mtl}} \)—It was suggested that HPr could play a dual role in the regulation of MtlR since maximal stimulation of DNA binding was observed when MtlR was both phosphorylated by HPr and dephosphorylated by IICB\( ^{\text{mtl}} \). Diphosphorylation of one of the PRDs by IICB\( ^{\text{mtl}} \) could be a possible explanation. When a site can be phosphorylated by HPr and subsequently dephosphorylated by IICB\( ^{\text{mtl}} \), P-enolpyruvate-dependent phosphoryl transfer from HPr to IICB\( ^{\text{mtl}} \) via this site on MtlR must be possible. The transfer of a phosphate group from P-enolpyruvate via EI, HPr, MtlR, and IICB\( ^{\text{mtl}} \) to mannitol can be followed by measuring the formation of \([^{3}H]\)mannitol phosphate as described under "Experimental Procedures." The formation of mannitol phosphate was followed against time in the presence (○) and absence (□) of P-enolpyruvate. The slope of the P-enolpyruvate-independent reaction was determined by linear regression and is a measure for the number of phosphoryl groups present on MtlR. A linear increase in the level of mannitol phosphate was observed (△) when the difference between the reaction with (○) and without (□) P-enolpyruvate was plotted against the reaction time. The P-enolpyruvate-dependent phosphoryl transfer rate can be calculated from the slope of this line and is dependent on the MtlR concentration used (data not shown). The phosphoryl transfer via MtlR is not efficient
The formation of \(^{3}H\)mannitol 1-phosphate as a measure of phosphoryl transfer by the PTS was followed against time as described in the legend of Fig. 5. The phosphoryl transfer rate was calculated from the slope of the difference between the reaction with and without P-enolpyruvate. The P-enolpyruvate-independent phosphorylation level is a measure of the number of phosphoryl groups present in the system, which was derived from the end level of \(^{3}H\)mannitol 1-phosphate formed in the absence of P-enolpyruvate. All reactions were carried out at comparable MtlR or MtlR mutant concentrations (~50 μg/ml). In the case of the H598A + H348A/H405A complementation experiment, both MtlR mutants were added to a concentration of 45 μg/ml. The activity in this case is expressed as the amount of \(^{3}H\)mannitol 1-phosphate formed per g of one of the mutants.

<table>
<thead>
<tr>
<th>Mutant</th>
<th>PEP(^{-}) dependent activity</th>
<th>PEP-independent phosphorylation level</th>
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<tbody>
<tr>
<td>Wild-type</td>
<td>29</td>
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<tr>
<td>H236A</td>
<td>18</td>
<td>0.6</td>
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<tr>
<td>H295A</td>
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<td>H405A</td>
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</tr>
<tr>
<td>H598A + H348A/H405A</td>
<td>37</td>
<td>0.8</td>
</tr>
<tr>
<td>II(^{A}_{\text{mut}})</td>
<td>7600</td>
<td>0.0</td>
</tr>
</tbody>
</table>

\(^{a}\) PEP, P-enolpyruvate.

**FIG. 6. Proposed model for the regulation of MtlR by the PTS.**

MtlR can be phosphorylated on several domains, leading to an increased (+) or decreased (−) affinity for the mannitol promoter region. Phosphorylation of PRD-II by HPr results in an increased affinity of MtlR for DNA, whereas phosphorylation of PRD-I by HPr and of the IIA-like domain by IICB\(^{mut}\) prevents binding to this region. Stimulation by phosphorylated PRD-II is only possible when the domains involved in negative regulation, PRD-I and the IIA-like domain, are dephosphorylated. A, in the absence of mannitol and other substrates, all PTS proteins will be in the phosphorylated state. MtlR will be phosphorylated by HPr and IICB\(^{mut}\) on all its domains and have a low affinity for the mannitol operator. Since MtlR is probably a transcriptional activator, the expression of the mannitol operon is not stimulated under these conditions. B, in the presence of mannitol, IICB\(^{mut}\) prevents binding to this region. Stimulation by phosphorylated PRD-II can take place. C, when rapidly metabolizable PTS substrates, including mannitol, are transported, the concentration of HPr decreases. Consequently, MtlR is no longer phosphorylated at PRD-II, and the expression of the mannitol operon is no longer stimulated.

### III. In these experiments, we determined the P-enolpyruvate-dependent phosphoryl transfer rate of a mixture of the inactive H598A mutant and the PRD-II H348A/H405A double mutant with an activity of 24% compared with the wild-type protein. Combination of equal amounts of the PRD-II H348A/H405A mutant and the inactive H598A mutant resulted in the recovery of phosphorylation activity. 127% of wild-type activity was found when the activity was calculated as the phosphorylation rate/mg of one of the mutants (Table III). The double amount of
interact PRD-I domain in the complementation reaction compared with the reaction of wild-type MtiR could explain a complementation beyond 100% activity.

**DISCUSSION**

Interplay between HPr- and IICB<sup>mut</sup>-dependent Phosphorylation and Dephotophosphorylation in the Regulation of MtiR—MtiR senses the presence of mannitol and the need to utilize this substrate by monitoring the phosphorylation state of HPr and IICB<sup>mut</sup>. Depending on the amounts of HPr, phospho-HPr, IICB<sup>mut</sup>, and phospho-IICB<sup>mut</sup>, phosphorylation or dephosphorylation of the individual domains of MtiR in the cell will lead to the stimulation or reduction of the expression of the mannitol operon as shown in Fig. 6. The phosphorylation level of HPr is dependent on the rate of uptake of all PTS carbohydrates, whereas that of IICB<sup>mut</sup> is dependent only on the uptake rate of mannitol. At low PTS activities, phospho-HPr accumulates. MtiR is phosphorylated on both PRDs, resulting only in a slight stimulation of binding to DNA (Fig. 6A). Before full stimulation of MtiR by phosphorylated PRD-II can take place, PRD-I needs to be dephosphorylated by IICB<sup>mut</sup> and mannitol (Fig. 6B). In the absence of phospho-HPr, MtiR is not phosphorylated on PRD-II and will not be stimulated to bind to the mannitol promoter region (Fig. 6C).

Location of the HPr- and IICB<sup>mut</sup>-dependent Phosphorylation Sites in MtiR—Phosphorylation reactions with MtiR mutants indicated that both His-348 and His-405 of PRD-II are involved in the stable HPr-dependent phosphorylation. Whether PRD-I was phosphorylated by HPr could not be concluded from such experiments. Instead, α-chymotrypsin cleavage of wild-type MtiR at a location in between PRD-I and PRD-II was employed. It revealed that PRD-I was phosphorylated by HPr; however, the process was dependent on a functional PRD-II. Single mutations in PRD-II strongly reduced the total HPr-dependent phosphorylation of MtiR, whereas PRD-I and PRD-II were phosphorylated to the same order of magnitude when phosphorylation of the wild-type protein was studied in the α-chymotrypsin digestion experiments. In contrast, mutations in PRD-I seemed not to affect phosphorylation of PRD-II by HPr. A similar relation between a mutation in one PRD and the phosphorylation of another PRD has been observed for SacY and BglG (20–22). These observations suggest that phosphorylation of PRD-I and that of PRD-II are not independent reactions. Even the two phosphorylation sites within one PRD are not phosphorylated independently. In the case of PRD-II, a single mutation of either of the two phosphorylation sites results in the loss of HPr-dependent phosphorylation.

HPr-dependent Regulation of MtiR—The affinity of MtiR for its DNA-binding site is regulated by phosphorylation via HPr or IICB<sup>mut</sup>. Our previous work suggested that phosphorylation by HPr could have two effects, one leading to an increase and the other to a decrease in the affinity of MtiR for DNA (7). The current study shows that HPr phosphorylates both PRD-I and PRD-II. The increased affinity is probably due to phosphorylation of PRD-II because, when phosphorylation sites are removed from this domain, phosphorylation of MtiR by HPr no longer results in an increased affinity for DNA. Similarly, the decreased affinity is due to the phosphorylation of PRD-I; removal of phosphorylation sites from this domain results in a protein that, when phosphorylated by HPr, possesses much higher affinities than wild-type MtiR for DNA.

The relationship between positive and negative regulation and phosphorylation of PRD-II and PRD-I, respectively, correlates with that of the anti-terminator SacT and probably LicT. SacT is involved in the activation of the sacPA operon of *Bacillus subtilis*. Mutations in PRD-I of SacT result in the loss of negative control by the PTS upon expression of the sacPA operon (23, 24). The involvement of PRD-II in the positive regulation of sacT was suggested by site-directed mutagenesis studies. For the anti-terminator LicT, the involvement of PRD-II with positive regulation has been confirmed (25). Whether PRD-I in this protein is responsible for the observed negative control by the PTS is still unclear (26–28). In contrast with LicT, SacT, and MtiR, phosphorylation of PRD-II in BglG and LevR is correlated with a negative regulation of these proteins (21). PRD-I is responsible for the positive regulation of LevR (29, 30).

**IICB<sup>mut</sup>-dependent Regulation of MtiR**—The affinity of wild-type MtiR is decreased by phosphorylation by IICB<sup>mut</sup>. Analysis of the chymotrypsin cleavage data indicated that PRD-II is phosphorylated in a IICB<sup>mut</sup>-dependent manner that is contingent on the presence of His-598 in the IIA-like domain. This same analysis showed that PRD-I is not phosphorylated by IICB<sup>mut</sup>. Nevertheless, the affinity of PRD-I mutants phosphorylated by HPr can be reduced by additional phosphorylation by IICB<sup>mut</sup>. This points to a negative control site outside of PRD-I that can be phosphorylated by IICB<sup>mut</sup>. The most likely candidate is His-598 in the IIA-like domain. Mutation of His-598 to alanine was expected to release the negative effect of phosphorylation by IICB<sup>mut</sup>, but instead resulted in low affinity under all phosphorylation conditions. The mutation of His-598 could influence the structure of MtiR, resulting in the low affinity of this mutant. The possible inability of this mutant to dephosphorylate the site involved in negative control, PRD-I, is a less likely explanation since the H236A/H598A double mutant gives a similar result compared with the H598A single mutant in DNA binding experiments (data not shown).

IICB<sup>mut</sup> is also needed for the release of negative control. Maximal HPr-dependent stimulation of MtiR-DNA binding is observed only in the presence of IICB<sup>mut</sup> and the substrate mannitol. Dephosphorylation of sites of MtiR involved in negative control such as PRD-I by IICB<sup>mut</sup> and mannitol could be an explanation. A relation between the response to an available substrate and the corresponding permease is also found for other PRD-containing proteins, as demonstrated for the combinations BglG/BglF, SacY/SacX, LicT/BglP, GlcT/IICBA<sup>Δh</sup>, and LevR/LevE (28, 31–34). Mutations affecting the phosphorylation of these permeases resulted in constitutive expression of the genes under control of the corresponding transcriptional activators. Whether a IICB<sup>mut</sup> mutation in *B. stearothermophilus* will lead to constitutive expression of the mannitol operon is questionable since the in vitro DNA binding of MtiR phosphorylated in the absence of IICB<sup>mut</sup> is only slightly stimulated by HPr compared with the non-phosphorylated protein.

Dephosphorylation of the PRDs by IICB<sup>mut</sup> via the IIA-like Domain in MtiR—The above-proposed dephosphorylation of PRD-I by IICB<sup>mut</sup> implies that phosphorylation sites on PRD-I are directly or indirectly accessible to both HPr and IICB<sup>mut</sup>. This is confirmed by the observed phosphoryl transfer from HPr to IICB<sup>mut</sup> via MtiR. Internal phosphoryl transfer from one site to the other within MtiR is likely since HPr and IICB<sup>mut</sup> have different phosphorylation targets on MtiR. Indeed, both the PRDs and His-598 appear to be involved in the phosphoryl transfer, as was demonstrated using MtiR mutants. His-598 is essential, indicating an important role for the IIA-like domain in this process. Mutations in PRD-I and PRD-II also affect phosphoryl transfer; however, phosphoryl transfer was not abolished for any of the PRD mutants, including the H236A/H295A and H348A/H405A double mutants, demonstrating that phosphoryl transfer is not solely dependent on one of the two PRDs. Probably both PRD-I and PRD-II can be dephospho-

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<sup>2</sup> M. Arnaud, unpublished results.
rylated by IICB<sup>mutl</sup>. Even PRD-I/PRD-II double mutants showed some phosphoryl transfer activity. At this point, direct phosphoryl transfer from HPr to the IIA-like domain cannot be excluded completely. Mutations in PRD-I and PRD-II could affect this process and explain the observed differences in the phosphoryl transfer rates of the various mutants.

An indication that phosphoryl transfer from the PRDs to the IIA-like domain takes place is the complementation observed when two mutant proteins, the PRD-II mutant H348A/H405A and the IIA-like domain mutant H598A, were combined. The low activity of the PRD-II double mutant could be restored by the inactive H598A mutant. This demonstrates that the phosphoryl groups can be transferred between MtlR molecules and could be seen as evidence for a functional interaction between two MtlR molecules with transfer occurring over the MtlR-MtlR interface. An increase in phosphoryl transfer activity was observed for the PRD-I mutant H295A compared with the wild-type protein, suggesting that the rate of transfer via PRD-II is affected by this mutation. Differences in the association state upon phosphorylation could be the mechanism controlling the affinity of the protein for DNA.

**Conclusion**—MtlR and the PTS provide a regulatory system that can monitor the presence of the substrate and the need to utilize it. MtlR is the first protein in the class of PRD-containing transcriptional regulators for which a dual effect on the activity of MtlR by HPr-dependent phosphorylation has been shown. Also, the phosphorylation of one or more sites by HPr could be responsible since they would affect the proposed phosphoryl transfer from one MtlR molecule to the other. Changes in the association state upon phosphorylation could be the mechanism controlling the affinity of the protein for DNA.

**REFERENCES**