Characterization of a Mannose Utilization System in *Bacillus subtilis*[▽]

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The mannose operon of *Bacillus subtilis* consists of three genes, *manP*, *manA*, and *yjdF*, which are responsible for the transport and utilization of mannose. Upstream and in the same orientation as the mannose operon a regulatory gene, *manR*, codes for a transcription activator of the mannose operon, as shown in this study. Both mannose operon transcription and *manR* transcription are inducible by mannose. The presence of mannose resulted in a 4- to 7-fold increase in expression of *lacZ* from the *manP* promoter (P_{manP}) and in a 3-fold increase in expression of *lacZ* from the *manR* promoter (P_{manR}). The transcription start sites of *manPA-yjdF* and *manR* were determined to be a single A residue and a single G residue, respectively, preceded by –10 and –35 boxes resembling a vegetative σ^A promoter structure. Through deletion analysis the target sequences of ManR upstream of P_{manP} and P_{manR} were identified between bp –80 and –35 with respect to the transcriptional start site of both promoters. Deletion of *manP* (mannose transporter) resulted in constitutive expression from both the P_{manP} and P_{manR} promoters, indicating that the phosphotransferase system (PTS) component EII^{Man} has a negative effect on regulation of the mannose operon and *manR*. Moreover, both P_{manP} and P_{manR} are subject to carbon catabolite repression (CCR). By constructing protein sequence alignments a DNA binding motif at the N-terminal end, two PTS regulation domains (PRDs), and an EIIA- and EIIB-like domain were identified in the ManR sequence, indicating that ManR is a PRD-containing transcription activator. Like findings for other PRD regulators, the phosphoenolpyruvate (PEP)-dependent phosphorylation by the histidine protein HPr via His15 plays an essential role in transcriptional activation of P_{manP} and P_{manR} . Phosphorylation of Ser46 of HPr or of the homologous Crh protein by HPr kinase and formation of a repressor complex with CcpA are parts of the *B. subtilis* CCR system. Only in the double mutant with an HPr Ser46Ala mutation and a *crh* knockout mutation was CCR strongly reduced. In contrast, P_{manR} and P_{manP} were not inducible in a *ccpA* deletion mutant.

Bacillus subtilis can use many different sugars as carbon sources. Some of these sugars are taken up via the phosphoenolpyruvate (PEP):carbohydrate phosphotransferase system (PTS), a mechanism that couples translocation with phosphorylation of the substrate. The PTS forms a protein phosphorylation cascade and is composed of the general cytoplasmic proteins enzyme I (EI) and histidine protein (HPr) and the substrate-specific membrane protein enzyme II (EII). The EII complexes are the sugar-specific permeases, which consist of three (EI-IABC) or four (EIIABCD) subunits that are either fused in single multidomain proteins or occur as separate individual polypeptides (3, 4, 33). The phosphoryl groups are transferred sequentially from phosphoenolpyruvate via histidine residues as phosphorylation sites from EI, HPr, and the hydrophilic EIIA domain usually to a cysteine residue of EIIB and from there finally to the sugar which is recognized and translocated by the hydrophobic membrane spanning the EIIC or EIICD domain.

In *Firmicutes*, as in *B. subtilis*, HPr plays the major role in carbon catabolite repression (CCR). In addition to phosphorylation at His15, HPr is subjected to further phosphorylation at a serine residue at position 46 by the ATP-dependent HPr kinase/phosphorylase (10, 14, 24). In the presence of glycolytic interme-

diates (glucose-6-phosphate and fructose-1,6-bisphosphate), HPr kinase is stimulated to phosphorylate HPr at Ser46, and P-Ser-HPr forms a complex with the catabolite control protein CcpA (5). This complex binds to an operator sequence (catabolite-responsive element [*cre*]) in front of catabolic genes to prevent transcription of the target operons (4, 16, 24, 45, 46). In recent studies the homologous protein Crh (catabolite repression HPr), a second catabolite corepressor, was discovered in *B. subtilis* (9). Crh exhibits 45% sequence identity with HPr, but the active site His15 residue of HPr is replaced by a glutamine residue in Crh. Crh can be phosphorylated only by the HPr kinase, and P-Ser46-Crh interacts with CcpA like P-Ser-HPr and provokes CCR. The main difference seems to be that two small effector molecules, glucose-6-phosphate and fructose-1,6-bisphosphate, which act as corepressors, strengthen the interaction between CcpA–P-Ser-HPr and *cre* but not the interaction between CcpA–P-Ser-Crh and *cre* (3, 19, 38). HPr also mediates CcpA-independent catabolite repression via phosphorylation at His15, which phosphorylates and activates, for example, glycerol kinase. Glycerol kinase generates glycerol-3-phosphate, the inducer of the glycerol operon. In the presence of a preferred PTS carbohydrate the level of P-His-HPr is reduced, resulting in catabolite repression of the glycerol operon (4, 7). In addition, there are other CcpA-independent catabolite repression systems involving transcriptional repressors, such as CggR and CcpN (6, 39). These CcpA-dependent and -independent catabolite repression systems constitute a carbon metabolism network, and they coordinate and regulate catabolism and anabolism to ensure optimal cell status (7).

In addition to CCR, primary regulation of catabolic carbo-

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TABLE 1. *B. subtilis* strains used in this study

Strain	Genotype	Source (reference)
3NA	<i>spo0A3</i>	J. F. Michel (29)
168	<i>trpC2</i>	Bacillus Genetic Stock Center
1A147	<i>ccpA1 trpC2 alsR1 ilvBDR1</i>	Bacillus Genetic Stock Center
1A250	<i>trpC2 alsR1 ilvBDR1</i>	Bacillus Genetic Stock Center
QB5223	<i>trpC ptsH1</i>	J. Stülke (27)
QB5350	<i>trpC ptsH-H15A amyE::(levD'-lacZ aphA3)</i>	J. Stülke (43)
TQ276	<i>spo0A3 ΔmanR::erm</i>	3NA transformed with pSUN276.2
TQ281	<i>spo0A3 ΔmanA::(lacZ-erm)</i>	3NA transformed with pSUN281
TQ303	<i>spo0A3 ΔccpA::erm</i>	3NA transformed with pSUN303.3
TQ338	<i>spo0A3 Δcrh::erm</i>	3NA transformed with pSUN338.3
TQ338_S46	<i>trpC ptsH1 Δcrh::erm</i>	QB5223 transformed with pSUN338.3
TQ356	<i>spo0A3 ΔmanP::erm</i>	3NA transformed with pSUN356.7
TQ361	<i>spo0A3 cre*-manR</i>	TQ276 transformed with pSUN361.3
TQ431	<i>spo0A3 ΔyjdF::(erm)</i>	3NA transformed with pSUN431.1
TQ432	<i>trpC ptsH-H15A amyE::(cm)</i>	QB5350 transformed with pSUN432.3

hydrate gene expression is normally accomplished by transcription factors that respond to the presence of the corresponding substrate or an intermediate in the degradation pathway. The most frequent and best-investigated transcription factors are DNA binding proteins belonging to the LacI repressor or AraC activator family, which interact with the substrates. This leads to a change in their conformation and concomitantly to a change in the DNA binding affinity. For another class of regulatory proteins the binding activity is modulated in response to phosphorylation. Here the two-component response regulator superfamily is well known. For a smaller family of transcriptional antiterminators and activators the activity is modulated via phosphorylation of PTS regulation domains (PRDs). PRD-containing regulators consist of an N-terminal RNA (for antiterminators) or DNA (for activators) binding domain, followed by duplicated regulatory domains designated PRD1 and PRD2. Some molecules have additional domains; for example, the activator MtlR of *Geobacillus stearothermophilus* (formerly *Bacillus stearothermophilus*) has sequences homologous to the EIIA and EIIB domains of the PTS transporters (17, 18). Alignment of the PRD sequences showed that each PRD contains one or two conserved histidines that are phosphorylation sites. Some of the sites have a positive effect on the activity, and some have a negative effect. In general, it seems that the regulators are active when PRD1 is dephosphorylated and PRD2 is phosphorylated. This is the case in the presence of the substrate and in the absence of glucose. So far there is no clear evidence indicating which components have the major role in phosphorylation and dephosphorylation *in vivo* (42). For the BglG antiterminator of *Escherichia coli* even a phosphorylation-independent mechanism has been observed (34).

D-Mannose is a 2-epimer of glucose and is present in mannan and heteromannan polysaccharides, glycoproteins, and numerous other glycoconjugates. Many bacteria can use D-mannose as a carbon source. In *B. subtilis* mannose enters carbohydrate metabolism in two steps. First, it undergoes phosphorylation to mannose-6-phosphate by a mannose-specific PTS transporter during uptake. Then it is converted to fructose-6-phosphate by mannose-6-phosphate isomerase. Three genes in the mannose operon were identified previously (22). The first gene, *manP*, encodes a putative PTS mannose-

specific enzyme IIBCA component (transporter) that has significant sequence similarity with the fructose-specific permeases of mycoplasmas (37) and therefore belongs to the Fru permease family. The second gene, *manA*, encodes a mannose-6-phosphate isomerase, whereas the function of the third gene, *yjdF*, is unknown. It has been postulated that upstream and in the same orientation as the mannose operon is a regulatory gene, *manR*, coding for a putative activator of the mannose operon (36). By using protein sequence alignments a DNA binding motif at the N-terminal end, two PRDs, and an EIIA-EIIB domain were identified (11), indicating that ManR is a PRD-containing activator similar to MtlR of *G. stearothermophilus*.

In this paper, we report characterization of the mannose utilization system in *B. subtilis*.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *E. coli* JM109 (48) and *B. subtilis* 3NA (29) were used as main hosts for cloning and expression. Other strains are listed in Table 1. *E. coli* was grown in LB (25) liquid medium and on LB agar plates supplemented with 100 $\mu\text{g ml}^{-1}$ ampicillin or 100 $\mu\text{g ml}^{-1}$ spectinomycin at 37°C. *B. subtilis* was grown in LB liquid medium and minimal medium C supplemented with 0.5% mannose, 0.05% Casamino Acids, and 0.02% yeast extract (26) at 37°C. Liquid media and agar plates were supplemented with 100 $\mu\text{g ml}^{-1}$ spectinomycin, 10 $\mu\text{g ml}^{-1}$ kanamycin, or 5 $\mu\text{g ml}^{-1}$ erythromycin. For induction of the mannose promoter, sterile filtered D-mannose was added to a final concentration of 0.2%.

Materials. All chemicals were obtained from Sigma-Aldrich (Taufkirchen, Germany), Fluka (Buchs, Germany), or Merck (Darmstadt, Germany). Synthetic DNA oligonucleotides (see Table 3) were purchased from MWG. Restriction enzymes and DNA-modifying enzymes were purchased from Roche Applied Science (Mannheim, Germany) or New England Biolabs. PCRs were performed with high-fidelity DNA polymerase from Fermentas (St. Leon-Rot, Germany) using a MiniCycler from Biozym.

Preparation of DNA and transformation. DNA was prepared from *E. coli* or *B. subtilis* or from an agarose gel using DNA preparation kits from Qiagen (Hilden, Germany) or Roche (Mannheim, Germany) as described by the manufacturers. Standard molecular techniques were used throughout this study. The plasmids used in this study are shown in Table 2. *E. coli* was transformed with plasmid DNA as described by Chung et al. (2). *B. subtilis* was transformed with plasmid DNA using the modified "Paris method" (15, 41).

Construction of the expression vectors. The expression vector pSUN279.2 and its derivatives were *E. coli-B. subtilis* shuttle vectors, in which the *lacZ* gene was included as a reporter gene. The *lacZ* gene was cut with NdeI and XmaI from pLA2 (13) and ligated into pJOE5531.1, a derivative of the rhamnose-inducible expression vector pWA21 (47) which contained the *B. subtilis* *tufA* transcription terminator at the XmaI site. Into the resulting plasmid (pSUN228.1) two oligo-

TABLE 2. Plasmids used in this study

Plasmid	Genotype
pLA2	<i>araBp7-lacZ tL3 attL oriRg kan</i>
pJOE4786.1	<i>amp</i>
pJOE5531.1	<i>rhaP-eGFP-tufA-ter bla</i>
pDG1730	<i>amyE'-spc-amyE' bla erm</i>
pMTLBS72	<i>repA cmR bla</i>
pSUN063.11	<i>manR-P_{manR}-amy-lip bla</i>
pSUN151.1	<i>cat amp</i>
pSUN178.4	<i>manR lss kan</i>
pSUN228.1	<i>rhaP-lacZ-tufA-ter bla</i>
pSUN235.1	<i>ter-lacZ-ter bla</i>
pSUN252.1	<i>spc ter-lacZ-ter bla</i>
pSUN263.1	<i>yjdB' bla</i>
pSUN264.4	<i>manP' bla</i>
pSUN269.4	<i>yjdB'-erm bla</i>
pSUN271.1	<i>spc ter-lacZ-ter</i>
pSUN272.1	<i>spc ter-lacZ-ter repA'</i>
pSUN276.2	<i>yjdB'-erm-manP' bla</i>
pSUN279.2	<i>spc ter-P_{manR}-manR-P_{manP}-lacZ-ter repA</i>
pSUN281	<i>ΔmanA-lacZ-erm-yjdF' bla spc</i>
pSUN284.1	<i>spc ter-ΔmanR-P_{manP}-lacZ-ter repA</i>
pSUN289.3	<i>spc ter-P_{manPD1}-lacZ-ter repA</i>
pSUN290	<i>spc ter-P_{manPD3}-lacZ-ter repA</i>
pSUN291	<i>spc ter-P_{manR}-lacZ-ter repA</i>
pSUN297.5	<i>spc ter-P_{manPD4}-lacZ-ter repA</i>
pSUN298.1	<i>spc ter-P_{manPD2}-lacZ-ter repA</i>
pSUN303.3	<i>aroA-erm-yxuD spc amp</i>
pSUN338.3	<i>yvcL-erm-yvcN bla spc</i>
pSUN356.7	<i>manR'-erm-manA' bla spc</i>
pSUN357.3	<i>cre*-manR amy-lip bla</i>
pSUN360.1	<i>spc ter-cre*-manR-P_{manP}-lacZ-ter</i>
pSUN361.3	<i>yjdB'-cre*-manR-manP' amp</i>
pSUN377	<i>spc ter-cre*-P_{manR}-lacZ-ter</i>
pSUN380.1	<i>spc ter-P_{manPD5}-lacZ-ter repA</i>
pSUN381.1	<i>spc ter-P_{manPD6}-lacZ-ter repA</i>
pSUN431.1	<i>manA'-erm-yjdG spc amp</i>
pSUN432.3	<i>amyE::cat, spc amp</i>
pUB110	<i>rep kan</i>

nucleotides (s4956 and s4957 [Table 3]) were inserted between the AflII/MunI restriction sites (pSUN235.1) in order to add the same *tufA* transcription terminator upstream of *lacZ*. Thus, read-through from plasmid promoters into *lacZ*, as well as read-through from *lacZ* into the flanking plasmid sequences, was eliminated by the terminators. A spectinomycin resistance gene (*spc*) was amplified for both *E. coli* and *B. subtilis* from plasmid pDG1730 (12) with oligonucleotides s4833 and s4835 and inserted into pSUN235.1 to obtain pSUN252.1. In addition, the *E. coli* vector portion was shortened by deleting a BspHI/HindIII fragment (pSUN271.1). Subsequently, an EcoRI/SphI fragment with the replication region of the *B. subtilis* pMTLBS72 plasmid (23) was ligated into plasmid pSUN271.1 described above. New plasmid pSUN272.1 with promoterless *lacZ* was used in this study for promoter analyses. After this, an approximately 2.3-kb fragment, including *manR* with 191 bp upstream of the *manR* start codon and the intergenic region between *manR* and *manP* (132 bp downstream of the stop codon), was amplified with primers s4693 and s4694 from chromosomal DNA of *B. subtilis* 168. This fragment was inserted in front of *lacZ* in plasmid pSUN272.1 by digestion with AflII and NheI and ligation (plasmid pSUN279.2) (Fig. 1). Plasmid pSUN284.1 was obtained by deleting the fragment between the SfoI and NruI sites from pSUN279.2 (for pSUN284.1 and the following plasmids also see Fig. 5). The other expression vectors with *lacZ* under control of the *manP* promoter (pSUN289.3, pSUN290, pSUN297.5, pSUN298.1, pSUN380.1, and pSUN381.1) were constructed in the same way by inserting the PCR products (amplified with the s4801/s5203, s4802/s5203, s5262/s5203, s5263/s5203, s5861/s5203, and s5862/s5203 primer pairs, respectively) into pSUN272.1 using restriction enzymes EcoRV and NheI. A fragment that included the putative *manR* promoter and about 600 bp upstream of *manR* was amplified with primers s5208 and s5209 and with linearized plasmid pSUN279.2 DNA as the template and inserted in front of *lacZ* in plasmid pSUN272.1, replacing *P_{manR}-manR-P_{manP}* by digestion with KpnI and AflIII and ligation (pSUN291). Three other expression vectors with *lacZ* under control of the *manR* promoter, pSUN384.1, pSUN385.2,

and pSUN386.9, were constructed in a similar manner by inserting the PCR products (amplified with primer pairs s5931/s5934, s5932/s5934, and s5933/s5934, respectively) into pSUN279.2 using restriction enzymes SacI and NheI. To construct the expression vector pSUN377 with the *cre* mutation in the *manR* promoter, a fragment from the KpnI site to the AflII site in plasmid pSUN360.1 was amplified with primers s5208 and s5209 and then inserted into pSUN279.2 using KpnI/AflII. The shuttle vector pSUN178.4 was used to isolate mRNA for primer extension. It was constructed from the *E. coli* pIC20HE plasmid (1) and the *B. subtilis* pUB110 vector (28) and contained the *lss* gene as a reporter gene, which codes for the mature form of lysostaphin from *Staphylococcus simulans* (35). The *P_{manR}-manR-P_{manP}* promoter cassette that was inserted in pSUN279.2 was inserted upstream of the *lss* gene.

Site-specific mutation of the *cre* site in the *manR* promoter. A *cre* mutation in the *manR* promoter was generated by PCR amplification of *manR* from chromosomal DNA of *B. subtilis* 168 with primers s5616 and s4694. Primer s5616 has 8 nucleotide changes compared with the *cre* sequence of *manR*, so 6 nucleotides differed from nucleotides in the consensus *cre* sequence and the -10 region remained unchanged, whereas two restriction sites, SfiI and BsrGI sites, were added for convenient cloning. The PCR product obtained with s5616 and s4694 was then inserted into pSUN063.11 using BstBI and PstI (pSUN357.3). The *manR* gene with a mutated *cre* sequence was designated *cre*-manR*. The *cre*-manR* fragment was then cut out of pSUN357.3 using PstI/BglII and inserted between the sequences flanking *manR* (*yjdB* and *manP*) in pSUN351.3, a derivative of pSUN276.2, to obtain the integrative vector pSUN361.3. The *ΔmanR* mutant TQ276 was transformed with pSUN361.3. Transformants were selected on CMM minimal plates with 0.5% mannose as the sole C source. The integration of *cre*-manR* by double crossover was verified by PCR amplification with corresponding primers.

Construction of *B. subtilis* knockout mutants. To construct the vector for chromosomal deletion of *manR*, two flanking fragments were amplified from chromosomal DNA of *B. subtilis*. The sequence from position -708 to position -194 upstream of the *manR* start codon was amplified with primers s5077 and s5078 and inserted into pJOE4786.1, a derivative of pJOE773 (1), which does not replicate in *B. subtilis*, by digestion with SmaI to obtain plasmid pSUN263.1. The sequence between position -26 upstream and position 650 downstream of the *manR* stop codon was amplified with primers s5079 and s5080 and inserted into pJOE4786.1 using SmaI to obtain plasmid pSUN264.4. Then the erythromycin resistance gene *erm* was amplified with primers s5069 and s5070 from linearized plasmid pDG1730 DNA and inserted into pSUN263.1 by digestion with EcoRI/MunI and ligation (pSUN269.4). Finally, the flanking fragment downstream of *manR* was cut out of pSUN264.4 and inserted into pSUN269.4 using EcoRI/MluI digestion and subsequent ligation. *B. subtilis* 3NA was transformed with plasmid pSUN276.2. Mutants were selected on LB agar plates with erythromycin, and the mutants with double crossovers were identified by colony PCR. The other plasmids used for gene knockout in the *B. subtilis* chromosome (pSUN356.7 for *manP* knockout, pSUN281 for *manA* knockout, pSUN431.1 for *yjdF* knockout, and pSUN303.3 and pSUN338.3 for *ccpA* and *crh* knockout, respectively) were constructed in the same way. An additional selection marker (*spc* from pDG1730, coding for spectinomycin resistance in *B. subtilis*) was inserted into these five plasmids using EcoRI/EcoRV sites in order to distinguish between single crossover and double crossover. Gene replacement in the chromosomes of the mutants was verified by PCR amplification with appropriate primers.

The *ptsH*(H15A) QB5350 mutant has a *lacZ* gene integrated into the *amyE* gene. To remove this gene, plasmid pSUN432.3 was constructed by insertion of the *cat* gene, obtained from pSUN151.1 as an EcoRV/HindIII fragment, between the EcoRV and HindIII sites of pDG1730. This new plasmid was used to replace the *levD'-lacZ* and *aphA3* genes in QB5350 with the chloramphenicol resistance gene.

Measurement of β-galactosidase activity (Miller's assay). The *lacZ* (β-galactosidase) activity was determined as follows. Cells were grown in LB medium with the appropriate antibiotics. In the exponential growth phase mannose alone or mannose and glucose were added, each at a final concentration of 0.2%, and the cultures were incubated further. One hour later 0.1 ml cells was treated with 10 μl toluene for 30 min at 37°C. The β-galactosidase activity in the toluene-treated cells was determined with *o*-nitrophenyl-β-galactopyranoside using Miller's method (30) at 22°C.

Primer extension. A *B. subtilis* strain carrying promoter-containing plasmid pSUN178.4 was grown in LB medium. In the exponential growth phase the culture was induced with 0.2% mannose. After 1 h of growth at 37°C, induced and noninduced cells were harvested. Total RNA was isolated with a Qiagen RNeasy minikit (Hilden, Germany). Primers labeled with Cy5 5' at the end were designed for plasmid pSUN178.4, which contained the complete *manR* gene with the putative *manR* promoter and the intergenic region between *manR* and *manP*.

TABLE 3. Oligonucleotides used in this study

Oligonucleotide	Sequence	Use
s4693	5'-AAA AAA ACG CGT GTT TAA ACT GAA TTT CTG CTG AAT ATA CA-3'	PCR amplification of <i>manR</i> from <i>B. subtilis</i>
s4694	5'-AAA AAA TCT AGA AAG TGT GAA TAA TAA GAT CTT G-3'	PCR amplification of <i>manR</i> from <i>B. subtilis</i>
s4801	5'-AAA AAA ACT AGT GTT TAA ACG ACA TTT TTA TCT CAT TTG GAT-3'	Forward primer for amplification of <i>P_{manPD1}</i>
s4802	5'-AAA AAA ACT AGT GTT TAA ACA GGG AAA AAT GCC TTT ATT AC-3'	Forward primer for amplification of <i>P_{manPD3}</i>
s4833	5'-AAA AAA GTT TAA ACC CCT GGC GAA TGG CGA T-3'	Amplification of <i>spc</i> from plasmid pDG1730
s4835	5'-AAA AAA GAA TTC ATT AGA ATG AAT ATT TCC CAA AT-3'	Amplification of <i>spc</i> from plasmid pDG1730
s4956	5'-AAT TGC GTC GAG ACC CCT GTG GGT CTC GTT TTT TGG ATC CGG CGC CCA CGT GGC TAG CC-3'	Insertion of <i>tufA</i> terminator
s4957	5'-TTA AGG CTA GCC ACG TGG GCG CCG GAT CCA AAA AAC GAG ACC CAC AGG GGT CTC GAC GC-3'	Insertion of <i>tufA</i> terminator
s5006	5'-Cy5-TAG CCT TTT TTA TAG TTG TTC AGC CAC TGT-3'	Labeled primer for primer extension
s5007	5'-Cy5-ATC CAC GCC ATA ATG CAT GCC GCC ATT AAT-3'	Labeled primer for primer extension
s5069	5'-AAA AAA GAA TTC GAT ATC AGA TCT ACG CGT TAA CCC GGG C-3'	Amplification of <i>erm</i> from plasmid pDG1730
s5070	5'-AAA AAA CAA TTG AAT CGA TTC ACA AAA AAT AGG-3'	Amplification of <i>erm</i> from plasmid pDG1730
s5071	5'-AAA AAA AGA TCT CAT GGC AGG GCT TGA GAA-3'	Amplification of sequence (<i>yjdF</i>) downstream of <i>manA</i>
s5072	5'-AAA AAA GAA TTC TTA TTT ACC TCT GTG CTT CTT-3'	Amplification of sequence (<i>yjdF</i>) downstream of <i>manA</i>
s5073	5'-AAA AAA GGC GCC AAG CTA ATG GAT GAC AAT TAT A-3'	Deletion of <i>manA</i> , amplification of upstream region
s5074	5'-AAA AAA CTT AAG CTT TTC CAA TCG CAT GAA CA-3'	Deletion of <i>manA</i> , amplification of upstream region
s5077	5'-AGATTAATAAACCGTTACATATA-3'	Amplification of the sequence upstream of <i>manR</i> (<i>yjdB</i>)
s5078	5'-AAAAAGAATTCAAAATCTCCAATTCTTCACC-3'	Amplification of the sequence upstream of <i>manR</i> (<i>yjdB</i>)
s5079	5'-AAAAAACGCGTCATTTTATCTCATTTGGATTA T-3'	Amplification of the sequence downstream of <i>manR</i> (<i>manP</i>)
s5080	5'-AAAAAGAATTCCAGAAGGAATCATCCG-3'	Amplification of the sequence downstream of <i>manR</i> (<i>manP</i>)
s5097	5'-Cy5-CACTGTACCTATCTGCGAAA-3'	Labeled primer for primer extension
s5098	5'-Cy5-ATTGAGATAATCCTCGATCACCT-3'	Labeled primer for primer extension
s5203	5'-GATATCCTGCACCATCGTC-3'	Backward primer for amplification of <i>P_{manP}</i> for promoter study
s5208	5'-GGTACCATTCTTGCTGAATA-3'	Amplification of <i>P_{manR}</i> region from pSUN279.2
s5209	5'-CTTAAGCCTGTCAGTATCTACTTGAG-3'	Amplification of <i>P_{manR}</i> region from pSUN279.2
s5262	5'-AAAAAAGCTAGCGTTTAAACAAAAGCGATT T AATGAGCTG-3'	Forward primer for amplification of <i>P_{manPD4}</i>
s5263	5'-AAAAAAGCTAGCGTTTAAACCTTGCTTTTTTT GT TATAGGGA-3'	Forward primer for amplification of <i>P_{manPD2}</i>
s5362	5'-ACTAGTGAATTCCTTTTCCAATCGCA-3'	Amplification of the sequence downstream of <i>manP</i> (<i>manA</i>) for knockout of <i>manP</i>
s5363	5'-ACTAGTGAATTCCTTTTCCAATCGCA-3'	Amplification of the sequence downstream of <i>manP</i> (<i>manA</i>) for knockout of <i>manP</i>
s5616	5'-TTA TAA TG CTA TAG GTG TAC AAT ATA GTA TAC TTA TAC TAT CAA T-3'	Amplification of <i>cre*-manR</i> in order to create a <i>cre</i> mutation
s5861	5'-ACT AGT GTT TAA ACT TTA TTA CCG GAA CCT ATG-3'	Forward primer for amplification of <i>P_{manPD5}</i>
s5862	5'-ACT AGT GTT TAA ACC TAT GGT AAA AAA AGC GA-3'	Forward primer for amplification of <i>P_{manPD6}</i>
s5931	5'-AAA AAA GCT AGC GTT TAA ACA TAG CAA ACT CAA AGA GTA-3'	Forward primer for amplification of <i>P_{manRD1}</i>
s5932	5'-AAA AAA GCT AGC GTT TAA ACA GTA TAA AAA TCG CTT TTT TCC-3'	Forward primer for amplification of <i>P_{manRD2}</i>
s5933	5'-AAA AAA GCT AGC GTT TAA ACC GGA AGC TTC GGT AAA AA-3'	Forward primer for amplification of <i>P_{manRD3}</i>
s5934	5'-GTG CAG GAG CTC GTT ATC-3'	Reverse primer for amplification of <i>P_{manRD1-3}</i>
s6463	5'-AAA AAA ACG CGT TCC AAA CAA AAG CAG GCG C-3'	Amplification of sequence (<i>yjdG</i>) downstream of <i>yjdF</i>
s6464	5'-AAA AAA GGA TCC ATG GTT CTC CTT GAA ACG AAT-3'	Amplification of sequence (<i>yjdG</i>) downstream of <i>yjdF</i>

FIG. 1. (A) Map of expression vector pSUN279.2. (B) β -Galactosidase activities of *B. subtilis* 3NA containing plasmids pSUN279.2, pSUN284.1, and pSUN291. Cells were grown in liquid LB medium at 37°C, and β -galactosidase activity was determined without induction or after 1 h of induction with 0.2% mannose, 0.2% mannose, and 0.2% glucose. The data in this and other figures are the means of at least three independent experiments, and the standard deviations were less than 20%. M.U., Miller units.

Following the putative *manP* promoter (*P_{manP}*) a gene coding for lysostaphin from *S. simulans* was added as a reporter for transcription. Primers s5006 and s5007 hybridized at positions 21 to 50 and positions 76 to 105 with respect to the start codon of the lysostaphin gene. Primers s5097 and s5098 hybridized at positions 81 to 101 and positions 131 to 153 with respect to the start codon of *manR*. The same primers were used for the sequencing reaction with plasmid pSUN178.4 DNA, which was used as a size standard. Avian myeloblastosis virus reverse transcriptase and T7 DNA polymerase from Roche (Mannheim, Germany) were used for reverse transcription and DNA sequencing, respectively. The products of reverse transcription and sequencing were analyzed on a denaturing polyacrylamide sequencing gel (GE Healthcare). Other reagents were obtained from a GE Healthcare AutoRead sequencing kit.

RESULTS

Deletion of the mannose utilization genes *manR*, *manP*, *manA*, and *yjdF*. Based on DNA and protein sequence analyses of the *B. subtilis* genome data it was presumed that the *manR*, *manP*, *manA* and *yjdF* genes as annotated by Reizer et al. (36) are involved in mannose utilization. Thus, all four of these genes were first separately deleted from the chromosome and replaced by an erythromycin resistance gene to ascertain their functions. As expected, the TQ276 (Δ *manR*), TQ356 (Δ *manP*), and TQ281 (Δ *manA*) mutants could not grow in minimal liquid medium with 0.5% mannose as the sole carbon source, which confirmed that *manR*, *manP*, and *manA* are genes necessary for mannose utilization. Deletion of *yjdF*, resulting in TQ431 (Δ *yjdF*), had no effect on mannose utilization.

Identification of promoters regulated by mannose. To study the regulation of the three mannose genes in the wild type and the knockout mutants, plasmid pSUN279.2 was constructed, which contained the *manR* gene, the upstream region of *manR* up to the next gene, and the intergenic region between *manR* and *manP* in front of *lacZ*. Plasmid pSUN279.2 (Fig. 1) and plasmid pSUN272.1, the precursor of pSUN279.2 without a promoter that was used as a background control, were transferred into *B. subtilis* 3NA. *B. subtilis* strains 3NA/pSUN279.2 and 3NA/pSUN272.1 were grown in LB medium with spectinomycin, and in the exponential growth phase either 0.2% mannose, 0.2% mannose plus 0.2% glucose, or no sugar (non-

induced control) was added to the cultures for induction. After 1 h of induction the β -galactosidase activity of the cells was determined by using Miller's assay. No β -galactosidase activity was detected in control strain *B. subtilis* 3NA/pSUN272.1 (not shown). In contrast, the noninduced culture of 3NA/pSUN279.2 exhibited a basal level of β -galactosidase activity that was quite high (Fig. 1); the presence of mannose resulted in a 4-fold increase in the β -galactosidase activity, whereas with mannose and glucose the activity was reduced but was still above the basal level.

The promoter activity seen with pSUN279.2 might have originated from the region between *manR* and *manP*, from the region upstream of *manR*, or from both regions. Therefore, the upstream region of *manR*, as well as most of *manR*, was deleted from pSUN279.2 to obtain pSUN284.1. *B. subtilis* 3NA containing the pSUN284.1 vector with *manR* deleted exhibited only about one-half the basal level of β -galactosidase activity compared to 3NA/pSUN279.2; there was an even greater increase when there was mannose induction (7-fold), and there was a greater reduction in the presence of glucose. These results prove that the *manP* promoter (*P_{manP}*) is located between *manR* and *manP* and show that the chromosomal copy of *manR* is sufficient for regulating all *P_{manP}* copies on the low-copy-number plasmids. This might be explained by auto-regulation of *manR* expression. To study *manR* promoter (*P_{manR}*) expression, a plasmid like pSUN284.1 was constructed and designated pSUN291. This plasmid contains the putative *manR* promoter region in front of *lacZ*. In *B. subtilis* wild-type strain 3NA with pSUN291 the basal level of expression of *lacZ* in was relatively high, and it increased 3-fold when 0.2% mannose was added. Furthermore, addition of glucose resulted in repression of β -galactosidase activity to a level that was nearly the same as the basal expression level (Fig. 1). This indicated that the *manR* promoter is not just a weak constitutive promoter but is subject to mannose and CCR regulation.

Regulation of the *P_{manR}* and *P_{manP}* promoters in *manR* and *manP* deletion strains. Addition of plasmid pSUN279.2 with *manR* and addition of plasmid pSUN284.1 without *manR* re-

FIG. 2. Expression of *lacZ* under control of the *manP* promoter (pSUN279.2 and pSUN284.1) and the *manR* promoter (pSUN291) in the $\Delta manR$ strain TQ276 (A) and in the $\Delta manP$ strain TQ356 (B). The strains were grown in LB medium at 37°C and induced for 1 h with 0.2% mannose or with 0.2% mannose and glucose, and the β -galactosidase activities were compared to those of the noninduced strains. M.U., Miller units.

sulted in similar β -galactosidase activities in *B. subtilis* 3NA. To obtain definite proof that ManR is indeed the *trans*-acting regulator of *P_{manP}* and a regulator of its own synthesis, plasmids pSUN279.2, pSUN284.1, and pSUN291 were introduced into strain TQ276, from which *manR* was deleted. When the pSUN279.2 expression vector was introduced into this mutant, growth on mannose was restored due to complementation by the ManR gene on the plasmid (not shown). Induction of this strain with mannose during exponential growth in LB medium resulted in β -galactosidase activity that was about the same as that of strain 3NA. Growth of TQ276 on D-mannose was not restored by addition of the other two plasmids containing no *manR* gene, and there was no induction of *lacZ* by D-mannose during growth in LB medium (Fig. 2A). The basal level of expression of *lacZ* controlled by *P_{manR}* and *P_{manP}* was more than 10-fold lower than the levels observed for wild-type strains 3NA/pSUN284.1 and 3NA/pSUN291. Actually, no β -galactosidase activity was observed for TQ276/pSUN284.1. This shows that ManR is a *trans*-acting activator of the two promoters and indicates that even in the absence of external mannose there is enough active activator in the *B. subtilis* wild type to allow a high basal level of expression.

When plasmids pSUN284.1 and pSUN291 were introduced into strain TQ356, from which the PTS-dependent mannose permease was deleted, there was constitutive *lacZ* expression, but the activity was slightly lower, as observed for the fully induced wild-type strains. Obviously, the permease had a negative effect on expression from the promoters. Nevertheless, the promoters were still subject to catabolite repression since addition of glucose led to a 2-fold reduction in β -galactosidase activity (Fig. 2B). When plasmids pSUN284.1 and pSUN291 were introduced into strain TQ431, from which *yjdF* was deleted, there was no difference from the wild-type strain in basal expression, maximal induction, and catabolite repression (data not shown).

Expression from *P_{manP}* and *P_{manR}* in *B. subtilis* HPr-Ser46, Crh, and CcpA mutants. The expression of most PTS-dependent sugar utilization genes and operons, such as *sacPA*, *licTS*,

bglPH, and *levDEFG* (20, 21, 26, 42, 44), in *B. subtilis* is repressed by glucose. The same is true for the mannose utilization. Repression was detected both at the *manP* promoter and at the *manR* promoter. The induction by mannose of 3NA/pSUN284.1 having the *manP* promoter on the plasmid was decreased 3.4-fold, and the induction by mannose of 3NA/pSUN291 containing the *manR* promoter was decreased 2.4-fold. Since most CCR is mediated through a complex of P-Ser-HPr and CcpA binding at *cre* sites in the promoter regions of catabolic operons, a search for *cre* sites was carried out using the whole mannose operon. One putative *cre* site was found in the promoter region of *manR*. This means that CCR of the *manPA-yjdF* operon might be due to repression of ManR synthesis by CcpA complexed with P-Ser46-HPr. Thus, the *manR* and *manP* promoters were tested for CCR in mutant QB5223, in which the HPr Ser46 residue is replaced by alanine. Only a slight decrease in CCR was observed for both promoters, as shown in Fig. 3A.

Crh is the second central element in CCR of *B. subtilis* (8) and might complement the Ser46 mutation of HPr. Therefore, a *crh* knockout mutant was constructed, and the CCR of the *manP* and *manR* promoters was determined again. The CCR in this mutant (TQ338) was not abolished (Fig. 3B). Subsequently, a double mutant, TQ338_S46 with a Ser46 HPr point mutation and a *crh* knockout mutation, was constructed. In this mutant the CCR was less distinctive for the *P_{manP}* and *P_{manR}* promoters. In addition, for the *manR* promoter a higher basal level of expression and a maximum level of induction were observed (Fig. 3C). This indicated that the CCR of the mannose operon is due in part to HPr as well as Crh. If one of the two was disabled, the other would exert CCR.

Plasmids pSUN284.1 and pSUN291 were also introduced into *ccpA* mutant 1A147. The basal level of expression for the *manP* promoter was similar to that of the wild-type strain, but no induction was observed with mannose. Addition of glucose reduced expression about 20-fold. The results were similar for the *manR* promoter; there was no induction by mannose, and addition of glucose reduced the β -galactosidase activity about

FIG. 3. Expression of *lacZ* under control of the *manP* promoter (pSUN284.1) and the *manR* promoter (pSUN291) in HPr-Ser46Ala mutant QB5223 (A), in Δ *crh* mutant TQ338 (B), in HPr-Ser46Ala Δ *crh* double mutant TQ338_S46 (C), and in *ccpA* mutant 1A147 (D). The cells were grown at 37°C and induced for 1 h with 0.2% mannose, 0.2% mannose, and glucose or were incubated without added sugars. M.U., Miller units.

5-fold (Fig. 3D). To verify these surprising results and to exclude the possibility that there were additional mutations in *ccpA* mutant strain 1A147, the experiments were repeated with a second, independent *ccpA* mutant. TQ303 is a *ccpA* deletion mutant of *B. subtilis* 3NA constructed in this work. The same results were obtained with this strain (data not shown).

HPr His15 plays an essential role in activation of the mannose operon. It has been shown that HPr transfers phosphoryl groups from His15 to the EIIA domains of the EII transporters, as well as to PRDs of regulators like LevR, LicR, and BglG. Whether HPr plays the same role in the mannose promoters was investigated in this study. *B. subtilis* strain TQ432 carrying an HPr His15Ala mutation was transformed with plasmid pSUN284.1 carrying the *P_{manP}* promoter upstream of *lacZ* and with pSUN291 carrying the *P_{manR}* promoter in a similar construction. The strains were grown in LB medium and induced for 1 h with 0.2% D-mannose. No increase in β -galactosidase activity was detected compared to the noninduced controls, and in both cases the basal level of expression was strongly reduced compared to the levels observed with plasmids in the HPr wild-type strains (Fig. 4). These results suggested that the PEP-dependent phosphorylation of HPr at the His15 residue plays an essential role in transcription activation of *P_{manP}* and *P_{manR}*. Similar results were obtained for the levanase operon (43).

Determination of the transcription start sites of *P_{manR}* and *P_{manP}*. The locations of the transcription start sites of *manR*

FIG. 4. Involvement of HPr His15 in regulation of the *manP* and *manR* promoters. Plasmids pSUN284.1 and pSUN291 were introduced into HPr His15Ala mutant strain QB5350, and the induction at *P_{manP}* and *P_{manR}* was determined by determining β -galactosidase activity. Cells were induced for 1 h under the standard conditions (note the difference between the scale in this figure and the scales in Fig. 1 to 3). M.U., Miller units.

and *manPA-yjdF* were determined by performing primer extension experiments. To obtain sufficient mRNA, the fragment with the *manR* upstream region, *manR* gene, and *manP* promoter region used in plasmid pSUN279.2 was cloned into a high-copy-number pUB110 derivative upstream of the lyso-staphin gene of *S. simulans* from which its own promoter and signal sequence for export had been deleted. *B. subtilis* 3NA with the resulting plasmid, pSUN178.4, was grown in LB medium with kanamycin. After 1 h of induction with D-mannose, total RNA was isolated from induced and noninduced cells. The transcription start site of *manPA-yjdF* was found to be a single A residue (Fig. 5) downstream of the -10 and -35 boxes. The putative -35 box showed a very low level of similarity to the consensus sequence of a *B. subtilis* vegetative σ^A -dependent promoter (32). The transcriptional start site of *manR* was found to be a single G residue (Fig. 6), which was preceded by -10 and -35 boxes resembling a σ^A vegetative promoter structure (32). Transcription from the *manR* promoter and particularly transcription from the *manP* promoter were strongly increased when the cells were induced by mannose, as shown by the much stronger signals in the primer extension experiment. This finding is in agreement with the data obtained using the transcriptional fusions of *P_{manR}* and *P_{manP}* with *lacZ*. The primer extension experiments were repeated with different primers, and the results were the same in terms of the transcriptional start sites, as well as the differences in the signal strengths of induced and noninduced cells (data not shown).

Deletion analysis of the *manP* and *manR* promoters. The primer extension experiment showed that the transcriptional start site of the *manP* promoter is located near the 3' end of the intergenic region between *manR* and the beginning of *manP*. Upstream of the deduced -10 and -35 boxes there was still about 300 bp, including the C-terminal end of *manR* in plasmid pSUN284.1, which was thought to contain the binding site of ManR for activating the transcription of *P_{manP}*. To

FIG. 5. Mapping of the transcriptional start site of P_{manP} . (A) RNA was isolated from *B. subtilis* 3NA/pSUN178.4 grown in LB medium in the presence (man) and in the absence (-) of mannose, and the transcriptional start site was determined by primer extension using primer s5006. DNA sequence reaction mixtures with plasmid pSUN178.4 and the same primer were prepared and run on the same denaturing gel for comparison. (B) DNA sequence around the *manP* promoter. The transcriptional start site at an adenine nucleotide and the deduced -10 and -35 boxes are indicated by bold type and italics. The region encoding the C-terminal end of *manR* is indicated by an arrow, the stop codon is indicated by bold type, and three inverted repeats are indicated by arrows above (IRIII-P) and below (IRII-P and IRI-P) the corresponding nucleotides. The bent arrows indicate the promoter regions integrated upstream of *lacZ* in the pSUN272.1 vector. Restriction sites for NruI and BglIII are underlined.

localize this operator sequence more precisely, the *manP* promoter region was shortened step by step by PCR amplification and cloned back into the same expression vector. In the first step 175 bp comprising sequences of the C-terminal end of *manR* (plasmid pSUN289.3) was deleted (Fig. 5). There was no difference between induction of *lacZ* by mannose in *B. subtilis* strain 3NA/pSUN289.3 and induction of *lacZ* by mannose in 3NA/pSUN284.1 (Fig. 7). A stem-loop structure which might act as a *manR* transcriptional terminator (IRIII-P) was found to overlap the TAA stop codon (Fig. 5). The next deletion step removed *manR* completely, as well as one-half of the IRIII-P stem-loop sequence. Again, strain 3NA with plasmid pSUN298.1

did not differ significantly in induction behavior from strains 3NA/pSUN289.3 and 3NA/pSUN284.1 (Fig. 7). Deletion of another 18 bp (pSUN290) did not affect P_{manP} regulation either. Interestingly, this deletion removed part of another putative stem-loop structure (IRII-P). Deletion of another 12 bp (pSUN380.1) reduced activity by about one-half. Finally, deletions ending 20 bp (pSUN381.1) and 7 bp (pSUN297.5) upstream of the putative -35 box completely eliminated mannose induction, and the basal level of expression was in the range of the levels observed for the strain from which *manR* was deleted. This indicated that the target sequence of ManR is presumably located between bp -80 and -35 with respect to

FIG. 6. Mapping of the transcriptional start site of P_{manR} . (A) RNA isolation, primer extension, and DNA sequencing were done as described in the legend to Fig. 5, except that primer s5098 was used, which binds in the *manR* gene. (B) DNA sequence of the *manR* promoter region. The transcriptional start site and the deduced -10 and -35 boxes are indicated by bold type and italics, and the start site of the *manR* gene is indicated by an arrow. The HindIII restriction site and the ribosomal binding site (RBS) are indicated by underlining and bold type, a putative *cre* site is underlined, and a long imperfect inverted repeat (IRI-R) is indicated by arrows.

FIG. 7. β -Galactosidase activities of *B. subtilis* 3NA containing the promoter probe vector pSUN284.1 and various derivatives of the *manP* promoter fragments, as shown in Fig. 5B. Induction of the cells with mannose was performed under standard conditions. M.U., Miller units.

the transcription start site of *manPA-yjdF*. The deletions in pSUN380.1 and pSUN297.5 affected a third putative stem-loop structure, IRI-P, which overlaps IRII-P on one side. A similar sequence was found upstream of the *manR* promoter. The *manR* promoter was analyzed in a similar way by shortening the promoter sequence from the 5' end. Three derivatives were constructed, pSUN384.1, pSUN385.2, and pSUN386.9 (Fig. 6). In the first two plasmids 23 bp and 40 bp were removed from the 5' end. This had no significant effect on *lacZ* expression (Fig. 8). For unknown reasons the basal level of expression of *lacZ* in pSUN385.2 nearly doubled compared to strains 3NA/pSUN291 and 3NA/pSUN384.1. Finally, the third deletion, a 60-bp deletion, had a drastic effect. In this case expression from the promoter was completely eliminated. This deletion ended in an imperfectly inverted sequence (IRI-R) which was identical to IRI-P at 21 of 26 bp and might be the binding site of ManR.

Effect of a *cre* mutation on expression of the mannose operon. Based on the consensus sequence WWTGNAARCG

FIG. 8. β -Galactosidase activities of *B. subtilis* 3NA containing the promoter probe vector pSUN291 and various derivatives of the *manR* promoter fragments, as shown in Fig. 6B. Induction of the cells with mannose was performed under standard conditions. M.U., Miller units.

NWWCAWW (where W is A or T, R is G or A, and N is any nucleotide) (31), a putative *cre* sequence overlapping the transcription start region of *manR* was identified in the mannose operon. By using site-specific mutagenesis 8 bp in this sequence was replaced, which resulted in six mismatches compared with the consensus *cre* sequence (Fig. 9A). The chromosomal *manR* wild-type promoter of *B. subtilis* 3NA was replaced by this mutated promoter (*cre**-*P_{manR}*) to obtain mutant strain TQ361. Catabolite repression of *P_{manP}* in TQ361 was tested by transforming the strain with plasmid pSUN284.1 and inducing it with mannose and glucose. Catabolite repression was strongly reduced in this mutant; instead of a 3.4-fold reduction in β -galactosidase activity there was only a 1.6-fold reduction when glucose was added together with mannose (Fig. 9B). When the same experiments were repeated with the *P_{manR}*-containing plasmid pSUN291, there was no significant difference in catabolite repression between TQ361 and the wild type (data not shown), presumably due to the intact *cre* site remaining in *P_{manR}* of pSUN291. Subsequently, the *cre* sequence in *P_{manR}* of pSUN291 was also replaced with the mutated sequence. With this *cre* mutation in the expression vector pSUN377 both the basal level and the induction by mannose in the *B. subtilis* *cre* wild-type strain were increased, whereas the catabolite repression resulting from glucose was reduced. When *cre**-*P_{manR}*-*lacZ* of plasmid pSUN377 was expressed in mutant TQ361 harboring *cre**-*P_{manR}* in the chromosome, the induction was increased further and the catabolite repression was reduced even more (Fig. 9B). This clearly demonstrates that the *cre* site of *P_{manP}* is functional in *B. subtilis* catabolite repression.

DISCUSSION

In this work, the regulation of a mannose utilization system in *B. subtilis* was studied. The regulator ManR belongs to the group of PRD-containing proteins called the BglG family. This is confusing since members of this family contain transcription antiterminators like BglG and transcription activators like ManR. The two types of regulators can be distinguished easily

FIG. 9. Mutation of *cre* in the *manR* promoter region. (A) Comparison of the consensus *cre* sequence and the original and mutated *cre* sequences in the *manR* transcription start region. Eight nucleotides were replaced (shaded), resulting in two new restriction sites (*SfcI* and *BsrGI*) and six mismatches (bold type) compared with the consensus *cre* sequence. W is A or T; R is G or A; and N is any nucleotide. "+1" indicates the transcription start site. (B) Effect of *cre* mutation on *manP* and *manR* promoters. β -Galactosidase activities were determined separately for *B. subtilis* 3NA and mutant TQ361 harboring *cre**-*P_{manR}* in the chromosome with the expression vector pSUN284.1 (*P_{manP}*-*lacZ*) or pSUN377 (*cre**-*P_{manR}*-*lacZ*). Induction of the cells with mannose was performed under standard conditions. M.U., Miller units.

because there is a helix-turn-helix motif for DNA binding at the N-terminal end of the activators and there is an RNA binding motif in the antiterminators. Another difference is the additional domains present in activators, like the EIIA domain of ManR, which are not present in the transcription antiterminators. Based on these findings, ManR is a transcription activator due to the helix-turn-helix motif at its N-terminal end and the EIIA and EIIB domains in the C-terminal region. In the BglG family phosphorylation of PRD1 depends on the presence of inducer and phosphorylation of PRD2 on the presence of other fast-metabolized sugars, particularly on the presence of glucose. So far it is not possible to predict from just the protein sequence which PRD sequence is domain 1 and which PRD sequence is domain 2. Analogous to the MtlR protein of *G. stearothermophilus*, it is likely that the first PRD after the DNA binding domain in ManR is PRD1 and the next PRD is PRD2. Most likely PRD1 of ManR is phosphorylated in the absence of mannose and dephosphorylated in the presence of mannose via the EIIA and EIIB domains present in ManR and ManP. This would explain the constitutive expression from the *manP* and *manR* promoters in the *manP* mutant strain.

A complete loss of activity was observed for the HPr His15Ala mutant. Presumably, HPr-His15-P phosphorylates PRD2 in the absence of glucose and the phosphorylated form is essential for activity. A mutation in HPr at residue His15 leads to a permanently inactive form. This is obviously not

compensated for by the simultaneous lack of phosphorylation at PRD1 via HPr or ManP, which is another requirement for the active form of ManR.

Surprisingly, ManR not only is the *trans*-acting regulator for the *manPA-yjdF*-operon but also is an autoregulator for *manR* itself. Two promoters (*P_{manR}* and *P_{manP}*) were found to be responsible for the regulated transcription of *manR* and of the *manPA-yjdF* operon, and the transcription start sites were determined by primer extension analysis. The target of ManR is located within -80 and -35 in relation to the transcription start site of the *manP* promoter. Since all of the experiments described here indicated that ManR binds at *P_{manR}* and *P_{manP}*, one would expect similar and maybe palindromic sequences in the two promoter regions. A 14-bp sequence that is present in both promoter regions was found, but the distances from the experimentally defined transcription start point were different. In *P_{manP}* this sequence overlaps the -35 box, whereas in *P_{manR}* it is in the inverse orientation and 28 bp from this box. In the *manP* promoter region three inverted repeat sequences were identified, one at the end of *manR* (IRIII-P) that has the typical structure of the rho-independent transcriptional terminator and two (IRII-P and IRI-P) in which the inverted repeats are separated by 23 bp and 3 bp, respectively. An inverted repeat similar to IRI-P was also identified in the *manR* promoter (IRI-R) at about the same distance from the -35 sequence. In both promoters activity was reduced or even eliminated when deletions removed part of IRI-P (*P_{manP}*) or IRI-R

(*P_{manR}*). This is a strong indication that these inverted repeats represent the ManR binding sites. So far it has not been possible to isolate enough active ManR from recombinant *B. subtilis* or *E. coli* to determine the exact binding sites of ManR in DNA mobility shift assays or DNA footprint analyses.

In addition to carbon catabolite repression acting on ManR via phosphorylation, the mannose genes seem to be target of a second type of CCR that acts via the CcpA-HPr-Ser46 complex. Surprisingly, the *cre* site was found to be not at the *manP* promoter but at the *manR* promoter. In the presence of excess glucose, glucose-6-phosphate and fructose-1,6-bisphosphate stimulate HPr kinase, which phosphorylates HPr-Ser46. The CcpA-HPr-Ser46-P complex binds to the *cre* site at the *manR* promoter, inhibiting the synthesis of more ManR activator. Mutations at 6 of the 18 bp in the *cre* sequence of *P_{manR}* resulted in strong decreases in CCR. There was also some relief of CCR in an HPr-Ser46Ala mutant. A stronger effect, comparable to that of the *cre* mutations, was observed when catabolite expression was tested with a HPr-Ser46Ala and *crh* knockout double mutant. Obviously, Crh can replace HPr in CCR of the mannose utilization system. Surprisingly, in two different *ccpA* mutants the *manP* and *manR* promoters could not be induced by mannose. CcpA negatively controls genes needed for alternative carbon sources and the Krebs cycle and activates genes for glycolytic enzymes and carbon overflow in *B. subtilis* (40). Maybe the lack of gene activation in the *ccpA* mutants decreases the pool of PEP. Since PEP is needed for mannose and glucose uptake, the level of PEP decreases even further when the sugars are added, leaving ManR in an unphosphorylated inactive conformation.

The unusual features of the mannose regulatory system and the strength of the *manP* promoter should make this system ideal for use in vectors for heterologous gene expression. Further studies are needed to completely understand this system in terms of the effects of the two CCR mechanisms and the phosphorylation and binding of ManR to the mannose promoters. With detailed knowledge workers should be able to use this system and improve it for applications in high-cell-density fermentations.

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