Regulation of the *Bacillus subtilis* mannitol utilization genes: promoter structure and transcriptional activation by the wild-type regulator (MtlR) and its mutants

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Expression of mannitol utilization genes in *Bacillus subtilis* is directed by P$_{mtlA}$, the promoter of the *mtlAFD* operon, and P$_{mtlR}$, the promoter of the MtlR activator. MtlR contains phosphoenolpyruvate-dependent phosphotransferase system (PTS) regulation domains, called PRDs. The activity of PRD-containing MtlR is mainly regulated by the phosphorylation/dephosphorylation of its PRDII and EIIB$_{Gat}$-like domains. Replacing histidine 342 and cysteine 419 residues, which are the targets of phosphorylation in these two domains, by aspartate and alanine provided MtlR-H342D C419A, which permanently activates P$_{mtlA}$ in vivo. In the mtlR-H342D C419A mutant, P$_{mtlA}$ was active, even when the mtlAFD operon was deleted from the genome. The mtlR-H342D C419A allele was expressed in an *Escherichia coli* strain lacking enzyme I of the PTS. Electrophoretic mobility shift assays using purified MtlR-H342D C419A showed an interaction between the MtlR double-mutant and the Cy5-labelled P$_{mtlA}$ and P$_{mtlR}$ DNA fragments. These investigations indicate that the activated MtlR functions regardless of the presence of the mannitol-specific transporter (MtlA). This is in contrast to the proposed model in which the sequestration of MtlR by the MtlA transporter is necessary for the activity of MtlR. Additionally, DNase I footprinting, construction of P$_{mtlA}$-P$_{licB}$ hybrid promoters, as well as increasing the distance between the MtlR operator and the $_{35}$ box of P$_{mtlA}$ revealed that the activated MtlR molecules and RNA polymerase holoenzyme likely form a class II type activation complex at P$_{mtlA}$ and P$_{mtlR}$ during transcription initiation.

**INTRODUCTION**

*Bacillus subtilis* mainly takes up carbohydrates via a phosphoenolpyruvate-dependent phosphotransferase system (PTS) (Reizer *et al.*, 1999). In the PTS, a carbohydrate is concomitantly taken up and phosphorylated by its specific transporter (also known as enzyme II or EII). In addition to the sugar-specific transporter, the PTS consists of general proteins, namely enzyme I (EI) and a histidine containing phosphocarrier protein (HPr). EI phosphorylates HPr at histidine 15 using phosphoenolpyruvate (PEP) as the donor of the phosphoryl group. HPr(H15∼P) then transfers the phosphoryl group to the sugar-specific EII. The PTS is not only used for sugar uptake. HPr of *B. subtilis* can also be phosphorylated at serine 46 by HPr kinase in response to the concentration of the glycolysis intermediate, fructose 1,6-biphosphate. In this case, it acts as a signal transduction system by which the cell regulates various operons in response to the presence/absence of specific carbohydrates in the milieu. This is mainly mediated by a global regulator, the carbon catabolite protein A (CcpA). CcpA binds in a complex with HPr(H346∼P) to a specific cis element (cre site) at the target promoters causing carbon catabolite repression/activation (CCR/CCA) (for reviews, see Deutscher *et al.*, 2006; Fujita, 2009; Sonenshein, 2007).

In *B. subtilis*, expression of the operons encoding the PTS components is generally regulated by transcriptional antiterminators, activators or repressors (Deutscher *et al.*, 2002, 2006). Amongst these, transcriptional antiterminators and activators have received particular attention due to their interactions (phosphoryl group transfer and/or direct protein–protein interaction) with the cognate sugar-specific transporters as well as with HPr, which allows a completely different, CcpA-independent, type of carbon catabolite repression (Joyet *et al.*, 2013). Antiterminators, such as LicT, consist of an RNA binding domain (CAT) and two PTS regulation domains (PRDI and PRDII)
The operons encoding the mannitol-, mannose- and oligo β-glucoside-PTSs are regulated by their specific activators, i.e. MtlR (Watanabe et al., 2003), ManR (Sun & Altenbuchner, 2010) and LicR (Tobisch et al., 1999), respectively. Similar to the PRD-containing antiterminators the PRD-containing activators MtlR, ManR and LicR carry two PRD domains, whereas the CAT domain is replaced by a DNA-binding domain of the DeoR type. All these activators carry additional EIIA and EIIB regulatory domains similar to the cytoplasmic domains of PTS transporters (Greenberg et al., 2002). Due to this difference, the activities of MtlR, ManR and LicR are modulated by the (de)phosphorylation of their PRDII, EIIA- and EIIB-like conserved regulatory domains, whereas the PRD1 domain does not seem to play any role (Joyet et al., 2013). There are two additional activators for PTS transporters in B. subtilis, LevR and MalR. LevR is a NifA/NtrC-type PRD-containing activator interacting with the RNA polymerase holoenzyme (RNAP) containing σL (Martin-Verstraete et al., 1992). The activity of MalR is supposed to be regulated by maltose 6-phosphate via a sugar isomerase domain (SIS) (Yamamoto et al., 2001).

The mannitol utilization system of B. subtilis consists of the mtlAFD operon (mtlA encodes EIICBMtl, mtlF encodes EIIAβMtl, and mtlD encodes mannitol 1-phosphate 5-dehydrogenase) and a separated mtlR locus (activator encoding gene) (Joyet et al., 2010; Watanabe et al., 2003). MtlR activates the promoters of both, the mtlAFD operon (PmtlA) and mtlR (PmtlR), each containing a σA-type promoter structure (Heravi et al., 2011; Watanabe et al., 2003). The prerequisite step for the activation of MtlR is the HPr(H15~P)-dependent phosphorylation of the MtlR PRDII domain (H342 residue). Any loss of phosphorylation in PRDII due to shortage of HPr(H15~P) renders MtlR completely inactive (Joyet et al., 2010). Mutation of H342 to aspartate compensates the dependency of MtlR on HPr(H15~P)-dependent phosphorylation (Heravi et al., 2011; Joyet et al., 2010). Complete activation (or induction) of MtlR additionally needs dephosphorylation of the EIIABγMtl-like domain of MtlR. Phosphorylation and dephosphorylation occurs via the cytoplasmic phosphocarrier EIIAβMtl (MtlF). The uptake of mannitol leads to the dephosphorylation of MtlF-P. As a result, the MtlF protein dephosphorylates the EIIABγMtl-like domain of MtlR (Joyet et al., 2010).

In addition to (de)phosphorylation of MtlR, it has been recently reported that MtlR sequestration by MtlA (EIIICMtl) might also be necessary for the activity of MtlR (Bouraoui et al., 2013; Joyet et al., 2013). Prior to that, the protein–protein interaction between a regulator and its cognate transporter was also reported for the (de)activation of the E. coli antiterminator, BglG (Lopian et al., 2003). To clarify whether the active form of MtlR needs the transporter for its function, we studied mtlR mutants in different backgrounds. In this way, we showed that a double-mutant MtlR was functional in vivo as well as in vitro, even when MtlA was absent. Likewise, the MtlR binding site at PmtlA and PmtlR was characterized by using the active double-mutant MtlR.

METHODS

Bacterial strains and growth conditions. The bacterial strains used in this study are listed in Table 1. Escherichia coli JM109 was used for plasmid propagation. B. subtilis 3NA was used as a host for expression vectors or gene integration. Unless otherwise specified, transformants of E. coli and B. subtilis were selected on LB agar (Luria et al., 1960) supplemented with ampicillin (100 μg ml−1) or spectinomycin (100 μg ml−1) depending on the plasmid antibiotic marker. Integration mutants of B. subtilis were selected on LB agar containing chloramphenicol (5 μg ml−1), erythromycin (5 μg ml−1) or spectinomycin (100 μg ml−1). Histidine prototroph transformants were selected on Spizizen’s minimal medium (Harwood & Cutting, 1990), while the histidine auxotroph mutants were tested on Spizizen’s minimal medium with 20 μg ml−1 i-histidine. Induction of the mtl promoters, the hybrid promoters, or the lic operon promoter was carried out by 1:50 inoculation of the strains in 42.5 ml LB in 250 ml Erlenmeyer flasks followed by overnight culture. All strains were incubated at 37 °C with shaking at 200 r.p.m. Aliquots of 8 ml with an OD600 of 0.4 were divided in 100 ml Erlenmeyer flasks and subsequently induced by the addition of different carbohydrates, namely mannitol, mannitol together with glucose, cellobiose, or cellobiose with glucose to a final concentration of 0.2% (w/v) for each carbohydrate. Cultures were harvested 1 h after the addition of the carbohydrates and used for β-galactosidase activity measurements. All experiments were repeated at least three times and mean values and standard deviations are presented.

DNA manipulation and bacterial transformation. Standard molecular techniques were used according to Sambrook et al. (1989). Natural transformation of B. subtilis was performed according to the ‘Paris Method’ (Harwood & Cutting, 1990). The oligonucleotides (synthesized by Eurofins MWG Operons) employed in this study are listed in Table 2. PCRs were performed using Phusion Hot Start II High-Fidelity DNA Polymerase (Fisher Scientific GmbH) on a PTC-200 Peltier Thermal Cycler (MJ Research). DNA preparation kits (Qiagen) were used for chromosomal DNA or plasmid extraction according to the manufacturers’ instructions. DNA constructs were sequenced by GATC Biotech AG.

Construction of mtlR mutants. Construction of different B. subtilis mtlR mutants was performed by replacement of a chromosomal erythromycin resistance gene via homologous recombination with a particular mtlR allele. To amplify PmtlA-mltR-H342D, PCRs were performed with the oligonucleotides s6949/s6865 and s6866/s6867 using B. subtilis 168 chromosomal DNA as a template. Next, fusion
### Table 1. Strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Genotype or relevant structure</th>
<th>Source or reference</th>
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<td><strong>E. coli strains</strong></td>
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<td>JM109</td>
<td>recA1, endA1, gyrA96, thi-1, hsdR17(16F−, mK−), mcrA, supE44, gyrA96, relA1, λ−, Δ(lac-proAB), F' (traD36, proAB+, lacI, Δ(lacZ)M15)</td>
<td>Yanisch-Perron et al. (1985)</td>
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<td>JW2409-1</td>
<td>F−, Δ araD-araB)567, ΔlacZ4787::ramB-3, λ−,  ΔptsI745::kan, rph-1, Δ(rhaD-rhaB)568, hsdR514</td>
<td>Baba et al. (2006)</td>
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<td><strong>B. subtilis strains</strong></td>
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<td>3NA</td>
<td>spo0A3</td>
<td>Michel &amp; Millet (1970)</td>
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<td>KM13</td>
<td>spo0A3 ΔmtlAFD::ermC</td>
<td>Heravi et al. (2011)</td>
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<td>spo0A3 ΔmtlR::ermC</td>
<td>Heravi et al. (2011)</td>
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<td>spo0A3 ΔmtlR::ermC hisI−, spc</td>
<td>This study</td>
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<td>KM176</td>
<td>spo0A3 amyE::[ter-P_mtl(lacZ, spc)]</td>
<td>This study</td>
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<td>spo0A3 mtlR-H342D</td>
<td>This study</td>
</tr>
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<td>KM213</td>
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<td>KM271</td>
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<td><strong>Plasmids</strong></td>
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<td>pDG1730</td>
<td>erm, bla, amyE−-spc-amyE</td>
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<td>pIC200HE</td>
<td>lacZ, bla, oriPJ1218</td>
<td>Altenbuchner et al. (1992)</td>
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<td>pOE6089.4</td>
<td>oriPB2322, rop, cer, rhpPAD, eGFP-Strep-tag II-rnnB, bla</td>
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<td>pMW363.1</td>
<td>ter, spc, manP−-cat-yidB-yidA′, bla</td>
<td>Heravi et al. (2011)</td>
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<td>pSUN279.2</td>
<td>ter-P_mtl(lacZ-ter, repA, oriPUC19, oriPB572, spc)</td>
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<td>pKAM145</td>
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<td>ycsN-P_mtl::mtlR-H342D C419A-ydaB′, bla, spc</td>
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<td>pKAM176</td>
<td>ter-P_mtlA+11-lacZ-ter, spc</td>
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<td>pKAM182</td>
<td>rhpPAD-::mtlR-H342D C419A</td>
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<td>pKAM191</td>
<td>hisF−-hisI-::mtlR-H342D C419A-P_mtl-yvcA-yvcB, bla</td>
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<td>pKAM223</td>
<td>yerL- mroxP-cat-mroxP-ycsA′', spc, bla</td>
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*The numbers show the base pair position with respect to the start codon of the wild-type gene.
oligonucleotides s6949/s6867. The generated P_mtlA−mtlR-H342D
C419A fragment was inserted into pKM4 via XmnI/SpeI digestion to
form pKM170. Afterwards, pKM170 was digested by SpeI/XmnI,
and pHM31 digested by NheI/HindIII. The 2.3 kb fragment from
pKM170 was then inserted into pHM31 vector constructing
pKM191. Integration of P_mtlA−mtlR-H342D in the chromo-
some was carried out according to the markerless integration
method of Motejadded & Altenbuchner (2007) based on histidine
auxotrophy. At first, strain KM15 (his−) was transformed with pKAM12
pDG1730 containing the operon in the strain KM231 and create strain KM271, plasmid
pDG1730 via HindIII/EcoRI restriction sites creating pKAM123. Integration of P
and pHM31 digested by NheI/HindIII into pKAM191. Integration of P_mtlA−mtlR-H342D
C419A into the chromo-
some was carried out according to the markerless integration
method of Motejadded & Altenbuchner (2007) based on histidine
auxotrophy. At first, strain KM15 (his−) was transformed with pKAM12
pDG1730 containing the operon in the strain KM231 and create strain KM271, plasmid
pKAM223 was employed in which the erythromycin resistance gene of
pKM6 was replaced with a chloramphenicol resistance gene. For this
purpose, pMW363.1 containing the chloramphenicol resistance gene
was used as a template for PCR employing oligonucleotides s6646 and
s6466. The resulting fragment was then inserted into pIC20HE via
XhdI/EcoRI restriction sites creating pKM19. Plasmid pKM19
was digested by Xhol/Nhel and the chloramphenicol resistance gene
was inserted into pKM6 which was cut by XmnI/SpeI to create
pKM223.

Construction of expression plasmids. PCR was performed for
amplification of the desired DNA fragment using chromosomal DNA of
B. subtilis 168 as a template. Unless otherwise specified, the
amplified promoter fragments were inserted into pSUN279.2
upstream of lacZ via Xhol and AflII restriction sites. Amplification
of the promoter of the licBCAH operon (P_licBA) was performed using
oligonucleotides s7614/s7615. The resulting fragment was then inserted into pSUN279.2 to create
pKM160. Hybrid promoter 41 (PHP41) was created by amplification of the truncated P_mtlA
fragment using oligonucleotides s6209/s6867, while oligonucleotides s7616
s7615 were employed to amplify the truncated P_mtlA
operon. The primary PCR products were digested by BsaI followed by ligation. The ligation
reaction was then used as a template for a PCR by
oligonucleotides s6209/s6867. By insertion of the PHP41 into
pSUN279.2, plasmid pKAM161 was constructed. To create PmtlR
oligonucleotides s6209/s7618 and s7619/s7615 were used in the PCRs
prior to the final fusion PCR by s6209/s7615. Insertion of PmtlR into
pSUN279.2 resulted in pKAM162. PmtlR was created by using
oligonucleotides s7714 and s7615 in a PCR. The final fragment was
inserted into pSUN279.2 to construct pKAM176. Promoter PmtlR+10
was created in a PCR using oligonucleotides s6209/s7548 the product of
which was inserted into pSUN279.2 to construct pKAM145. To create
PmtlR+11 and PmtlR+9 oligonucleotides s6209/s7678 and s6209/
s7679 were used in PCR, respectively. Insertion of PmtlR+11 into
pSUN279.2 created pKAM167, and PmtlR+9 was inserted into
pSUN279.2 to construct pKAM168. The mtlR-H342D C419A
double-mutant was created by mutation of the C419 to alanine
in mtlR-H342D in order to purify MtlR-H342D C419A. For this
purpose, plasmid pKAM66 containing mtlR-H342D was used as the
template in two PCRs using oligonucleotides s7303/s7301 and s7302/
s7304. Primary PCR products were used in the fusion PCR with
oligonucleotides s7303/s7304. The mtlR-H342D C419A fragment was
inserted into pJOE6089.4 via AffiX/mL digestion to create pKAM182
where the expression of mtlR allele was controlled by rbadBAD.
The 35 upstream sequence of PmtlR was exchanged with its counterpart
from Pmap to construct hybrid promoter 7 (P7) in which the Mtr
binding site was replaced with the putative ManN binding site. P7
was constructed by amplification of the Pmap truncated fragment
using oligonucleotides s6504/s6505, and amplification of the PmtlR
fragment with oligonucleotides s6506/s6507. The primary PCR
products were used in fusion PCR with oligonucleotides s6504/
s6507 and the resulting fragment (P7) was inserted into pSUN279.2
to create pKAM21. P7 was then applied as a template in PCR for
generation of a Cy5-labelled P7 DNA fragment used as a negative
control in electrophoretic mobility shift studies.

Overexpression and purification of MtlR-H342D C419A in E. coli. For overexpression and purification of MtlR-H342D C419A,
200 ml LB in a 1 l Erlenmeyer flask was inoculated (1:100 dilution)
with an overnight culture of E. coli JW2409-1 containing pKAM182.
The bacterial culture was then cultivated at 37 °C with shaking at
200 r.p.m. When the culture reached an OD600 of 0.4, 0.2% L-
rhamnose was added in order to induce the rbadBAD promoter
located upstream of the mtlR-H342D C419A allele of pKAM182.
Afterwards, the induced cells were further incubated at 30 °C
in order to purify MtlR-H342D C419A. The bacterial culture was harvested 5 h
after the addition of L-rhamnose at an OD600 of approximately 2.8.
After centrifugation for 5 min at 5800 × g, the bacterial pellet was
washed twice with Buffer A (50 mM HEPES pH 7.4, 1 mM tris(2-
carboxyethyl)phosphine (TCEP)), anion-exchange chromatography was carried out with a flow
rate of 1 ml min
−1. Using Buffer A and Buffer B (50 mM HEPES pH 7.4, 1 M NaCl, 1 mM
TCEP), anion-exchange chromatography was carried out with a flow
rate of 1 ml min
−1 and a gradient of 0–50 % Buffer B in 50 min
after the addition of L-rhamnose at an OD600 of approximately 2.8.
Constitutive activity of MtlR-H342D C419A in the absence of MtlA

Electrophoretic mobility shift assays (EMSA) and DNase I
footprinting. Cy5-labelled 5′-end DNA fragments of PmtlR and PmtlR
were synthesized in PCRs using Cy5-labelled oligonucleotides, i.e.
s5960 and s8365. For labelling the non-coding strand, PmtlR
was amplified from pKAM1 with oligonucleotides s8447 and s5960. The
PmtlR coding strand was labelled with oligonucleotides s8365 and
s6213 in a PCR with pKAM1 as a template. Plasmid pKAM12 was also
employed as the template for generation of Cy5-PmtlR DNA using
oligonucleotides s8447 and s5960 in a PCR. The same oligonucleotides

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RESULTS

Constitutive activity of MtlR-H342D C419A in the absence of MtlA

To investigate the interaction between the promoters of the B. subtilis
mannitol utilization system and their activator,
MtlR, it was necessary to obtain MtlR functional in vivo and in vitro. Such MtlR, which was expected to be active independently of the phosphorylation state of its regulatory
domains, was obtained by specifically mutating the mtlR
gene. In strains that produce the single-mutant proteins
MtlR-C419A or MtlR-H342D, expression of the mtlR
operon either occurs constitutively or is not subject to
CCR, respectively (Joyet et al., 2010). To test whether combining the two mutations by constructing the mtlR-
H342D C419A allele would provide a functional activator
with both specified characteristics, the PmtlR-mtlR-H342D
C419A cassette was integrated into its wild-type locus within
the chromosome of B. subtilis KM15, a ΔmtlR::ermC
mutant. Next, the PmtlR-lacZ fusion was separately inte-
grated into the amyE loci of the chromosomes of B. subtilis
3NA (wild-type), PmtlR-mtlR-H342D, PmtlR-mtlR-H342D
C419A and ΔmtlR strains in order to measure the

β-Galactosidase activity. β-Galactosidase activities were measured by using o-nitrophenyl-β-galactopyranoside (ONPG) as a substrate
according to Miller (1972) with the modification of Wenzel &
Altenbuchner (2013).
β-galactosidase activity of these strains in the presence of different sugars. Subsequently, the mtlR mutants and wild-type strains were cultivated in LB and the β-galactosidase activity was measured 1 h after the addition of 0.2% (w/v) mannitol alone or together with 0.2% (w/v) glucose at an OD<sub>600</sub> of 0.4. As a negative control, no sugar was added to the bacterial culture. Addition of mannitol increased the β-galactosidase activity by about 11-fold compared to the wild-type strain, the <i>P<sub>mtlA</sub></i> activity was induced with mannitol in the <i>P<sub>mtlA</sub></i>-H342D strain; however, CCR was considerably reduced in the presence of glucose as reported before (Heravi et al., 2011; Joyet et al., 2010).

The double mutation <i>mtlR-H342D C419A</i> resulted in strong constitutive production of β-galactosidase by <i>P<sub>mtlA</sub></i>. Likewise, the presence of glucose had no influence on the β-galactosidase production in the <i>mtlR-H342D C419A</i> strain compared to the β-galactosidase production with mannitol (Fig. 1). An even higher β-galactosidase activity was observed in the <i>mtlR-H342D C419A</i> strain in LB (uninduced). This difference could be due to the uptake of a PTS sugar, mannitol or glucose, resulting in a strong CcpA-dependent CCR of <i>P<sub>mtlA</sub></i>. Altogether, <i>mtlR-H342D C419A</i> is a functional regulator activating <i>P<sub>mtlA</sub></i> in vivo regardless of the presence of an inducing (mannitol) or repressing sugar (glucose). Prior to purifying doubly mutated MtlR-H342D C419A and using it in vitro, it was important to understand whether it functions independently of MtlA. It has recently been suggested that the function of MtlR requires sequestration by the components of the mannitol-specific transporter, i.e. the domain B of the EIIC<sup>MtlA</sup> (Bouraoui et al., 2013; Joyet et al., 2013). To test whether <i>mtlR-H342D C419A</i> needs sequestration by MtlA, the <i>mtlAFD</i> operon was deleted in the wild-type strain as well as in the strain producing doubly mutated MtlR-H342D C419A. Deletion of the <i>mtlAFD</i> operon in the wild-type strain resulted in a weak constitutive <i>P<sub>mtlA</sub></i> activity, while this weak activity was increased when <i>mtlR-H342D C419A</i> was expressed in the Δ<i>mtlAFD</i> background (Fig. 1). In the presence of mannitol, the β-galactosidase activity of the <i>mtlR-H342D C419A</i> strain without the mannitol-specific transporter was even higher than its parental strain containing <i>mtlAFD</i> (Fig. 1). This could be due to loss of mannitol uptake and metabolism. As a consequence, the CcpA-dependent CCR will not be activated and will not lower <i>P<sub>mtlA</sub></i> activity. In conclusion, these results indicate that the MtlR-H342D C419A protein acts independently of MtlA, whereas wild-type MtlR probably needs to interact with MtlA to gain full activity.

**Identification of the MtlR-H342D C419A binding site by DNA footprinting**

The <i>mtlR</i> double-mutant, i.e. <i>mtlR-H342D C419A</i>, encodes a functional protein activating <i>P<sub>mtlA</sub></i> in vivo. To identify the

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**Fig. 1.** β-Galactosidase activity of the <i>B. subtilis</i> mutants. All strains carry a <i>P<sub>mtlA</sub>-lacZ</i> cassette integrated at the amyE locus. The wild-type strain (KM176) and mutant strains <i>mtlR-H342D</i> (KM213), <i>mtlR-H342D C419A</i> (KM231), Δ<i>mtlR</i> (KM241), Δ<i>mtlAFD</i> (KM240) and Δ<i>mtlAFD</i> <i>mtlR-H342D C419A</i> (KM271) were induced at an OD<sub>600</sub> of 0.4 by the addition of 0.2% (w/v) mannitol (inducer) alone or together with 0.2% (w/v) glucose (repressing sugar). No sugar was added to the negative control. The enzyme activity was measured 1 h after the addition of sugars. All measurements were performed three times. The mean and standard deviation (error bars) values are represented.
MtIR binding site at P_{mtlA} and P_{mtlR}, the mtIR-H342D C419A allele was expressed under control of rhaP_{BAD} in E. coli strain JW2409-1, in which the El of the PTS is disrupted. The theoretical pl of MtIR-H342D C419A was approximately 5.2. Therefore, anion-exchange chromatography was conducted and the resulting fractions were tested for the presence of an active MtIR by electrophoretic mobility shift assays (data not shown). The DNA fragments Cy5-P_{mtlA} and Cy5-P_{mtlR} were subsequently used for DNase I footprinting to determine the MtIR binding sequence in both coding and non-coding strands of P_{mtlA} and P_{mtlR} (Fig. 2a). The results indicated that the MtIR operator at the coding strand of P_{mtlA} is located between −85 and −39 with respect to the transcription start site of P_{mtlA}, while the protected region of the non-coding strand extended from −88 to −41 (Fig. 2b). Similar results were obtained by P_{mtlR} footprinting where a region between −81 and −37 remained protected during the DNase I digestion on the coding strand, while the MtIR bound to the region between −86 and −40 on the non-coding strand (Fig. 2c). These results indicate that MtIR binds adjacent to the −35 box in both P_{mtlA} and P_{mtlR} promoters.

**Construction of P_{mtlA}−P_{licB} hybrid promoters and confirmation of the MtIR operator in vivo**

To confirm the identified 3′-end of the MtIR binding site by DNase I footprinting, hybrid promoters were constructed using the promoter core elements of P_{licB} as a platform. P_{licB} is the promoter of the licBCAH operon which is also activated by a PRD-containing activator, called LicR. Previously, a LicR binding site immediately upstream of the −35 sequence of P_{licB} was postulated (Tobisch et al., 1999). Accordingly, the hybrid promoters

![Fig. 2. Identification of the MtIR binding site at P_{mtlA} and P_{mtlR} by DNase I footprinting. (a) The sequencing reactions (shown by A, C, G and T) with oligonucleotides s5960 (non-coding strand) and s8365 (coding strand) were carried out using pKAM1 (P_{mtlA}) and pKAM18 (P_{mtlR}) as templates. The DNA protection pattern was analysed in the absence (−) and presence (+) of MtIR-H342D C419A. The protected regions of each DNA strand is shown by marks on the left and by bold lines on the right side of each gel. (b) Promoter sequence (−10 and −35; shown by boxes), transcription start site (+1; capital letters), and MtIR binding site (bold lines) in P_{mtlA} and (c) in P_{mtlR} are demonstrated. The arrows represent the incomplete inverted repeats. Bold lines show the protected regions of DNA and the binding site of MtIR-H342D C419A.](http://mic.sgmjournals.org)
were constructed by substitution of the complete upstream region of the PlicB −35 box with its counterpart from PmtlA (construct PlicB61, Fig. 3a). PlicB was also used as a control in this study. Both constructs were inserted into pSUN279.2, a derivative of pMTLBS72 (Titok et al., 2003), upstream of the lacZ reporter gene. In this way, plasmid pKAM160 (PlicB) and pKAM161 (PlicB61) were created. Induction of the B. subtilis strains containing PlicB-lacZ with cellobiose and PlicB61-lacZ with mannitol resulted in 1657 and 859 Miller units β-galactosidase activity, respectively (Fig. 3b). On the other hand, the uninduced cells showed only 300 Miller units (PlicB-lacZ) and 86 Miller units (PlicB61-lacZ). Indeed, PHP42 was inducible with mannitol similar to the wild-type PmtlA on plasmid pKAM1 (reported by Heravi et al., 2011). Further replacement of 3 bp located adjacent to the −35 box of PHP43 with their counterparts from PlicB created PHP43 (Fig. 3a). Induction of PHP43 showed no significant changes in the β-galactosidase activity compared to PHP43 (Fig. 3b), whereas substitution of 7 bp of PHP43 with its PlicB counterparts rendered PHP42 uninducible with mannitol (Fig. 3b). Altogether, these results indicate that the MtlR operator in PmtlA extends towards the −35 box at the position −38 with respect to the transcription start site.

**Increasing the distance between the −35 box and the MtlR binding site**

Given that the MtlR operator begins about 3 to 4 bp upstream from the −35 box in both PmtlA and PmtlR, the distance between the MtlR binding site and −35 box was increased in order to confirm the class II type activation by MtlR. For this purpose, 9 (PmtlA + 9), 10 (PmtlA + 10) and 11 (PmtlA + 11) bp were inserted between the −35 box and the MtlR binding site in order to separate the MtlR binding site from the −35 box with a complete turn of B-DNA double helix (Fig. 4a). Insertion of the newly constructed promoters in front of the lacZ gene was followed by induction of the B. subtilis 3NA containing these constructs. Although PmtlA + 10 and PmtlA + 11 respectively showed eight- and fourfold induction, the β-galactosidase activity was considerably lower than in the wild-type strain (Fig. 4b). In contrast, the PmtlA + 9 activity was non-inducible (Fig. 4b). EMSA showed that MtlR was able to interact with its operator in the PmtlA sequence or its derivatives, although the MtlR binding site was relocated further from the −35 box (Fig. 4c). This suggests that the distance between the MtlR binding site and the −35 box is not flexible with respect to activation of gene expression.

**DISCUSSION**

The mannitol utilization system of B. subtilis is regulated by two promoters, PmtlA and PmtlR. Transcription from these promoters, which have conserved −35 and −10 boxes similar to the σA-type housekeeping promoters, depends on MtlR, a PRD-containing activator. The activation complex of simple housekeeping promoters is formed by RNAP and an activator. Generally, transcription activation has been categorized into two classes, class I and II. In the class I type of activation, the activator usually binds between positions −61 to −91 and interacts with the carboxy terminal domain of the RNAP α subunit (αCTD). In the class II type activation, the activator binding site overlaps the −35 box, where it contacts domain 4 of the α subunit of RNAP (Browning & Busby, 2004; Lee et al., 2012). This situation is found in the promoters of the B. subtilis mannose utilization system, PmanF and PmanR, where the operators completely overlap the −35 region (Wenzel & Altenbuchner, 2013). Identification of the MtlR operator at PmtlA and PmtlR revealed a structure somewhere between class I and class II type transcription activation where MtlR binds a few bases adjacent to the −35 box (Figs 2 and 3). Changing the distance between the MtlR operator and the −35 box significantly reduced the activity of PmtlA showing the necessity of the proximity of the MtlR molecules to the RNAP/αCTD holoenzyme as required in class II type transcription activation (Fig. 4). Besides, the activation of PlicB by LicR, a PRD-containing activator, also depends on a dyad symmetry located 8 bp upstream of the −35 box (Tobisch et al., 1999). Obviously, the PRD-containing activators of B. subtilis usually form a class II type activation complex at their target promoters.
Prior to the activation of P$_{mtlA}$ and P$_{mtlB}$, MtlR molecules must be activated. There are two reported activation processes for MtlR: (i) regulator activation by post-translational protein modifications (phosphorylation) and (ii) a direct protein–protein interaction between regulator and the cognate transporter. It is assumed that the nascent MtlR PRDII domain is phosphorylated by HPr(H15~P), and the EIIB$^{Gal}$-like domain of MtlR by phosphorylated EIIA$^{Mtl}$ in the absence of glucose and mannitol. In the presence of mannitol, MtlF dephosphorylates the EIIB$^{Gal}$-like domain of MtlR which renders MtlR active (Joyet et al., 2010). The results obtained with the mtlR-H342D C419A double-mutant are in line with the previous studies showing that MtlR-H342D C419A is a functional protein activating P$_{mtlA}$ independent of the presence of mannitol or glucose. However, the MtlR-H342D C419A mutant protein was fully functional in vivo when MtlA was deleted from the B. subtilis chromosome. Besides, the purified B. subtilis MtlR-H342D C419A isolated from E. coli cells could also bind to the P$_{mtlA}$ DNA fragments without MtlA in vitro (Fig. 4). These mutations probably induce structural changes which render the mutant MtlR independent of activation by the mannitol transporter. In fact, the modulation of the regulator and transporter phosphorylation state could alter their direct interaction. Up until now, the localization of E. coli BglG and B. subtilis LicT antiterminators has been investigated. BglG is sequestered by the cognate phosphorylated transporter, BglF, in the absence of salicin (inducer). Upon addition of salicin, BglF becomes dephosphorylated and BglG will be released into the cytosol (Lopian et al., 2003). B. subtilis LicT, however, is homogeneously distributed in the cytoplasm in the absence of inducer. Upon addition of salicin, LicT localizes in the subpolar region. No LicT membrane sequestration was observed in the presence/absence of inducer (Rothe et al., 2013). Also, in the B. subtilis mannose system there seems to be no need for such sequestration since ManR is fully active in manA deletion mutants (Wenzel & Altenbuchner, 2013). In conclusion, it might be possible that the hypothesized MtlR sequestration by MtlA takes place during MtlR activation by the formation of the MtlA/MtlF/MtlR(PRDII–P/EIIB$^{Gal}$–P) complex. However, the active form of MtlR(PRDII–P/EIIB$^{Gal}$) functions independently of the presence of MtlA in the cell (Fig. 1). The exact dynamic of the MtlR localization and its relationship to control mtl gene expression must be further studied.
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