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Regulation of the *glv* Operon in *Bacillus subtilis*: YfiA (GlvR) Is a Positive Regulator of the Operon That Is Repressed through CcpA and *cre*

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Maltose metabolism and the regulation of the *glv* operon of *Bacillus subtilis*, comprising three genes, *glvA* (6-phospho-α-glucosidase), *yfiA* (now designated *glvR*), and *glvC* (EIICB transport protein), were investigated. Maltose dissimilation was dependent primarily upon the *glv* operon, and insertional inactivation of either *glvA*, *glvR*, or *glvC* markedly inhibited growth on the disaccharide. A second system (MalL) contributed to a minor extent to maltose metabolism. Northern blotting revealed two transcripts corresponding to a monocistronic mRNA of *glvA* and a polycistronic mRNA of *glvA-glvR-glvC*. Primer extension analysis showed that both transcripts started at the same base (G) located 26 bp upstream of the 5′ end of *glvA*. When *glvR* was placed under control of the *spac* promoter, expression of the *glv* operon was dependent upon the presence of isopropyl-β-D-thiogalactopyranoside (IPTG). In regulatory studies, the promoter sequence of the *glv* operon was fused to *lacZ* and inserted into the *amyE* locus, and the resultant strain (AMGLV) was then transformed with a citrate-controlled *glvR* plasmid, pHYCM2VR. When cultured in Difco sporulation medium containing citrate, this transformant [AMGLV(pHYCM2VR)] expressed LacZ activity, but synthesis of LacZ was repressed by glucose. In an isogenic strain, [AMGLVCR(pHYCM2VR)], except for a mutation in the sequence of a catabolite-responsive element (*cre*), LacZ activity was expressed in the presence of citrate and glucose. Insertion of a citrate-controlled *glvR* plasmid at the *amyE* locus of *ccpA*$^+$ and *ccpA* mutant organisms yielded strains AMCMVR and AMCMVRCC, respectively. In the presence of both glucose and citrate, AMCMVR failed to express the *glv* operon, whereas under the same conditions high-level expression of both mRNA transcripts was found in strain AMCMVRCC. Collectively, our findings suggest that GlvR (the product of the *glvR* gene) is a positive regulator of the *glv* operon and that glucose exerts its effect via catabolite repression requiring both CcpA and *cre*.

Bacteria have evolved a highly sophisticated multiprotein sugar transport and phosphorylation system, the phosphoenolpyruvate-sugar phosphotransferase system (PTS) (11, 14). *Bacillus subtilis*, whose whole genome sequence was established in 1997 (8), encodes 15 complete PTS permeases, of which only 7 have been characterized (15). We previously reported that the *glv* operon of *B. subtilis* encodes 6-phospho-α-glucosidase (GlvA), an unknown product (YfiA, now designated GlvR [regulatory protein of the *glv* operon]), and a PTS permease (GlvC), in that order, in the 76° region (36). GlvA is a polypeptide consisting of 449 amino acid residues, and this enzyme requires NAD(H) and divalent metal ions for activity (30). This glucosidase is assigned to the ~20-member family 4 of the glycosylhydrolase superfamily (30, 32; SWISS-PROT protein sequence data bank [http://www.expasy.ch/cgi-bin/lists?glycosid.txt]). GlvR is a polypeptide consisting of 254 amino acid residues, and its amino acid sequence exhibits high similarity to those of RpiR/YebK/YfhH family members (SWISS-PROT). RpiR is involved in the regulation of *rpiB* expression in *Escherichia coli*, and its N-terminal region contains a helix-turn-helix DNA binding motif characteristic of many regulatory proteins (22). GlvC is a polypeptide consisting of 527 amino acid residues and is a PTS transport component with 12 transmembrane segments (36). Maltose is transported into the cytoplasm simultaneously with phosphorylation by GlvC, and maltose-6-phosphate is hydrolyzed intracellularly to glucose-6-phosphate and glucose by GlvA (30). Recent studies by Dahl and coworkers have identified a cluster of nine genes that promote the non-PTS-catalyzed transport and metabolism of maltose in *B. subtilis* (18, 19). Included in this putative operon is the gene *malL*. The gene encodes MalL (YvdL), a maltose-inducible α-glucosidase that exhibits high amino acid sequence similarity with several α-glucosidases and hydrolyzes various disaccharides such as maltose, sucrose, and isomaltose and longer maltodextrins (18). The *malL* gene is located in the deduced gene cluster (*yvdE* to *yvdM*) in the 302.8° to 303.9° region (18). Upstream of *yvdE* there is a ρ-independent terminator, and downstream of *yvdM* there is a *clpA* gene in the opposite orientation, followed by a ρ-independent terminator (BSORF database). In the cluster, *yvdE* is assumed to be a transcriptional regulatory gene belonging to the helix-turn-helix LacI family (BSORF database), and the *yvdF* and *yvdG* products are homologues of glucan 1,4-α-maltohydrolase and maltose- and maltodextrin-binding protein, respectively (SWISS-PROT). *yvdH* and *yvdI* are genes for maltodextrin transport system permeases, and *yvdJ* is a membrane protein with an ATP- and GTP-binding motif. The *yvdK* product has no identifiable mo-

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TABLE 1. Bacterial strains used in this study

Strain	Relevant genotype	Source[a]
B. subtilis		
168	*trpC2*	S. D. Ehrlich
GLVAd	*trpC2 glvA*::pMV1	This study
GLVRd	*trpC2 glvR*::pMVR	This study
GLVCd	*trpC2 glvC*::pMV2	This study
MALLdd	*trpC2 malL*::*kan*	This study
MLGLVAd	*trpC2 malL*::*kan glvA*::pMV1	This study
GLVR-PSP	*trpC2 glvR*::pMVRSD (*spac-glvR glvC*)	This study
AMGLV	*trpC2 amyE*::(P_{glvA} [−240 to +32]′-′*lacZ cat*)[b]	pDHΔglv→168
AMGLVCR	*trpC2 amyE*::(P_{glvA} [−240 to +32 region carrying a CG-to-AT dinucleotide change at positions +6 and +7 relative to the *glvA* start point]′-′*lacZ cat*)[b]	pDHΔglvCR→168
AMCMVR	*trpC2 amyE*::(P_{citM} [−209 to +14]-*glvR cat*)[c]	pDAFBCMVR→168
1A1	*trpC2 ccpA*::*neo*	Y. Fujita
AMCMVRCC	*trpC2 amyE*::(P_{citM} [−209 to +14]-*glvR cat*) *ccpA*::*neo*[c]	1A1→AMCMVR
E. coli		
JM109	*recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi-1* Δ(*lac-proAB*)/F′ (*traD36 proAB*$^+$ *lacI*^{q} *lacZ* Δ *M15*)	Takara
C600	*supE44 hsdR17 thi-1 thr-1 leuB6 lacY1 tonA21*	Laboratory stock

[a] Arrows indicate construction by transformation.
[b] Nucleotide numbers correspond to the *glvA* promoter regions relative to the *glvA* start point.
[c] Nucleotide numbers correspond to the *citM* promoter regions relative to the *citM* start point.

tif, but *yvdL* (*malL*) and *yvdM* are the genes for sucrase-isomaltase-maltase and β-phosphoglucomutase, respectively (19; SWISS-PROT). Thus, the gene cluster may be a polycistronic operon associated with transport (as free sugar) and metabolism of maltose.

Catabolite repression is a global regulatory mechanism which, in *B. subtilis* and other gram-positive bacteria, comprises three major components (6, 23). First, the expression of many catabolic genes is repressed in the presence of a readily metabolizable carbon source such as glucose, fructose, or mannitol. Second, *cis*-acting sequences called catabolite responsive elements (*cre*) mediate catabolite repression of many genes (7,

TABLE 2. Plasmids used in this study

Plasmid	Relevant genotype	Source or Reference
pMUTIN2	*bla erm lacZ lacI spac*	33
pMUTIN4	*bla erm lacZ lacI spac oid*	33
pBluescript II SK(+)	*bla lacZ*	Stratagene
pGEM-3Zf(+)	*bla lacZ*	Promega
pUC119	*bla lacZ*	Takara
pDG782	*bla kan*	BGSC[a]
pHY300PLK	*bla tet*	Takara
pMV1	pMUTIN2::Δ*glvA*	This study
pMVR	pMUTIN2::Δ*glvR*	This study
pMV2	pMUTIN2::Δ*glvC*	This study
pMVR-SD	pMUTIN4::Δ*glvR* (containing *glvR* SD sequence)	This study
pGV1	pGEM-3Zf(+)::Δ*glvA*	This study
pGVR	pGEM-3Zf(+)::Δ*glvR*	This study
pGV2	pGEM-3Zf(+)::Δ*glvC*	This study
pUCMALL	*bla malL*	This study
pUCMALLKm	*bla malL*::*kan*	This study
pBCM2	pBluescript II SK(+) with a 242-bp *citM* promoter region	This study
pHYCM2	pHY300PLK with a 223-bp *citM* promoter region	This study
pDHAFBLZ	*bla amyE*::(*lacZ cat*)	34
pHYCM2LZ	pHY300PLK with a 223-bp *citM* promoter region and *lacZ*	This study
pBVR-SD	*bla glvR*	This study
pHYCM2VR	pHY300PLK with a 223-bp *citM* promoter region and *glvR*	This study
pUCΔglv	pUC119 with a 272-bp *glvA* promoter region	This study
pUCΔglvCR	pUC119 with a 272-bp *glvA* promoter region (carrying a CG-to-AT dinucleotide change at positions +6 and +7 relative to the *glvA* start point)	This study
pDHΔglv	pDHAFBLZ with a 272-bp *glvA* promoter region fused to *lacZ*	This study
pDHΔglvCR	pDHAFBLZ with a 272-bp *glvA* promoter region fused to *lacZ* (carrying a CG-to-AT dinucleotide change at positions +6 and +7 relative to the *glvA* start point)	This study
pDHAFB	*bla amyE*::*cat lacI*	34
pDHAFB2	*bla amyE*::*cat*	This study
pDAFBCMVR	pDHAFB2 with a 242-bp *citM* promoter region and *glvR*	This study

[a] BGSC, *Bacillus* Genetic Stock Center, The Ohio State University, Columbus.

TABLE 3. Primers used in this study

Primer	Sequence (5′→3′) or source[a]	Restriction site
V1-EF	gccg<u>GAATTC</u>CATTCTCAATCGTAATAGCG	*Eco*RI
V1-BR	gcgc<u>GGATCC</u>TTCCCTACTCTGATGTGC	*Bam*HI
VR-EF	gccg<u>GAATTC</u>GAAGAACTGATCAATCAGC	*Eco*RI
VR-BR	gcgc<u>GGATCC</u>TCTTCCGGCTGATCTTCC	*Bam*HI
V2-HF	gccg<u>AAGCTT</u>TCGTCGGTATCAGCACG	*Hind*III
V2-BR	gcgc<u>GGATCC</u>GACCTCTTGATTCATGTCG	*Bam*HI
VR-PSPE1	gccg<u>GAATTC</u>TATAATAGAAAGAAAATGGGG	*Eco*RI
VR-PSPB2	cgc<u>GGATCC</u>ACTGTAACCGCTGAAACC	*Bam*HI
GLVR-SDB	gccg<u>GGATCC</u>AAATGGGGGGATCTGATAT	*Bam*HI
GLVR-PROE2	gcgc<u>GAATTC</u>GCTTCCAAAGCGCTGAAT	*Eco*RI
CMUD-F4	gccg<u>GAATTC</u>TAAACGAACAGGACTGGG	*Eco*RI
CMUD-PH2B	gcgc<u>GGATCC</u>GTCTTGCCTTTTTGCCATC	*Bam*HI
GLV-UPF	gccg<u>GAATTC</u>GGCATGTATCCGAATCG	*Eco*RI
GLV-UPR	gccg<u>GTCGAC</u>CTTCATATGACGACCTCC	*Sal*I
GLV-creF	ATAAATGGAATTGTAAAATTTATCAAGGAGGTCGTC	
GLV-creR	GACGACCTCCTTGATAAATTTTACAATTCCATTTAT	
Mal-EF	gccg<u>GAATTC</u>GTGGAAAGAAGCTGTCG	*Eco*RI
Mal-PB	gccg<u>CTGCAG</u>CTAATGCCCATCACAGC	*Pst*I
CCPA-F1	AGCGAGAGAAGCTAATGTAA	
CCPA-R2	GTGCGGCAGTTCGACGA	
V1-PEX	AGAGCATGAGTACGATCC	
−21M13	Universal primer (Takara)	
M13RV	Universal primer (Takara)	
PM-FK	cgg<u>GGTACC</u>GTGTGGAATTGTGAGCG	*Kpn*I
PM-T7	**TAATACGACTCACTATA**GTGTATCAACAAGCTGG	

[a] The additional sequence (lowercase), restriction site (underlined), and the T7 promoter sequence (bold) are indicated. The PM-FK and PM-T7 primers were annealed to the outside of the multicloning site of pMUTIN derivatives.

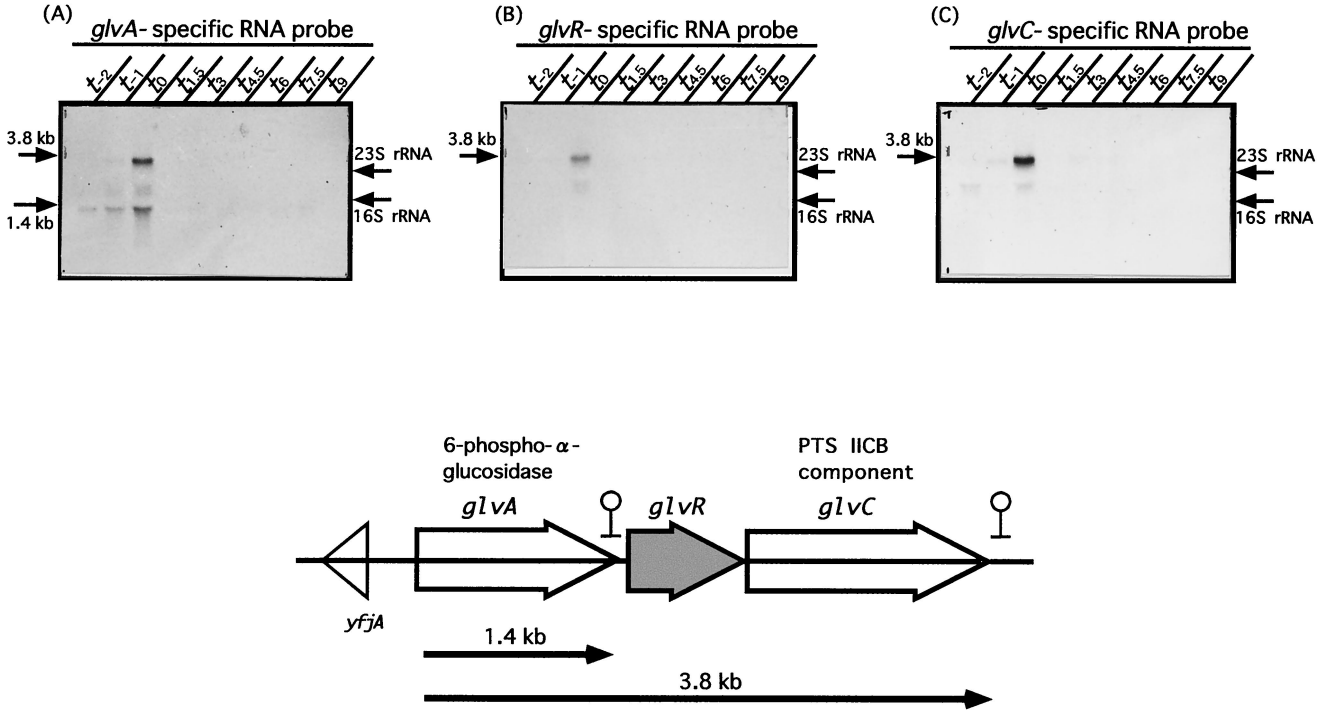

FIG. 1. Transcriptional analysis of the *glvA, glvR*, and *glvC* genes of *B. subtilis, B. subtilis* 168 cells were cultured in a rich sporulation medium (DSM) at 37°C for various periods (t_0 means the time of onset of sporulation, and t_{-x} and t_x mean x hours before and after t_0, respectively). mRNAs were prepared (see Materials and Methods) and subjected to Northern blot analysis. Ten micrograms of each RNA was separated on a 1% formaldehyde–agarose gel. Signals were detected with DIG-labeled RNA probes (panel A, probe A; panel B, probe R; panel C, probe C) specific to the *glvA, glvR*, and *glvC* mRNAs, respectively. The positions of mRNA signals and rRNAs are indicated by arrows on the left and right, respectively. A map of the three genes encoded by the *glv* operon is shown below the panels.

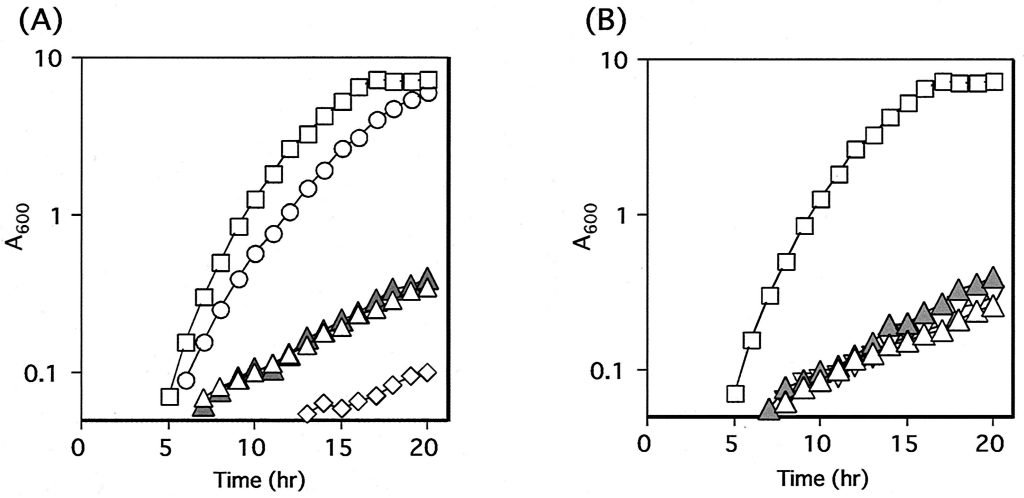

FIG. 2. Growth of various mutants in MMM. (A) Open squares, 168 (wild type); open triangles, GLVAd (*glvA*::pMV1); filled triangles, GLVAd with 1 mM IPTG; open circles, MALLdd (*malL*::*kan*); open diamonds, MLGLVAd (*glvA*::pMV1 *malL*::*kan*). (B) Open squares, 168 (wild type); open triangles, GLVRd (*glvR*::pMVR); filled triangles, GLVRd with IPTG; inverted triangles, GLVCd (*glvC*::pMV2).

12, 23). Mutations that result in release from *cre*-dependent catabolite repression occur in the gene encoding the catabolite control protein, CcpA, which belongs to the LacI family of transcriptional regulators (6, 23). The third important factor is HPr, an intermediate phosphoryl transfer protein in the PTS (4, 6, 23, 24). HPr phosphorylates not only EII for sugar transport but also certain catabolic enzymes such as glycerol kinase and transcriptional regulators for modulation of their activities (23).

In this report we show that regulation of the *glv* operon in *B. subtilis* requires a positive factor (GlvR), catabolite repression through CcpA and *cre*, and induction by maltose. We also discuss the function of *malL* in maltose metabolism.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The bacterial strains used in this study are listed in Table 1. The bacterial strains were cultured in Luria-Bertani (LB) medium (5 g of yeast extract, 10 g of polypeptone, 5 g of NaCl per liter, pH 7.2) at 37°C. When required, ampicillin, erythromycin, tetracycline, kanamycin, chloramphenicol, or neomycin was added to a final concentration of 50, 0.3, 5, 5, 5, or 15 μg per ml, respectively. *B. subtilis* strains were grown in Difco sporulation medium (DSM) (17), Spizizen's minimal medium (SMM) (1), modified SMM (mSMM; glucose was replaced by maltose), or maltose minimal medium (MMM; C medium [13] supplemented with 50 mM L-glutamic acid and 10 mM maltose). When *B. subtilis* strains were grown in minimal medium, L-tryptophan was added to a final concentration of 50 μg per ml.

Construction of plasmids. The plasmids and primers used in this study are listed in Tables 2 and 3, respectively. Derivatives of pMUTIN2 (33) and pGEM-3Zf(+) were used to construct mutants of *B. subtilis* 168 and to prepare gene-

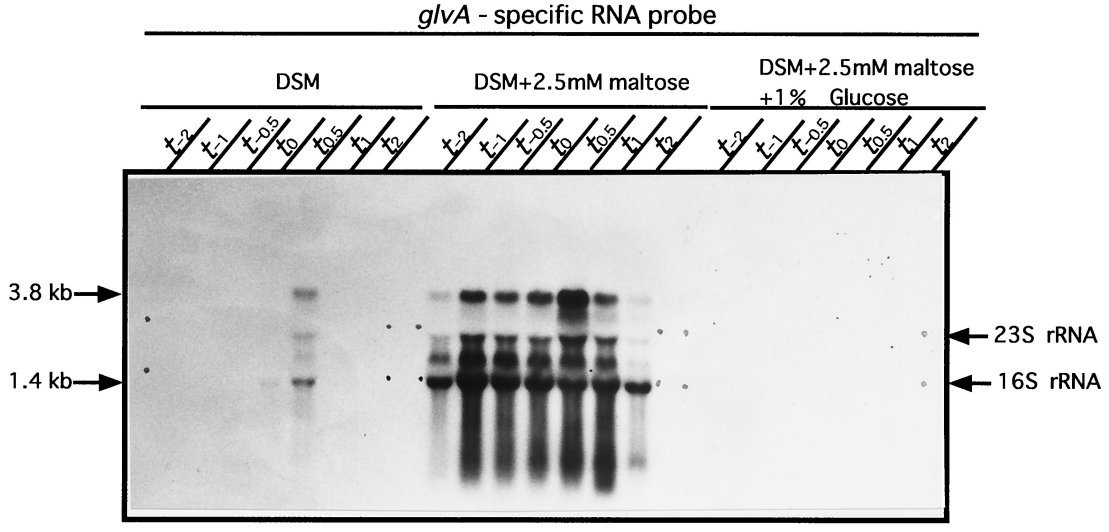

FIG. 3. Effects of maltose and glucose on transcription of the *glv* operon. Maltose (2.5 mM) and/or 1% glucose was added to *B. subtilis* 168 cells at the beginning of growth in DSM. Northern blot analysis was carried out with probe A.

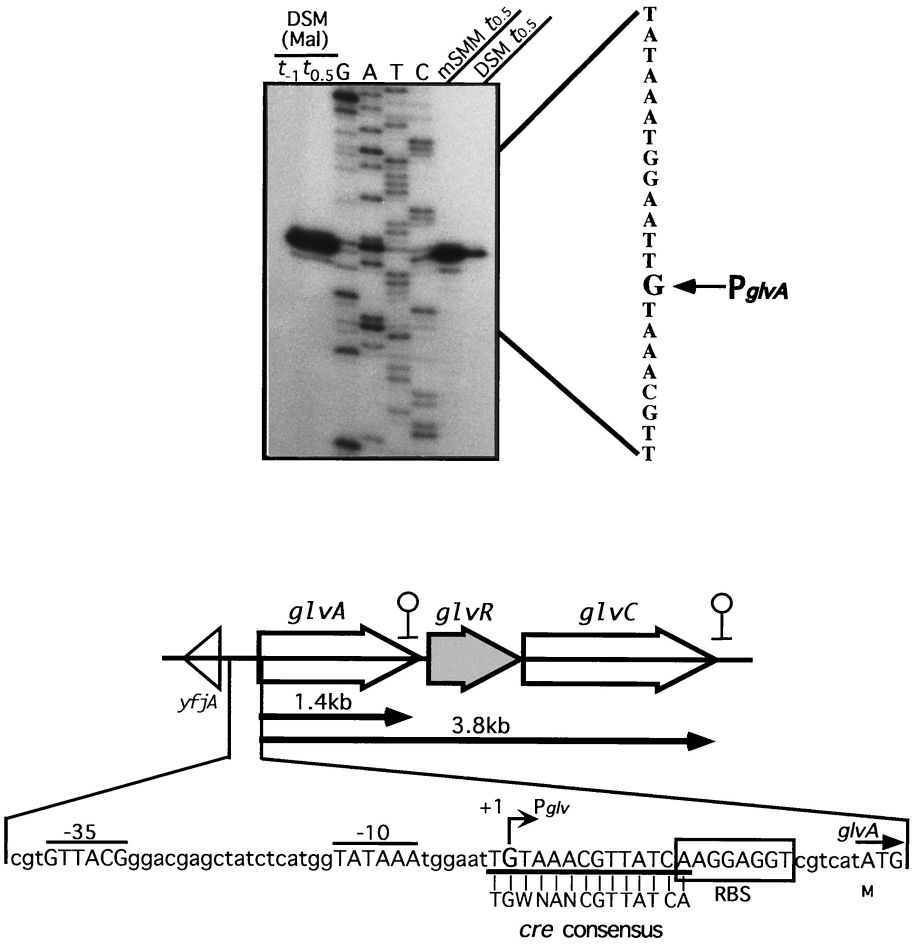

FIG. 4. Determination of the transcriptional start site by primer extension analysis. Total RNAs (40, 10, 10, and 10 μg) from *B. subtilis* 168 cells cultured in DSM at $t_{0.5}$, DSM with 2.5 mM maltose [DSM (Mal)] at t_{-1} and $t_{0.5}$, and mSMM at $t_{0.5}$, respectively, were used as RNA samples. Signals were detected with ^{32}P-labeled primer V1-PEX. Dideoxy DNA sequencing reaction mixtures with the same primer were electrophoresed in parallel (lanes G, A, T, and C). The nucleotide sequence of the transcribed strand is given beside the sequence ladder and the arrow indicates the nucleotide at the transcriptional start site. A map of the *glv* operon and the nucleotide sequence of the upstream region of *glv* are shown below the primer extension analysis results.

specific RNA probes, respectively. For the construction of gene-disrupted mutants, pMUTIN2 derivatives containing an internal region of each gene were used. For example, a *glvA* internal DNA fragment amplified with 168 DNA and primers V1-EF and V1-BR was digested with *Eco*RI and *Bam*HI. Then the digested fragment was ligated to the *Eco*RI and *Bam*HI sites of pMUTIN2, and *E. coli* JM109 was transformed with the mixture to obtain pMV1. In the same way, to obtain plasmids pMVR and pMV2, internal fragments of *glvR* and *glvC* were amplified with 168 DNA and primers VR-EF and VR-BR and V2-HF and V2-BR, respectively. *E. coli* C600 cells were transformed with these ligated DNAs. The nucleotide sequences of PCR products were always confirmed with a DNA sequencer (model 373A; Applied Biosystems). To prepare digoxygenin (DIG)-labeled RNA probes, *Hin*dIII- or *Eco*RI- and *Bam*HI-digested PCR fragments were cloned into pGEM-3Zf(+) to generate plasmids pGV1, pGVR, and pGV2.

Construction of *glvARC* and *malL* disruptants. GLVAd, GLVRd, and GLVCd were constructed by means of Campbell-type integration with plasmids prepared from *E. coli* C600 cells harboring pMV1, pMVR, and pMV2, respectively. To construct the *B. subtilis* MALLdd (*malL*::*kan*) strain, a fragment containing *malL* was amplified with primers Mal-EF and Mal-PB and then digested with *Eco*RI and *Pst*I, followed by ligation to the corresponding sites of pUC119. The resultant plasmid, pUCMALL, was digested at the *Hin*cII site in the *malL* gene and then ligated to a *Stu*I-*Sma*I fragment containing the kanamycin-resistant cassette from pDG782, followed by transformation of *E. coli*. Plasmids were isolated from the Km^r transformants, and a plasmid (pUCMALLKm) containing the Km^r cassette in the reverse direction with respect to the *malL* gene was selected. The pUCMALLKm plasmid was linearized with *Aat*II and used for transformation of *B. subtilis* 168. A *malL* null mutant, MALLdd, was selected on agar medium containing kanamycin. To construct *B. subtilis* MLGLVAd, *B. subtilis* GLVAd was transformed with chromosomal DNA prepared from the MALLdd strain, and kanamycin-resistant transformants were selected.

Construction of a strain containing isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible *glvRC*. A fragment containing the Shine-Dalgarno sequence and the 5′ end of *glvR* was amplified with primers VR-PSPE1 and VR-PSPB2, followed by digestion with *Eco*RI and *Bam*HI. The digested fragment was ligated to the corresponding sites of pMUTIN4, resulting in pMVR-SD. The plasmid from *E. coli* C600(pMVR-SD) was used for transformation of *B. subtilis* 168. The resultant Em^r transformant (GLVR-PSP) contains *glvRC* which is regulated by the *spac* promoter. Proper integration of the plasmids was confirmed by Southern hybridization.

Construction of plasmids integrated into the *amyE* locus. A 272-bp fragment containing the *glvA* promoter region was amplified with primers GLV-UPF and GLV-UPR, followed by digestion with *Eco*RI and *Sal*I. The digested fragment was ligated to the corresponding sites of pUC119, resulting in pUCΔglv. An *Eco*RI-*Sal*I fragment of pUCΔglv was cloned into the corresponding site of an integration vector, pDHAFBLZ, resulting in pDHΔglv. Then *B. subtilis* 168 was transformed with the linear fragment of pDHΔglv obtained with *Pst*I, and a Cm^r strain (AMGLV) was selected. Proper integration was confirmed by PCR, and moreover, the amylase deficiency due to integration into the *amyE* locus was

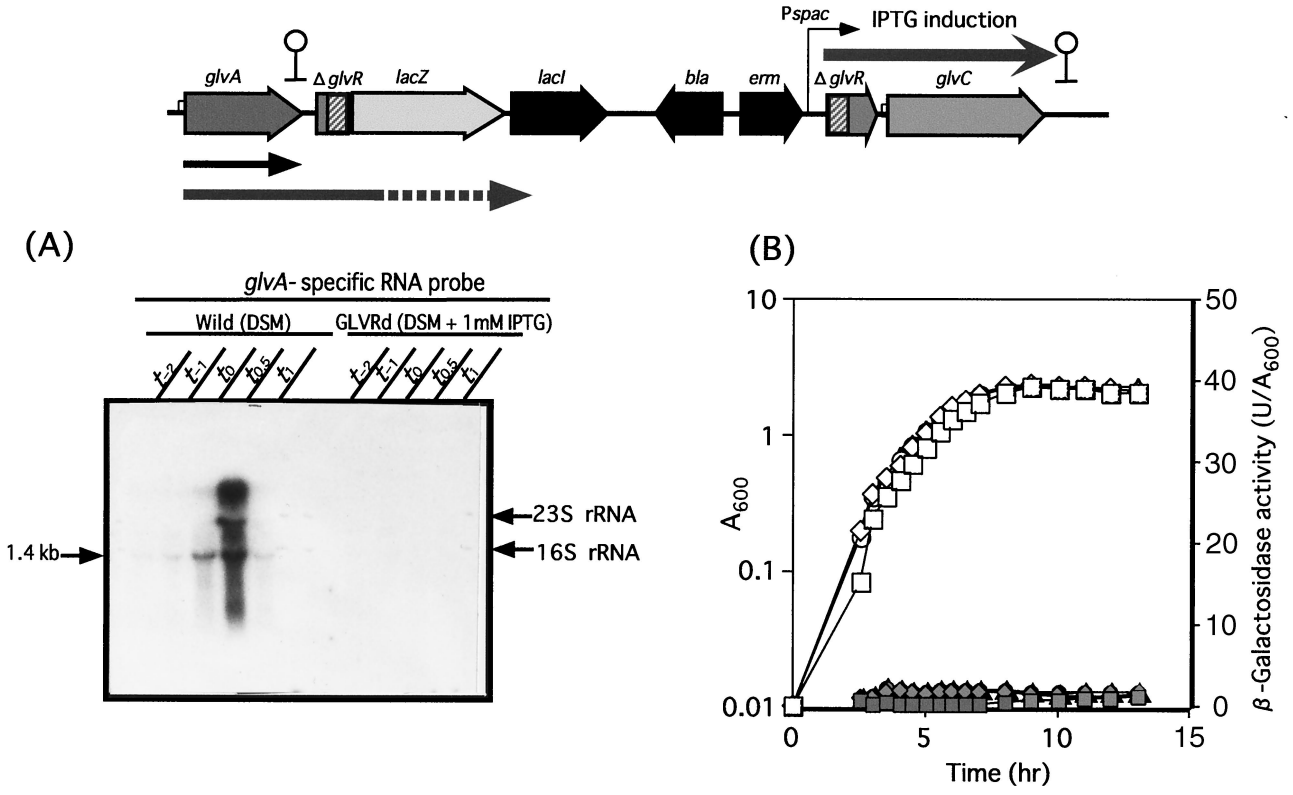

FIG. 5. Northern blot analysis (A) and β-galactosidase activity (B) of the *glvR-lacZ* transcriptional fusion strain constructed in the *B. subtilis* chromosome. (A) Wild and GLVRd strains were grown at 37°C in DSM without and with 1 mM IPTG, respectively. RNAs prepared from cells were separated on a gel, and signals were detected with a DIG-labeled specific RNA probe (probe A). (B) Cell growth (A_{600}) and β-galactosidase activity (units per A_{600}) of the *glvR-lacZ* transcriptional fusion strain (GLVRd) are shown by open and filled symbols, respectively. Squares, *B. subtilis* 168 (wild type); diamonds, GLVRd; circles, GLVRd with IPTG; triangles, GLVRd with IPTG plus maltose. A map of the insertionally inactivated *glv* operon of GLVRd is shown at the top.

confirmed by plating on LB agar medium with starch, followed by the addition of the I_2-KI solution (20).

Construction of strains containing citrate-controlled genes. The *citM* gene is regulated by the two-component system, CitS (sensor) and CitT (positive regulator), and the target site of phosphorylated CitT is located in the upstream region of the 5′ end of *citM* (35). *citM* is repressed by glucose via a *cre* sequence just upstream of the ribosome-binding site of *citM* (35). A 242-bp fragment containing the *citM* promoter region without the *cre* sequence, but with the phosphorylated CitT target site (35), was amplified with 168 DNA and primers CMUD-F4 and CMUD-PH2B, followed by digestion with *Eco*RI and *Bam*HI. The digested fragment was ligated to the corresponding sites of pBluescriptII SK(+). The resultant plasmid, pBCM2, was digested with *Hin*dIII and *Bam*HI and the resultant 223-bp fragment was cloned into the *Hin*dIII and *Bgl*II sites of pHY300PLK, resulting in pHYCM2. To construct a strain containing a P_{citM}-*lacZ* gene, pHYCM2 was digested with *Sal*I, followed by a fill-in reaction with T4 DNA polymerase (DNA blunting kit; Takara). After digestion with *Bam*HI, the plasmid DNA fragment was ligated to the *Sma*I-*Bam*HI fragment containing *lacZ* of pDHAFBLZ, resulting in pHYCM2LZ. After transformation of *B. subtilis* 168 with pHYCM2LZ, transformants expressed β-galactosidase activity only on the addition of 2 mM citrate (data not shown).

To construct strains containing a citrate-controlled *glvR* gene in the *amyE* locus, the *Bam*HI-*Bgl*II fragment of pDHAFB was self-ligated to produce pDHAFB2. Then the 242-bp *Eco*RI-*Bam*HI fragment containing the *citM* promoter region from pBCM2 was mixed with the *Bam*HI-*Eco*RV fragment (containing *glvR*) from pBVR-SD and the *Eco*RI-*Sma*I fragment from pDHAFB2, followed by ligation. The resulting plasmid, pDAFBCMVR, was digested with *Pst*I, followed by transformation of *B. subtilis* 168. The resultant Cm^r strain, AMCMVR, was plated on LB agar medium with starch for the amylase assay, and proper recombination at the *amyE* locus was confirmed by PCR. AMCMVR was transformed with *B. subtilis* 1A1 DNA and then selected on LB agar medium containing neomycin and chloramphenicol. The resultant transformant, AMCMVRCC, was a *ccpA*-deficient strain and contained the citrate-controlled *glvR* gene. Correct recombination was confirmed by PCR with primers CCPA-F1 and CCPA-R2.

Construction of a plasmid to produce GlvR in *B. subtilis* cells. A *glvR*-containing fragment was amplified with *B. subtilis* 168 DNA and primers GLVR-SDB and GLVR-PROE2 and then digested with *Bam*HI and *Eco*RI. The digested fragment was cloned into the corresponding sites of pBluescriptII SK(+), resulting in pBVR-SD. After digestion of pBVR-SD with *Bam*HI and *Eco*RI, the digested fragment was ligated to the corresponding sites of pHYCM2, resulting in pHYCM2VR.

Site-directed mutagenesis. Two-base replacement in the center of the consensus *cre* sequence from CG to AT at positions 6 and 7 upstream of the translational start point was performed with a Quick Change site-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions. pUCΔglvCR was constructed by PCR using plasmid pUCΔglv DNA and primers GLV-creF and GLV-creR. After confirmation of the two-base replacement by sequencing, the *Eco*RI-*Sal*I fragment from pUCΔglvCR was ligated to the corresponding sites of pDHAFBLZ, resulting in pDHΔglvCR. This plasmid was linearized with *Pst*I and then used for transformation of *B. subtilis* 168. Transformants (AMGLVCR) were selected on LB medium containing chloramphenicol, and proper recombination was confirmed by PCR and amylase assaying.

Transformation of *E. coli* and *B. subtilis*. Transformation of *E. coli* cells was performed as described by Sambrook et al. (16). Conventional transformation of *B. subtilis* cells was performed according to the procedure of Anagnostopoulos and Spizizen (1).

Northern blot and primer extension analyses. RNA preparation and probe labeling were performed as described previously (34). Northern blot analysis of RNAs fractionated by electrophoresis in agarose-formaldehyde gels was performed as described by Sambrook et al. (16). Probe labeling was performed with a DIG RNA labeling kit (Roche Diagnostics) according to the manufacturer's instructions, with some minor modifications. Briefly, the internal regions inserted

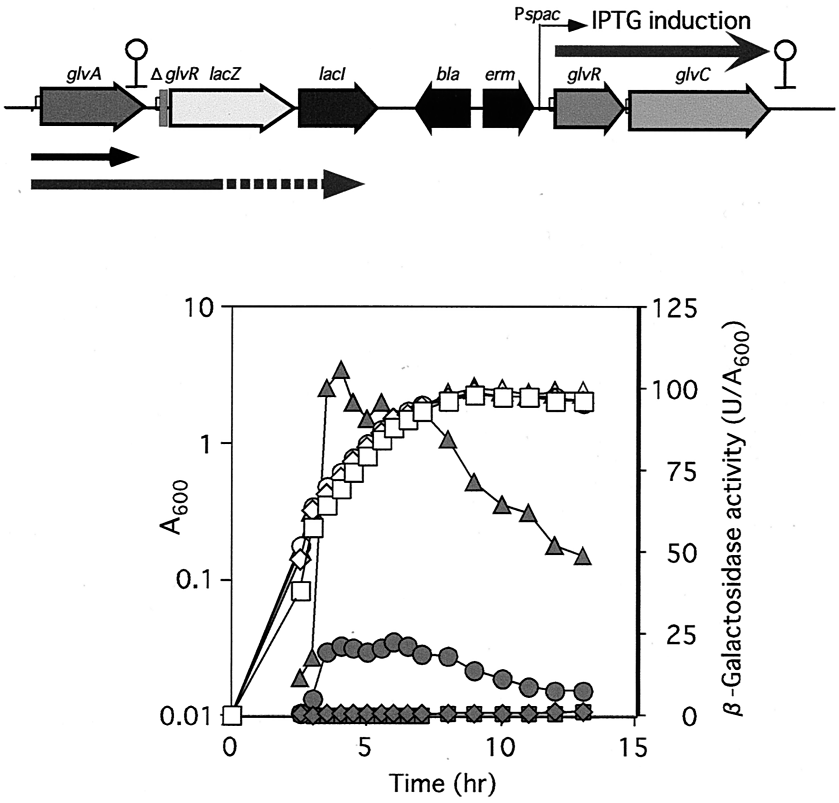

FIG. 6. β-Galactosidase activity of the *glvR-lacZ* transcriptional fusion strain (GLVR-PSP) with the intact *glvR* gene. Strain GLVR-PSP was grown in DSM with or without 1 mM IPTG and 2.5 mM maltose at 37°C. Growth (A_{600}) and β-galactosidase activity (units per A_{600}) are shown by open and filled symbols, respectively. Squares, *B. subtilis* 168 (wild type); diamonds, GLVR-PSP; circles, GLVR-PSP with IPTG; triangles, GLVR-PSP with IPTG plus maltose. A map of the *glv* operon of GLVR-PSP is shown at the top.

into pGEM-3Zf(+) derivatives (pGV1, pGVR, and pGV2) were amplified by PCR with −21M13 and M13RV as primers. The amplified fragments were digested with *Hin*dIII or *Eco*RI and then used as templates for in vitro runoff transcription with T7 or SP6 RNA polymerase, yielding probes A, R, and C, respectively. The internal region inserted into a pMUTIN derivative (pMVR-SD) was amplified by PCR with PM-FK and PM-T7 as primers. The amplified fragments were digested with *Eco*RI and then used as templates for in vitro runoff transcription with T7 RNA polymerase. Hybridization and detection were performed with a DIG luminescent detection kit (Roche Diagnostics) according to the manufacturer's instructions.

Primer extension analysis was performed as described previously (9) with an end-labeled V1-PEX primer.

β-Galactosidase assay. After shaking at 37°C, samples were withdrawn at various times to assay β-galactosidase activity. Measurement and calculation of β-galactosidase activity (expressed as units per milligram of protein or optical density at 600 nm) were carried out as described by Shimotsu and Henner (21). One unit of β-galactosidase activity was defined as the amount of enzyme necessary to release 1 nmol of 2-nitrophenol from *O*-nitrophenyl-β-D-galactopyranoside (ONPG) in 1 min at 28°C.

RESULTS

Transcriptional analysis of the *glvARC* genes of *B. subtilis*. Analysis of the *B. subtilis* genome sequence shows that *glvA, glvR*, and *glvC* are transcribed in the same direction. In addition, deduced ρ-independent terminators are located between *glvA* and *glvR* and downstream of *glvC*. *yfjA* that is transcribed in the opposite direction is located upstream of *glvA* (Fig. 1). To determine whether or not these three genes are transcribed as a polycistronic mRNA, we performed Northern blot analysis using specific probes A, R, and C for *glvA, glvR*, and *glvC*, respectively. Figure 1 shows that all three probes hybridized to a 3.8-kb mRNA at t_0 (time [0 h] after onset of sporulation) and that the *glvA*-specific probe hybridized only to a 1.4-kb mRNA throughout all growth phases (Fig. 1A). These results indicate that the 1.4-kb mRNA is a *glvA* transcript and the 3.8-kb one is a polycistronic (*glvARC*) mRNA.

Cell growth of mutants deficient in the *glv* operon. Previous results suggested that maltose is transported into cells and then metabolized through at least two systems; the PTS system related to the *glv* operon and the ABC transporter related to the *malL* gene cluster (18). Therefore, we examined the growth of organisms disrupted with respect to *glvA, glvR, glvC, malL*, and *glvA malL* in MMM (Fig. 2). The *glvA, glvR*, and *glvC* genes were disrupted by a single crossover of pMUTIN2 derivative plasmids (pMV1, pMVR, and pMV2), with the GLVAd, GLVRd, and GLVCd strains being obtained, respectively. In these mutants, the downstream genes can be expressed in the presence of IPTG. The GLVAd strain grew very poorly in MMM with or without IPTG. The MALLdd strain lacking *malL*, due to a double crossover as described in Materials and Methods, grew quite well in MMM (Fig. 2A). The GLVRd and GLVCd strains also grew very poorly in MMM with or without IPTG (Fig. 2B). Therefore, all the genes in the *glv* operon are required for growth in MMM, but *malL* is not required, although some growth inhibition was observed (Fig. 2).

Maltose induction and glucose repression of expression of the *glv* operon. The effects of maltose and glucose on *glv* ex-

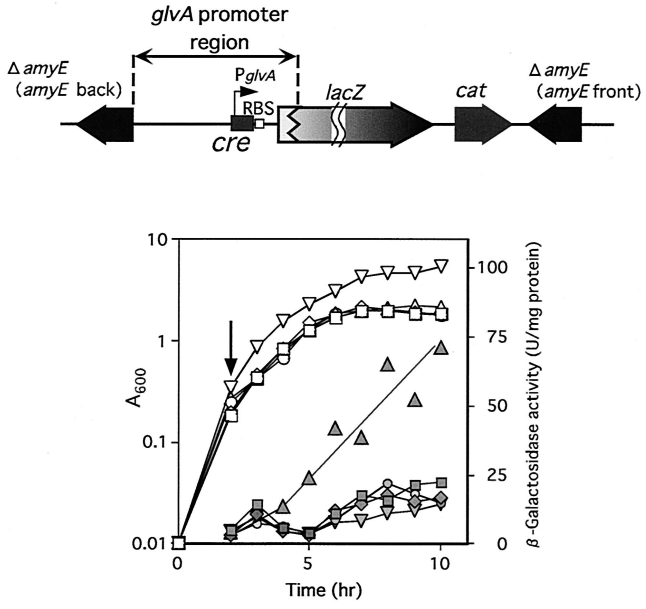

FIG. 7. β-Galactosidase activity of a P_{glvA}-*lacZ* translational fusion localized at the *amyE* locus. The AMGLV strain having the P_{glvA}-*lacZ* fusion at the *amyE* locus was transformed with a citrate-regulated *glvR* plasmid, pHYCM2VR, and then β-galactosidase activity of the transformant cultured in DSM with or without 1% glucose and 2 mM citrate was measured. Glucose was added at 0 h and citrate was added at the time indicated by an arrow. Growth (A_{600}) and β-galactosidase activity (units per A_{600}) are shown by open and filled symbols, respectively. pHYCM2 is a control plasmid without the *glvR* gene. Squares, *B. subtilis* 168(pHYCM2); diamonds, AMGLV(pHYCM2); circles, AMGLV(pHYCM2VR); triangles, AMGLV(pHYCM2VR) with citrate; inverted triangles, AMGLV(pHYCM2VR) with citrate and glucose. A map of the *amyE* locus of AMGLV is shown at the top.

pression in the wild-type strain are shown in Fig. 3. Transcripts (1.4 and 3.8 kb) were produced in DSM at $t_{0.5}$, but much stronger expression was observed for both transcripts in DSM supplemented with 2.5 mM maltose throughout the growth phase from t_{-2} to t_2. But the addition of 1% glucose completely repressed *glv* expression (Fig. 3). These results indicate that maltose is an inducer and glucose is a strong repressor of expression of the *glv* operon.

Primer extension analysis of the *glv* operon. The transcriptional start point of the *glv* operon of the wild-type strain was determined as described in Materials and Methods. The primer was designed for the sequence from +85 to +102 with respect to the 5′ end of *glvA*. The transcriptional start point was G for RNA transcripts from cells cultured in DSM supplemented with maltose at t_{-1} and $t_{0.5}$, mSMM at $t_{0.5}$, and DSM at $t_{0.5}$ (Fig. 4). We could not detect any other significant transcripts within a region 150-bp upstream of the 5′ end of *glvA*. These results suggest that the 1.4- and 3.8-kb transcripts start at the same position. The −35 region (GTTACG) and the −10 region (TATAAA), with a spacing of 18 bp, were similar to those of the σ^A consensus sequence (TTGACA for the −35 region and TATAAT for the −10 region, with a spacing of 17 bp) (Fig. 4) (5). Between the −10 region and the ribosome binding site, there is a sequence (TGTAAACGTTATCA) identical to the *cre* consensus sequence (TGWNANCGTTAT CA) (Fig. 4) (7). This sequence suggests that the *glv* operon is regulated by carbon catabolite repression through the *ccpA* gene and the *cre* sequence.

Effect of a *glvR* mutation on expression of the *glv* operon. The *glvR*-deficient disruptant (GLVRd) containing a *glvR-lacZ* transcriptional fusion was cultured in DSM with or without IPTG. A *glvA*-specific probe hybridized to the 1.4 and 3.8 kb transcripts for the wild-type strain cultured in DSM (Fig. 5A). In contrast, no significant hybridization with transcript was observed for the GLVRd culture in DSM plus 1 mM IPTG (Fig. 5A). These results indicated that GlvR is essential for transcription of the *glv* operon. The LacZ assay results for GLVRd with or without IPTG supported the above finding that there is no transcription of the *glvA* gene even with no polarity effect (Fig. 5B). Maltose failed to induce expression of the *glv* operon in the GLVRd strain. To further confirm the positive effect of GlvR on *glv* expression, a GLVR-PSP strain containing the intact *glvR* gene controlled by the *spac* promoter and a *glvR-lacZ* transcriptional fusion was constructed (Fig. 6). LacZ activity was not observed for GLVR-PSP without IPTG, but there was a significant level of LacZ activity with IPTG, and the activity considerably increased in the presence of both IPTG and maltose. These results indicated that GlvR is a positive regulator and that maltose is also required for induction of the *glv* operon.

To further investigate the glucose repression, we constructed an AMGLV strain containing the *glvA* promoter region fused with *lacZ* in the *amyE* locus (Fig. 7). Additionally, we constructed a GlvR-producing plasmid controlled by the *citM* promoter (pHYCM2VR). The *glvR* gene of pHYCM2VR was only expressed in the presence of citrate (data not shown). The AMGLV strain harboring the pHYCM2VR plasmid was cultured in DSM, followed by the addition of 2 mM citrate at t_{-2} (Fig. 7). GlvA-LacZ activity was observed on *glvR* expression due to citrate. If we added glucose to the citrate-containing AMGLV(pHYCM2VR) culture, the LacZ activity was completely repressed. This indicates that glucose represses the *glvA* operon and that the target site for the sugar is located between −240 and +32 with respect to the transcriptional start point of the *glv* operon (covering the −35 and −10 promoter regions and the translational start codon of *glvA*).

Effect of a *cre* mutation on expression of the *glv* operon. Since the target region contains the deduced *cre* sequence, we changed the *cre* sequence of the wild type to a mutated *cre* sequence (with a CG-to-AT change in the center of *cre*) (Fig. 8). The mutant strain AMGLVCR harboring pHYCM2VR expressed LacZ activity after citrate addition, and this activity was not repressed by glucose (Fig. 8). The maximal expression level in a medium containing citrate and glucose was very similar to that in a medium containing citrate, and thus the *cre* sequence was essential for glucose repression of the *glv* operon. There was a 3-h delay of *glvA* expression in the medium containing citrate and glucose (Fig. 8). Although the reason for this phenomenon has not been determined, the mutated *cre* sequence of AMGLVCR might retain weak binding for CcpA.

Effect of a *ccpA* mutation on expression of the *glv* operon. Since the *cre* sequence is known to be a target of CcpA for glucose repression (6), a *ccpA* mutation was introduced into AMCMVR containing the citrate-controlled *glvR* gene in the *amyE* locus of the chromosome. The resultant *ccpA* mutant strain, AMCMVRCC, was cultured in DSM containing 2 mM

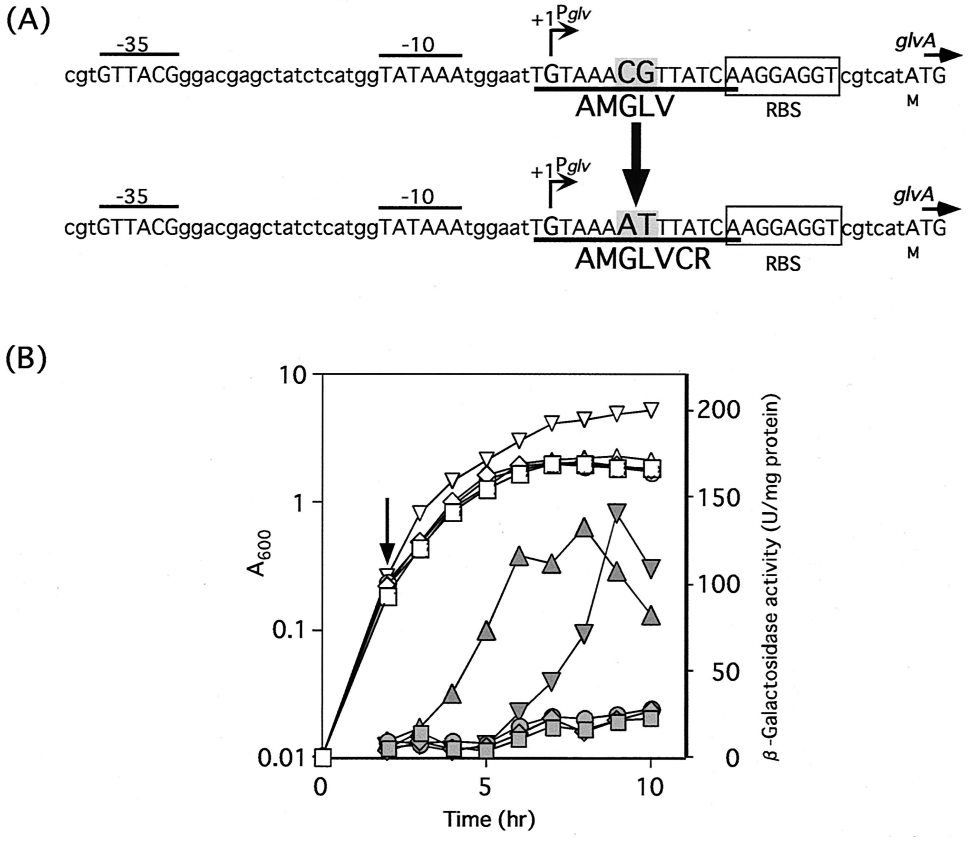

FIG. 8. The deduced *cre* and mutated *cre* sequences of AMGLV and AMGLVCR, respectively (A), and β-galactosidase activity of AMGLVCR(pHYCM2VR) (B). AMGLVCR was constructed by changing the *cre* sequence to a mutated *cre* sequence upstream of the *glvA-lacZ* fusion at the *amyE* locus. β-Galactosidase activity of AMGLVCR(pHYCM2VR) cultured in DSM with or without 1% glucose and 2 mM citrate was measured. Glucose was added at 0 h and citrate was added at the time indicated by an arrow. Growth (A_{600}) and β-galactosidase activity (units per A_{600}) are shown by open and filled symbols, respectively. Squares, *B. subtilis* 168(pHYCM2); diamonds, AMGLVCR(pHYCM2); circles, AMGLVCR(pHYCM2VR); triangles, AMGLVCR(pHYCM2VR) with citrate; inverted triangles, AMGLVCR(pHYCM2VR) with citrate and glucose.

citrate and 1% glucose, and then transcripts were submitted to Northern blot analysis (Fig. 9). AMCMVR failed to generate the 1.4- and 3.8-kb transcripts of *glv*, whereas AMCMVRCC clearly produced both transcripts. These results indicate that glucose repression of the *glv* operon requires both CcpA and the *cre* sequence.

DISCUSSION

Many *Bacillus* strains utilize maltose as sole carbon and energy source, but whether a maltose-specific phosphoenolpyruvate-PTS was present in members of this genus has long been unclear. Indeed, early studies with *Bacillus popilliae* (28) and later investigations with *Bacillus licheniformis* (27) and *B. subtilis* (26) had failed to detect maltose-PTS activity in these species. However, discovery of the three-gene *glv* operon in 1996 (36) during sequencing of the *B. subtilis* genome led to suggestions that this operon might participate in the PTS-mediated dissimilation of α-glucosides (including maltose) in *B. subtilis*. Two significant findings provided experimental support for this proposal. First, the gene *glvA* was shown to encode a unique NAD^+- and metal ion-dependent phospho-α-glucosidase that catalyzes the hydrolysis of maltose-6-phosphate (30). Second, the gene *glvC* encodes an $EII(CB)^{mal}$ component of the PTS, and mutation of this membrane protein (designated MalP by Reizer et al. [15]) severely curtailed the growth of the organism on maltose. In the present communication we have defined the regulatory function of the protein GlvR encoded by the second gene (*glvR*) in the *glv* operon. Additionally, we demonstrate that mutational inactivation of any of these genes almost abolishes growth of *B. subtilis* in the minimal medium containing maltose. A second route for transport (via an ATP-binding cassette) and metabolism of maltose is also present in *B. subtilis*, and Dahl and his colleagues have described a maltose-inducible α-glucosidase, MalL (19), whose gene is located in a large operon that also encompasses genes *yvdE* to *yvdM* (8, 19). Cells defective in *malL* grew poorly in MMM, but growth was not inhibited to the degree noted in the *glvA*-deficient organism (Fig. 2). The PTS–phospho-α-glucosidase would appear to be the more significant of the two metabolic routes for disaccharide metabolism. Inspection of the *glv* operon (Fig. 1) reveals the absence of a gene whose sugar-specific product (EIIA) is required for functional operation of all sugar-phosphotransferase systems. Interestingly, operons for the PTS-mediated translocation of both sucrose and trehalose by *B. subtilis* also lack the corresponding (and expected)

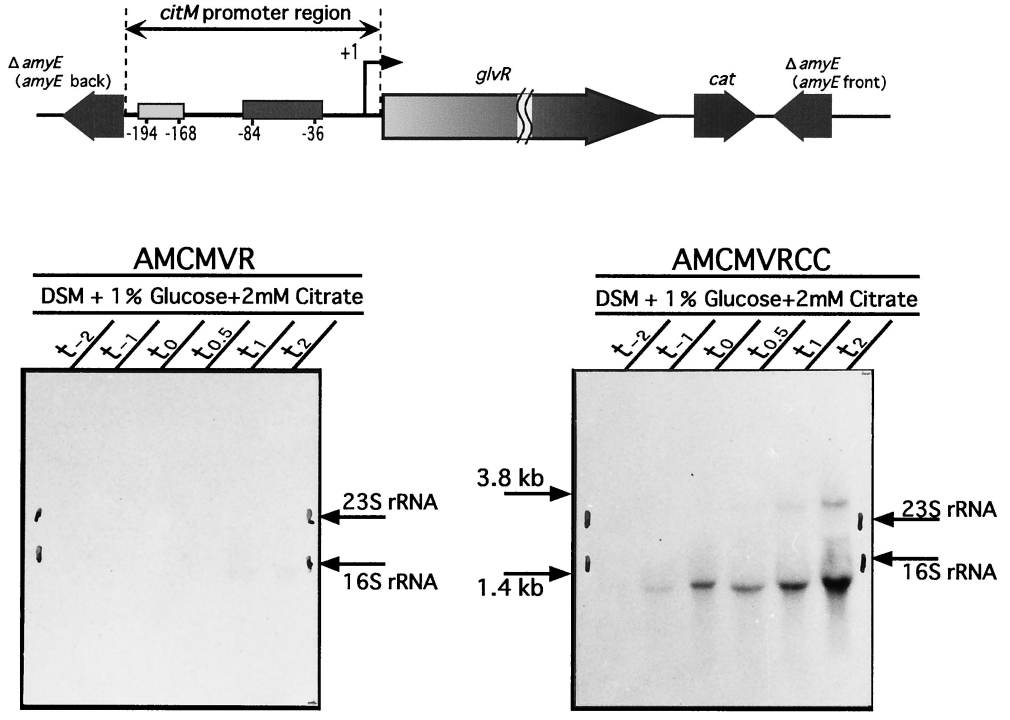

FIG. 9. Northern blot analysis of the *ccpA*$^+$ strain, AMCMVR, and the *ccpA* mutant, AMCMVRCC, in DSM with 2 mM citrate and 1% glucose. AMCMVR and AMCMVRCC contain P_{citM}-*glvR* at the *amyE* locus (top). Glucose was added at t_{-4} and citrate was added at t_{-2}. Probe A was used for Northern blotting.

disaccharide-specific EIIA genes. Evidence presented by Sutrina et al. (25) and Dahl (3) indicates that $EIIA^{glc}$ can serve as a substitute for these disaccharide-specific PTSs. A similar cross-complementation may also occur between $EIIA^{glc}$ and $EII(CB)^{mal}$ components to yield an operational maltose-PTS in *B. subtilis*.

Primer extension analysis indicated that the two transcripts (1.4 and 3.8 kb) start at the same point in the *glv* operon (Fig. 4). Only the σ^A consensus sequence was found in the −10 and −35 promoter regions, the former being highly conserved. Since the major transcript was the *glvA* transcript, it is considered that transcription mainly stopped between *glvA* and *glvR*. That the *glvARC* operon has a strong stem-loop structure ($\Delta G = -30.1$ kcal/mol) at 59 to 6 bp upstream from the *glvR* translational start point and a weaker stem-loop structure ($\Delta G = -18.6$ kcal/mol) downstream of *glvC* may be reflected in the relative amounts of the two transcripts.

Our findings indicate that the *glv* operon is autoregulated by the positive regulator GlvR, which is a potential helix-turn-helix DNA-binding protein (N-terminal amino acid residues 1 to 106; Pfam software; Sanger Centre). GlvR has the sugar isomerase (SIS) domain in the C-terminal region (residues 107 to 243; Pfam software). The SIS domain is a phosphosugar-binding domain found in many phosphosugar isomerases and phosphosugar binding proteins (22). SIS domains are also found in proteins that regulate the expression of genes involved in the synthesis of phosphosugars. It is therefore likely that maltose-6-phosphate binds to GlvR to exert a positive effect on *glvARC* transcription. The upstream region (−240 to +32 with respect to the transcriptional start point) of the *glvA* gene seems to be the target for GlvR, because the β-galactosidase activity of P_{glvA}-*lacZ* of AMGLV(pHYCM2VR) was completely dependent on the citrate-induced expression of GlvR (Fig. 7). Maltose is an inducer of the *glv* operon, and this induction may be caused by GlvR being strongly activated by the higher accumulation of maltose-6-phosphate. Actually, induction of *glvA* on the plasmid yielding the decrease of maltose-6-phosphate led to repression of the *glv* operon (data not shown). These proposed mechanisms for regulation of the *glv* operon are illustrated in Fig. 10. The role of the *malL* operon for maltose metabolism is not presented in Fig. 10, because the precise function(s) and contributions of this system in *B. subtilis* have yet to be resolved.

The *cre* sequence (TGTAAACGTTATCA), which is completely identical to the consensus sequence, located between the −10 sequence and a ribosome-binding site of the *glv* operon was important for catabolite repression by glucose (Fig. 8). A CG-to-AT change in the center of *cre* was made in the AMGLVCR strain. Expression of the *glv* operon in the mutated *cre* strain was not severely affected by glucose (Fig. 8). The lack of CcpA also led to expression of the *glv* operon even in the presence of glucose (Fig. 9). Therefore, glucose repression of the *glv* operon is mediated by CcpA and *cre*. Recently, Marino et al. (10) reported the two-dimensional gel electrophoretic patterns of proteins formed during adaptation of *B. subtilis* under the shift from aerobic to anaerobic conditions. Together with proteins of inositol and melibiose operons, GlvA was induced during this transition. The *glv* operon is also regulated through anaerobic stress, but the molecular basis for this response has yet to be defined.

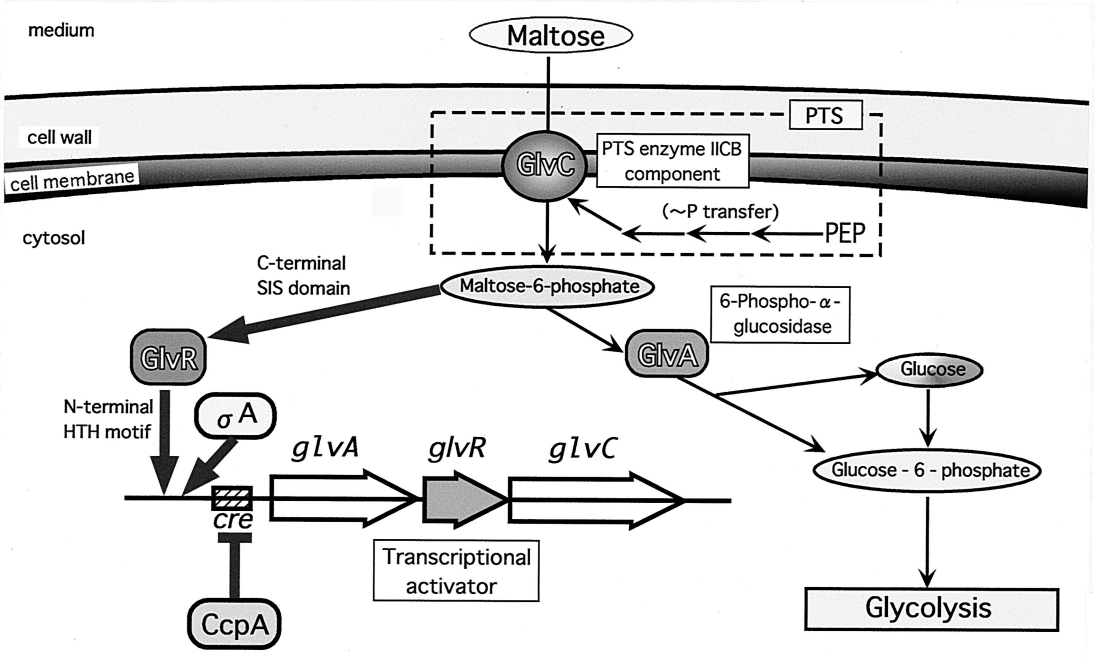

FIG. 10. Illustration of proposed mechanisms of PTS-dependent maltose transport and metabolism. Maltose is also incorporated into cells via an ABC transporter, whose system is explained with the PTS system in Discussion. Thin arrows indicate the metabolic pathway, and thick arrows and perpendicular ones indicate positive and negative controls, respectively. PEP, phosphoenolpyruvate; HTH, helix-turn-helix.

Our descriptions of the genetic, biochemical, and regulatory components of the *glv* operon in *B. subtilis* provide the first unequivocal evidence for the PTS-catalyzed metabolism of maltose in any bacterial species. However, the recent discovery of homologous genes for both PTS proteins and NAD^+- and metal-dependent phospho-α-glucosidase in such diverse organisms as *Fusobacterium mortiferum* (2, 29), *Klebsiella pneumoniae* (31), and *Clostridium acetobutylicum* (Thompson et al., unpublished data) suggests that the maltose (α-glucoside)-PTS may be considerably more widespread than is presently envisaged.

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