

Gene Organization and Transcription Analysis of the *Agrobacterium tumefaciens* Glycogen (*glg*) Operon: Two Transcripts for the Single Phosphoglucomutase Gene

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The gene organization and transcription of the *Agrobacterium glg* operon differ from those in other bacteria. *Agrobacterium tumefaciens* A348 contains a 9.1-kb gene cluster harboring genes for glycogen metabolism. The nucleotide sequence and gene organization of a region containing ADP-glucose pyrophosphorylase (*glgC*), glycogen synthetase (*glgA*), and phosphoglucomutase (*pgm*) genes have been previously described (A. Uttaro and R. A. Ugalde, *Gene* 150:117–122, 1994). In this work we report that the glycogen phosphorylase (*glgP*) and branching enzyme (*glgB*) genes are located immediately upstream of this region. The complete nucleotide sequences of the *glgP* and *glgB* genes were obtained, and mutants were constructed by targeted insertional mutagenesis with a kanamycin cassette. Enzymatic assays and reverse transcription PCR carried out with the wild type and with *glgP* and *glgB* mutants, as well as primer extension experiments and β -galactosidase fusions, revealed that this region containing five open reading frames (*glgPBCA* and *pgm*) is transcribed unidirectionally as a single operon under the control of a promoter located upstream of the glycogen phosphorylase gene (*glgP*). An alternative transcript was identified starting 168 bp upstream of an internal ATG start codon of the *pgm* gene, which is translated as a 71-amino-acid-shorter Pgm protein which complements in vivo a *pgm* mutant. This alternative transcript has a promoter with the motif TATCAAN₅G, identified in octopine Ti plasmid as an autoinducible TraR promoter. This promoter is >200 times more efficient in *A. tumefaciens* than in *Escherichia coli*, as judged by the level of enzymatic activity of a *lacZ-pgm* fusion.

Glycogen is produced and accumulates in many bacteria. Although it is known that this polysaccharide is used as a stored source of energy, the precise role that it may play in bacteria is still not clear. *Escherichia coli* glycogen synthetase mutants have no relevant growth phenotype. However, some authors have suggested that the accumulation of glycogen may give advantages under starvation conditions, providing a stored source of energy (23).

The reactions that lead to the synthesis of glycogen in bacteria have been extensively studied (23, 24). ADP-glucose provides the donor sugar nucleotide, whose synthesis is catalyzed by the enzyme ADP-glucose pyrophosphorylase (EC 2.7.7.27). The glucosyl moiety of ADP-glucose is transferred, in a reaction catalyzed by a specific ADP-glucose-glycogen synthetase (EC 2.4.1.21), to either a maltodextrin or a glycogen primer to form a new α -1,4-glucosidic bond. Subsequently, a branching enzyme (EC 2.4.1.18) catalyzes the formation of branched α -1,6-glucosidic linkages. All these reactions were observed to occur in extracts of more than 40 species of bacteria (25). The metabolic pathway that leads to the release of the energy stored in glycogen starts with the enzyme glycogen phosphorylase (EC 4.4.1.1), which releases glucose-1-phosphate from the nonreducing terminus of the α -1,4 chain (9).

The genetic organization of the glycogen operon (*glg*) was determined in *E. coli* (28, 38), *Bacillus stearothermophilus* (34), and *Bacillus subtilis* (18). The *glg* operon is located at approximately 75 min on the *E. coli* K-12 chromosome map (25). The

arrangement and nucleotide sequence of the entire *glg* cluster revealed that a continuous DNA fragment of over 15 kb flanked by the genes *asd* (17) and *glpD* (1) contains the genes encoding the branching enzyme (*glgB*), ADP-glucose pyrophosphorylase (*glgC*), and glycogen synthetase (*glgA*) and two genes, *glgX* (homologous to genes encoding α -amylases) and *glgP* (homologous to the rabbit glycogen phosphorylase gene) (25, 28). None of the latter genes are required for glycogen synthesis, but they are needed for glycogen metabolism. Detailed inspection of the organization of the *E. coli glg* cluster suggests that *glg* genes may be transcribed as two operons, *glgBX* and *glgCAP* (25). The coding regions of the *glgB* and *glgX* open reading frames (ORFs) overlap by 1 bp. ORFs *glgC* and *glgA* are separated by 2 bp, and *glgA* and *glgP* are separated by 18 bp. This close proximity suggests translational coupling of the two operons *glgBX* and *glgCAP* (25).

The *glgCAP(Y)* operon is under the positive control of cyclic AMP (cAMP) and the cAMP receptor protein (CRP); both the *cya* gene, encoding adenylate cyclase (EC 4.6.1.1), and *crp*, encoding CRP, are required for optimal synthesis of glycogen (9). CRP binds to a site located upstream of the *glgC* gene, and consensus DNA sequences between the *E. coli* and *Salmonella typhimurium glgC* upstream regions were found (25). Glycogen synthesis in *E. coli* is also positively regulated by ppGpp, which stimulates the transcription of the *glgCAP* operon; neither ppGpp nor CRP affects the transcription of the *glgB* gene (25).

In *E. coli*, glycogen synthesis is also down-regulated at the level of transcription (25). Regulatory mutants designated *glgR* and *glgQ* mutants were identified (29).

The organization of the *glg* operons in *B. stearothermophilus* and *B. subtilis* is different from that in *E. coli*. In *B. subtilis* the operon is located downstream of *trnB*, which maps at 275 min

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or characteristics	Reference or source
Strains		
<i>E. coli</i> K-12		
DH5 α -F'IQ	F' ϕ 80d <i>lacZ</i> Δ M15 Δ (<i>lacZYA-argF</i>)U169 <i>deoR recA1 endA1 hsdR17</i> ($r_K^- m_K^+$) <i>phoA supE44</i> λ^- <i>thi-1 gyrA96 relA1/F' proAB⁺ lacI^qZ</i> Δ M15 <i>zfp::Tn5</i> [Km ^r]	37
DH5 α	F' ϕ 80d <i>lacZ</i> Δ M15 Δ (<i>lacZYA-argF</i>)U169 <i>deoR recA1 endA1 hsdR17</i> ($r_K^- m_K^+$) <i>phoA supE44</i> λ^- <i>thi-1 gyrA96 relA1</i>	14
XL1-Blue MRF'	Δ (<i>mrcA</i>)183 Δ (<i>mcrCB-hsd SMR-mrr</i>)173 <i>endA1 supE44 thi-1 recA1 gyrA96 relA1 lac</i> [F' <i>proAB lacI^qZ</i> Δ M15 Tn10(Tet ^r)]	3
<i>A. tumefaciens</i>		
A348	Wild type, pTiA6 Rif ^r Cm ^r	12
A1120	<i>glgB::Km^r</i>	This study
A1121	<i>glgP::Km^r</i>	This study
A5129	<i>pgm::Tn5</i>	35
Plasmids		
pFC6251	30-kb fragment of genomic DNA of A348 in pVK102 that complements <i>exoC</i> mutant	4
pXH11	1.1-kb <i>XhoI-HindIII</i> fragment of pFC6251 containing 5' end of <i>glgB</i> and 3' end of <i>glgP</i>	35
pBH26	2.6-kb <i>BamHI-HindIII</i> fragment of pFC6251 containing <i>glgB</i> in pBluescript II KS (+)	This study
pBH26K	pBH26, <i>glgB::Km^r</i>	This study
pBB3	3-kb <i>BglII-BamHI</i> fragment of pFC6251 containing <i>glgP</i> in pBluescript II KS (+)	This study
pBB3K	pBB3K, <i>glgP::Km^r</i>	This study
pFus96	<i>lacZ</i> in pBBR1MCS-2	This study
pFus97	Translational fusion <i>pgm-lacZ</i> in pBBR1MCS-2	This study
pFus98	Translational fusion <i>glgP-lacZ</i> in pBBR1MCS-2	This study
pPGM1	756-bp fragment from bases 7768 to 8522 corresponding to 5' end of <i>pgm</i> product in pGEX2	This study
pCC15	1,642-bp fragment from bases 7733 to 9375 corresponding to <i>pgm2</i> in pBBR1MCS-4	This study
pBBR1MCS	Broad-host-range cloning vector (Km ^r or Amp ^r)	19
pAB5002	Vector for construction of translational fusions with β -galactosidase (Amp ^r Gm ^r)	2a

on the chromosome. The operon *glgBCDAP* has extensive homologies to genes encoding enzymes involved in glycogen and starch metabolism in both prokaryotes and eukaryotes (18). *glgD*, not present in other bacteria, has high homology to *glgC*. This operon is presumably expressed under the control of a sporulation promoter. The same operon organization was described for *B. stearothersmophilus* (34). Purification of ADP-glucose pyrophosphorylase (EC 2.7.7.27) demonstrated that it was heterotetrameric, formed by the GlgC and GlgD proteins (33). In this bacterium, as is the case in *B. subtilis*, the operon is preceded by a sporulation promoter (34).

The genes coding for ADP-glucose pyrophosphorylase, glycogen synthetase, and phosphoglucomutase in the plant pathogenic bacterium *Agrobacterium tumefaciens* were found to form a continuous cluster on the chromosome (35). Nucleotide sequence analysis of this cluster revealed that it is transcribed in the same orientation with no intergenic region, suggesting that it might be transcribed as a single operon (35).

In this work, we show that the genes encoding five enzymes (glycogen phosphorylase, branching enzyme, ADP-glucose pyrophosphorylase, glycogen synthetase, and phosphoglucomutase) are transcribed from a single operon in *A. tumefaciens*. However, an independent transcript for *pgm* was identified that is translated as a Pgm protein 71 amino acids shorter than the protein produced by the polycistronic messenger. The sequences of glycogen phosphorylase and branching-enzyme genes were obtained, completing the information for the whole *glg* operon in *A. tumefaciens*.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1.

Cloning, DNA sequencing, and gene disruption. Plasmid pFC6251 was digested with *HindIII* and *BglII* restriction enzymes, and a 2.6-kb fragment con-

taining the *glgB* gene was recovered and ligated into pBluescript II KS (+) digested with *BamHI* and *HindIII*, in order to eliminate the *BamHI* site in the polylinker of the recombinant plasmid. In the resulting recombinant plasmid, pBH26, a kanamycin cassette (22) was ligated into a unique *BamHI* site. Plasmid pBH26::Km (pBH26K in Table 1) was recovered and electroporated into the *A. tumefaciens* A348 wild-type strain. Double recombination events (Km^r Cb^r) were selected and confirmed by PCR with a set of primers that amplifies a 500-bp DNA fragment from the wild type and a 1.8-kb DNA fragment from the double recombinant. A mutant clone named A1120 was selected for further studies.

In order to clone and mutagenize the *glgP* gene, plasmid pFC6251 was digested with *BglII* and *BamHI* and a 3.0-kb DNA fragment that hybridized with a 0.6-kb probe containing the 3' end of the *glgP* gene was isolated. This 3.0-kb DNA fragment was ligated into pBluescript II KS (+) digested with *BamHI* to obtain plasmid pBB3. In order to eliminate the *PstI* site of the pBluescript II KS (+) polylinker, pBB3 was digested with *BamHI* and *EcoRV*, filled in, and religated. The kanamycin cassette was introduced into a unique *PstI* site of the *glgP* gene (Fig. 1), the plasmid was transformed into *E. coli* and selected with kanamycin (50 μ g/ml), and then pBB3::Km (pBB3K in Table 1) was recovered and electroporated into the *A. tumefaciens* A348 wild-type strain. Double recombination events were selected (Km^r Cb^r) and confirmed by PCR with a set of primers that amplified a fragment of 400 bp from the wild-type gene and a 1.7-kb fragment from the kanamycin interrupted gene. A mutant clone named A1121 was selected for further studies. Both strands of the *glgP* and *glgB* genes and flanking DNA regions were sequenced by the dideoxy terminator method as described elsewhere (33).

Construction of pCC15 and complementation of *A. tumefaciens* A5129. For the construction of the recombinant plasmid pCC15, a PCR was carried out with oligonucleotides 5'-CGGGATCCATGATCAAGACTATCAAGAC-3' (positions 7733 to 7753) and 5'-AACTGCAGCGGGCGGACGTTATCAGGTA-3' (positions 9355 to 9375), having *BamHI* and *PstI* sites (underlined) in their 5' ends. This PCR amplified a DNA fragment of 1,642 bp, spanning from the ATG₁ (Fig. 1) to the stop codon of the *pgm* gene. PCR product was digested with *BamHI* and *PstI* and ligated into pBBR1MCS-4 (19) digested with the same enzymes. This vector replicates in *Agrobacterium* and confers resistance to carbenicillin. It is worth noting that *pgm* was cloned in the opposite direction with respect to the transcription of the endogenous promoter of the multiple cloning site of pBBR1MCS-4. Recombinant plasmid pCC15 was introduced into *A. tumefaciens* A5129 by electroporation, and cells were plated on Luria-Bertani (LB) agar, containing carbenicillin (100 μ g/ml), kanamycin (50 μ g/ml), and Calcofluor (0.02%). Screening for complementation was carried out by searching for bright colonies under UV light.

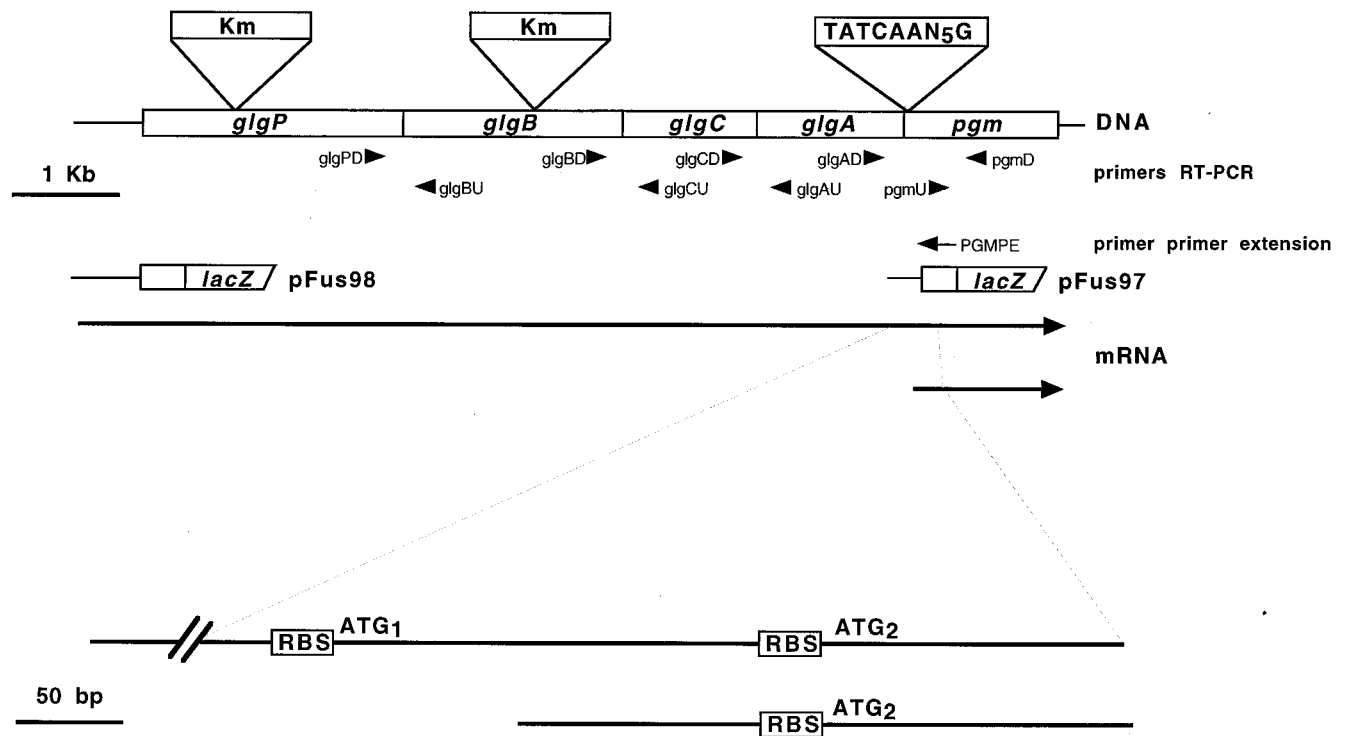


FIG. 1. Scheme of *A. tumefaciens* *glg* operon organization and transcription. The genomic arrangement with the positions of the kanamycin cassette insertions and the putative alternative *pgm* promoter are shown in the upper part. Oligonucleotides used for RT-PCRs and the primer extension experiment are indicated by arrows. The DNA regions used for the construction of β -galactosidase fusion plasmids pFus98 and pFus97 are shown. The transcriptional organization of the operon, indicating the polycistronic mRNA and the alternative *pgm2* mRNA (different scale), is shown in the lower part of the figure. RBS, Shine-Dalgarno conserved sequences; ATG₁ and ATG₂, initiation codons of Pgm1 and Pgm2, respectively.

RT-PCR experiments. RNA was extracted from 10-ml stationary-phase cultures by a previously described protocol (2). RNA was treated with DNase, RNase free (Promega, Madison, Wis.), before use. Reverse transcription-PCRs (RT-PCRs) were carried out with primers glgPD (5'-CAGCGACTGGTTCATGGT-3') (positions 2609 to 2626) and glgBU (5'-AATTTCCGTCCAGCGTC A-3') (positions 2970 to 2987) for the *glgP-glgB* region, glgBD (5'-GCACGGC GCCTGGTGAAAAA-3') (positions 4749 to 4768) and glgCU (5'-AATGTCG AAGCTTCGTTAC-3') (positions 5280 to 5299) for the *glgB-glgC* region, glgCD (5'-GCCGAGTGTGAAGATCGGGCG-3') (positions 6100 to 6120) and glgAU (5'-CGCCGCAAAACGCTTCCAGT-3') (positions 6605 to 6624) for the *glgC-glgA* region, glgAD (5'-AAATGCAGAACTCGGAATG-3') (positions 7631 to 7650) and pgmD (5'-GACGTCGTCATAAAGCTCT-3') (positions 8502 to 8521) for the *glgA-pgm* region, and pgmU (5'-AAAGATCACCGACG CGATCTA-3') (positions 8140 to 8160) and pgmD for the amplification of an internal *pgm* gene region (Fig. 1).

Construction of *lacZ* fusions. A *pgm-lacZ* fusion (pFus97) was constructed by amplifying by PCR with primers glgAD and pgmD (Fig. 1) a 872-bp DNA fragment, with cosmid pFC6251 as the template. The amplified fragment was ligated into pGEMT-easy (Promega Corporation) and transformed into *E. coli*. The recombinant plasmid was recovered, digested with *NotI* and *SalI*, and ligated into pAB5002 (Table 1). This construct leaves a DNA region of 315 bp upstream of the second ATG and the sequence for the first 104 amino acids encoded by *pgm2* (Fig. 1) in frame with the promoterless *lacZ* of pAB5002. From this plasmid a 5.5-kb fragment, containing the *pgm-lacZ* fusion and *accI*, was recovered by digesting with *XbaI* and *XhoI* and ligated into pBBR1MCS-2 (19). The resulting plasmid, named pFus97, was recovered from *E. coli* and electroporated into the *A. tumefaciens* A348 wild-type strain. The *glgP-lacZ* fusion (pFus98) consists of a 1.1-kb *XbaI-SalI* DNA fragment obtained from plasmid pBB3 (Table 1) cloned into pAB5002. This construct leaves a region of 650 bp upstream of the *glgP* ATG initiation codon with the sequence for the first 146 amino acids in frame with the promoterless *lacZ* of pAB5002. The subsequent steps for constructing pFus98 were the same as those used for pFus97. The control fusion plasmid pFus96 was constructed by cloning the *XbaI/XhoI* fragment obtained from pAB5002 in pBBR1MCS-2.

Primer extension. The transcription start site was determined by primer extension analysis (32). Experiments were carried out with total RNA (20 μ g) obtained from stationary-phase cultures of the *A. tumefaciens* A348 wild-type strain as described above. The synthetic oligonucleotide PGMPE (5'-ACTGGA

TGAAGTCTCTCGG-3') (positions 7830 to 7847 of the glycogen operon) was used as a primer (Fig. 1). The primer was labeled with [γ -³²P]ATP catalyzed by T4 kinase (32). For primer-RNA annealing, the samples were heated at 90°C for 4 min, left standing at room temperature for 5 min, and then put in an ice bath. Synthesis of cDNA employing avian myeloblastosis virus reverse transcriptase (Promega Corporation) was carried out at 42°C for 1 h, and the reaction was stopped by heating at 99°C for 5 min. Products were subjected to polyacrylamide gel electrophoresis in parallel with the sequencing ladder.

Determination of enzymatic activities. Cells from stationary-phase cultures were harvested by centrifugation at 10,000 \times g for 20 min and resuspended (2 ml of buffer per g [wet weight] of cells) with 40 mM Tris-acetate buffer (pH 7.5)-5 mM EDTA-5 mM dithiothreitol (Sigma Chemical Co., St. Louis, Mo.). Lysozyme (2 μ g/ μ l; Sigma Chemical Co.) was added. After incubation for 1 h at 4°C, the cells were disrupted by three compression-decompression cycles in a French press and 2 mM (final concentration) phenylmethylsulfonyl fluoride (Sigma Chemical Co.) was added. Extracts were centrifuged for 15 min at 11,000 \times g, and the supernatants (crude extracts) were used for enzymatic assays.

(i) **Branching enzyme (GlgB).** Assays for GlgB were carried out as described elsewhere (16), based on the ability of crude extracts to stimulate the formation of glycogen from glucose-1-phosphate (Glc-1-P) as the donor substrate by rabbit phosphorylase.

(ii) **ADP-glucose pyrophosphorylase (GlgC).** Assays for GlgC were carried out following the synthesis of ATP as described previously (13).

(iii) **Glycogen synthetase (GlgA).** Assays for GlgA were carried out following the synthesis of [¹⁴C]glycogen by using ADP-[¹⁴C]glucose as the substrate as described elsewhere (8).

(iv) **Phosphoglucomutase (Pgm).** Assays for Pgm were carried out in a coupled reaction following the reduction of NADP at 340 nm as described previously (27).

(v) **Glycogen phosphorylase (GlgP).** Assays for GlgP were carried out as described elsewhere (5), with some modifications. The reaction mixtures contained crude extract, in a final volume of 0.1 ml; 100 mM Na-citrate, pH 6; and glycogen (1.5 mg/ml). The reaction was started by the addition of 49.6 mM [¹⁴C]Glc-1-P (30 μ Ci/mmol), and the mixture was incubated for 45 min at 37°C. The reaction was stopped by heating at 100°C for 1 min, 1% glycogen was added as a carrier, and the mixture was precipitated with 75% methanol-1% KCl. The precipitates were washed three times with methanol-KCl and then resuspended

in water, and the radioactivity incorporated into glycogen was counted in a liquid scintillator.

(vi) **β -Galactosidase assays.** β -Galactosidase assays were carried out with whole cells as described elsewhere (32).

Preparation of phosphoglucomutase antibodies. A 765-bp region spanning positions 7768 to 8522 of the *glg* operon was amplified by PCR and cloned into plasmid pGEX-2T (Pharmacia-Biotech, Uppsala, Sweden). The recombinant plasmid pPGM1 (Table 1) was introduced into *E. coli* DH5 α and induced with isopropyl- β -D-thiogalactopyranoside, and inclusion bodies were subjected to polyacrylamide gel electrophoresis. Gels were stained with Coomassie brilliant blue R-250 (0.05% in water), and the recombinant protein was cut out of the gel and used to prepare rabbit antibodies by a standard protocol of immunization.

Western blot analysis. Cultures (500 ml) of the *A. tumefaciens* wild-type strain A348, the *pgm* mutant A5129, and the *glgB* mutant A1120 were grown until stationary phase and harvested by centrifugation. Cell pellets were resuspended in 50 mM Tris-HCl buffer, pH 8.2, with 3 mM EDTA, 20% sucrose, 1 mM phenylmethylsulfonyl fluoride, and 200 μ g of lysozyme per ml and incubated for 1 h at 4°C. Cells were recovered by centrifugation, resuspended in 50 mM Tris-HCl (pH 8.2)–20% sucrose–10 mM MgCl₂–20 μ g of DNase per ml and sonicated. Supernatants obtained after centrifugation for 30 min at 15,000 \times g were salted out with (NH₄)₂SO₄ at a final saturation of 30%, and the pellets were dissolved in 50 mM Tris-HCl (pH 8.2)–5% glycerol–3 mM β -mercaptoethanol and dialyzed overnight against the same buffer. Samples (30 μ g of protein) were subjected to 8% polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes, and subjected to Western blot analysis using anti-Pgm antibodies. Blots were developed afterwards with anti-rabbit immunoglobulin G-peroxidase (DAKO, Glostrup, Denmark).

Nucleotide sequence accession number. The sequence comprising the genes for the *A. tumefaciens* glycogen phosphorylase (*glgP*) and branching enzyme (*glgB*) and the complete *glg* operon has been assigned GenBank accession no. AF033856.

RESULTS

Nucleotide sequences of the branching enzyme (*glgB*) and glycogen phosphorylase (*glgP*) genes. Sequence analysis of the region located upstream from the *A. tumefaciens* A348 ADP-glucose pyrophosphorylase (*glgC*) gene (34) revealed the presence of two contiguous ORFs (ORF1 and ORF2) coding for two proteins, one 46.4% identical to the *E. coli* glycogen phosphorylase (EC 2.4.1.1) and the other 56% identical to the *E. coli* branching enzyme (EC 2.4.1.1.8). The complete organization of the *A. tumefaciens* glycogen operon, as deduced from the nucleotide sequence data, is shown in Fig. 1. The data shown in this figure were partially taken from our previous publication (35) and the results described below.

Comparison of the amino acid sequences deduced from ORF1 and ORF2 with GlgP and GlgB protein sequences. *A. tumefaciens* ORF1 encodes a protein of 821 amino acids which is 46.4% identical to the *E. coli* glycogen phosphorylase protein, 43.8% identical to *E. coli* maltodextrin phosphorylase, and 43.2% identical to rat glycogen phosphorylase. Four of the eight glycogen storage sites described elsewhere (6) are conserved in the four proteins (residues Q394, N400, I424, and E426) (data not shown). Residue Y397 is not conserved in *E. coli* glycogen GlgP and *E. coli* maltodextrin GlgP, residues L404 and S422 are not conserved in *E. coli* maltodextrin GlgP, and residue S420 is not conserved in any of the phosphorylases. Seven regions of the protein were identified as belonging to the active site (6); five of them are conserved in *A. tumefaciens* GlgP. The region between residues Y282 and T287 contains two nonconservative changes in the *E. coli* and rat GlgP proteins. Sequences from A663 to G671 are conserved in rat GlgP but have one nonconservative change in the *E. coli* GlgP proteins (M665 replaced by T in glycogen phosphorylase and M665 replaced by K in maltodextrin phosphorylase).

A. tumefaciens ORF2 encodes a protein of 734 amino acid residues which is 56% identical to the *E. coli* *glgB* gene product. The four putative active-site residues H352, D417, H537, and D538 are all conserved (21). The regions with similarity among α -amylases, α -glucosidases, and other glucantransferases are also highly conserved. The percentages of identity between

TABLE 2. Enzymatic activities of *A. tumefaciens* wild-type and mutant strains

Strain	Enzyme activity (%) ^a					Colony color ^b
	GlgP	GlgB	GlgC	GlgA	Pgm	
A348	100	100	100	100	100	+
A1120	100	0	0	1.47	26	– ^c
A1121	0.88	0	0	1.57	24	–
A5129	100	ND ^d	74	100	0	–

^a Enzymatic activities were determined as described in Materials and Methods and expressed as percentages of the wild-type strain activity. Wild-type activities (in units per milligram of protein) were as follows: glycogen phosphorylase (GlgP), 0.0047; branching enzyme (GlgB), 0.021; ADP-glucose pyrophosphorylase (GlgC), 0.0156; glycogen synthase (GlgA), 0.091; and phosphoglucomutase (Pgm), 0.041. One unit of activity is defined as follows: for GlgB, the amount of enzyme that stimulated rabbit phosphorylase at 1 μ mol/min; GlgC, the amount of enzyme that catalyzed the formation of 1 μ mol of ATP per min; GlgA, the amount of enzyme that catalyzed the incorporation of glucose into insoluble glycogen at 1 μ mol/min; Pgm, the amount of enzyme that catalyzed the formation of 1 μ mol of NADPH per min, and GlgP, the amount of enzyme that catalyzed the incorporation of 1 μ mol of Glc-1-P per min into insoluble glycogen.

^b Color of the colonies was detected after exposing a petri dish culture to iodine solution (30). *Agrobacterium* was grown at 28°C in AB medium (7) for 48 h, iodine solution was poured into the plate, and the color was scored after 2 to 5 min. +, brown color; –, no color.

^c A pale green color developed.

^d ND, not determined.

A. tumefaciens ORF2 and *E. coli* *glgB* are 80% for residues D307 to G316, 75% for residues T321 to G329, 90% for residues G343 to F353, 88% for residues W405 to M422, 90% for residues M466 to W475, and 100% for residues E529 to K544.

The homologous proteins were aligned by the program DNASTAR by using the algorithm developed by Lipman and Pearson (20).

The five *glg* genes form a single operon. Insertional mutagenesis of *glgP* and *glgB* genes and enzymatic activity assays were carried out as described in Materials and Methods. A kanamycin cassette was introduced into a unique *Pst*I site of the *glgP* gene and into a unique *Bam*HI site of the *glgB* gene (Fig. 1). *A. tumefaciens* mutants A1120 (*glgB*::Km^r) and A1121 (*glgP*::Km^r) were obtained. Crude extracts were prepared from stationary-phase cultures of the wild-type strain A348 and mutant strains A1120, A1121, and A5129 (*pgm* mutant) (35). The activities of glycogen phosphorylase (EC 2.4.1.1), branching enzyme (EC 2.4.1.18), ADP-glucose pyrophosphorylase (EC 2.7.7.27), glycogen synthetase (EC 2.4.1.21), and phosphoglucomutase (EC 2.7.5.1) were determined as described in Materials and Methods. All strains were also scored for the accumulation of glycogen by the iodine assay (30). Table 2 shows that the mutant strain A1121 (*glgP*::Km^r) displayed a polar effect on the enzymatic activity of downstream mapping genes *glgB*, *glgC*, and *glgA*, with no detectable activity of branching enzyme, ADP-glucose pyrophosphorylase, or glycogen synthetase. This mutation, however, had only a partial effect on phosphoglucomutase activity (24% of the wild-type activity). Mutant A1120 (*glgB*::Km^r) had wild-type phosphorylase activity and a polar effect on the enzymatic activities of downstream mapping genes *glgC* and *glgA*. This mutant also displayed a partial polar effect on phosphoglucomutase (26% of wild-type activity). These results revealed that the region containing *glgP*, *glgB*, *glgC*, *glgA*, and *pgm* genes is organized as a single operon transcribed from *glgP* to *pgm*. The fact that mutant strains A1121 (*glgP*::Km^r) and A1120 (*glgB*::Km^r) had only a partial polar effect on *pgm* activity suggested that this downstream gene might be transcribed as part of this operon and also as a separate transcript. In order to further analyze the latter pos-

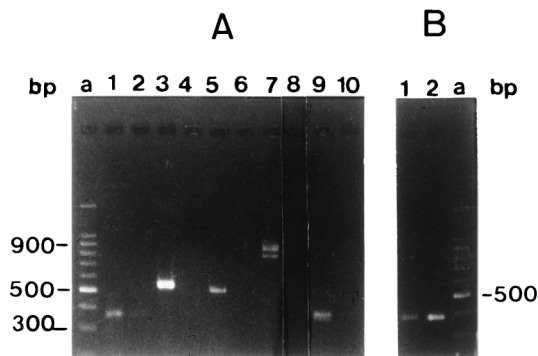


FIG. 2. RT-PCRs carried out with total RNA obtained from wild-type A348 and A1120 (*glgB*) mutant strains. (A) Reactions carried out with total RNA of wild-type strain A348. Lane 1, *glgP-glgB* intergenic region; lane 3, *glgB-glgC* intergenic region; lane 5, *glgC-glgA* intergenic region; lane 7, *glgA-pgm* intergenic region; lane 9, internal region of *pgm* gene. Lanes 2, 4, 6, 8, and 10 represent control reactions carried out without the addition of reverse transcriptase. Lane a, molecular size markers. (B) Reactions carried out with total RNA of strain A1120 (*glgB* mutant). Lane 1, *glgP-glgB* intergenic region; lane 2, internal region of *pgm* gene. Lane a, molecular size marker. All the other intergenic regions gave negative results (data not shown).

sibility, RT-PCR, primer extension experiments, and β -galactosidase fusions were carried out as described below.

Two possible transcripts for the *pgm* gene. The nucleotide sequence of the entire *A. tumefaciens glg* region, as well as enzymatic assays of *glg* enzymes in two mutants obtained by gene disruption with a kanamycin cassette, suggested that in *A. tumefaciens* the glycogen genes *glgP*, *glgB*, *glgC*, *glgA*, and *pgm* form a single operon. In order to confirm these results, RT-PCR experiments were carried out as described in Materials and Methods. Fig. 2B shows that, when RNA extracted from mutant A1120 (*glgB::Km^r*) was used as the template, the expected product was amplified after RT-PCR with primers homologous to the 3' and 5' ends of the coding regions of *glgP* and *glgB* genes, respectively. On the other hand, no products were recovered after amplification with primers homologous to the 3' and 5' ends of the coding regions of *glgB-glgC*, *glgC-glgA*, or *glgA-pgm* genes (data not shown), thus indicating that the specific mRNA was absent in this mutant strain. However, when the amplification reaction was carried out with a set of primers internal to the *pgm* gene, an amplified product of the expected size (384 bp) was obtained (Fig. 2B). These results are consistent with the fact that a decreased but detectable level of Pgm activity was present in this mutant (Table 2), and with our previous observation that a plasmid (pH21) containing a DNA fragment expanding the *glgA* 5' region and the *pgm* gene complemented a Tn5 *pgm* mutant (A5129 [35]) (Table 2). Control RT-PCRs carried out with wild-type RNA as the template produced the expected amplified products (Fig. 2A). These results suggested that the *Agrobacterium glg* operon is transcribed as a single mRNA containing the five genes *glgPBCA* and *pgm*, and that an alternative promoter might produce an mRNA leading to an active Pgm protein.

Identification of two promoters in the *glg* operon. In order to study the activity of the regions identified by sequencing as putative promoters, β -galactosidase fusions were constructed as described in Materials and Methods. pFus97, a fusion of a 315-bp DNA region located upstream of the *pgm2* ATG codon (Fig. 1), introduced in the *A. tumefaciens* wild-type background expressed β -galactosidase activity at 11.76 units of optical density at 420 nm (OD_{420}) \cdot mg of protein⁻¹; on the other hand, in

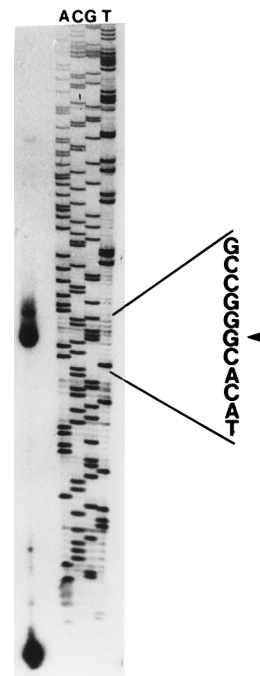


FIG. 3. Mapping of the 5' end of the *pgm* gene by primer extension analysis. The transcription start site is indicated by the arrow. Total RNA of the *A. tumefaciens* A348 wild-type strain was used to synthesize cDNA by employing avian myeloblastosis virus reverse transcriptase and the oligonucleotide PGMPE labeled with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. The products were subjected to polyacrylamide gel electrophoresis in parallel with the sequencing ladder.

E. coli the β -galactosidase activity of this fusion was 0.05 OD_{420} unit \cdot mg of protein⁻¹. This indicates that the DNA region of 315 bp upstream of the putative ATG codon of *pgm2* contains an active promoter that is more than 200 times stronger in *A. tumefaciens* than in *E. coli*. Fusion pFus98, containing a 650-bp DNA fragment located upstream of the *glgP* ATG start codon, introduced in the *A. tumefaciens* wild-type background expressed β -galactosidase activity at 37.07 OD_{420} units \cdot mg of protein⁻¹, which is 67 times higher than that observed in an *E. coli* background (0.55 OD_{420} unit \cdot mg of protein⁻¹). DNA sequence analysis of this 650-bp fragment revealed no homology to any gene in the database. The control fusion pFus96 expressed β -galactosidase activity neither in *E. coli* (0.02 OD_{420} unit \cdot mg of protein⁻¹) nor in *A. tumefaciens* (less than 0.01 OD_{420} unit \cdot mg of protein⁻¹), thus indicating that the β -galactosidase activity observed with pFus97 and pFus98 was indeed the result of the presence of active promoters upstream of the *glgP* and *pgm2* genes. In order to define the transcription start site of the alternative promoter of *pgm*, a primer extension study was carried out. Total RNA was hybridized with an excess of single-stranded synthetic oligonucleotide PGMPE labeled with ³²P at the 5' terminus (see Materials and Methods and Fig. 1). As shown in Fig. 3, the transcription start site (the nucleotide shown by the arrow) of this alternative transcript is located 168 bp upstream of a second in-frame putative ATG codon, 8 bp upstream of which is a Shine-Dalgarno conserved sequence (Fig. 4). This transcript may produce a shorter Pgm protein, indicated as *pgm2* in Fig. 4, that may account for the 24 and 26% remaining phosphoglucomutase activity observed in *glgP* and *glgB* polar mutants. This *pgm* promoter region contains 58% AT and has two copies of the sequence TATCAAN₅G (Fig. 4), described to be present in the promoter regions of TraR autoinducible genes



FIG. 4. Nucleotide sequence of the *glg* gene cluster. Only regions that contained the putative promoters and transcriptional and translational start sites of the 9,100-bp DNA are shown. The sequence from positions 7716 to 7916 has been reported previously (35). Putative ribosome binding sites (RBS) are underlined, start codons are shown with an angled arrow, stop codons are shown with an asterisk, the transcriptional start site is shown with a dot, and putative promoters are in bold type. The complete sequence has been deposited in EMBL GenBank.

of octopine Ti plasmids (10, 11). It is noteworthy that the A5129 mutant completely lacks Pgm activity (Table 2). This is consistent with the fact that in A5129, the Tn5 insertion is located 430 bp downstream of the first ATG codon (35) and, consequently, downstream of both alternative promoters.

Detection of two phosphoglucomutase proteins by Western blot analysis and functional complementation. Primer extension and β -galactosidase fusion suggested that there is an alternative promoter located between two in-frame ATG codons of the *pgm* gene (Fig. 4) that might produce, if translated, a Pgm protein 71 amino acids shorter than the protein translated from the polycistronic *glgPBCApgm* mRNA (Fig. 1 and 4). It is shown in Fig. 5 that antibodies raised against a recombinant *A. tumefaciens* A348 Pgm recognized in extracts of the *A. tumefaciens* wild-type strain A348 two proteins with apparent molecular masses of 66 and 58 kDa (Fig. 5B, lane 2). Both proteins were absent in *A. tumefaciens* *pgm* mutant A5129 (Fig. 5B, lane 3), thus indicating that the two proteins represent two forms of Pgm with the molecular masses expected for Pgm1 and Pgm2 (Fig. 4). It can be observed that in cell extracts prepared from *glgB* mutant A1120, the amount of the smaller

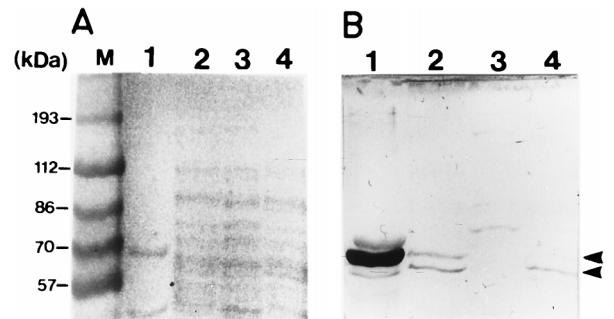


FIG. 5. Western blot analysis. Cell extracts were subjected to polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes as described in Materials and Methods. (A) Protein stained with Ponceau S; (B) Western blot carried out with rabbit polyclonal antibody raised against recombinant *A. tumefaciens* Pgm developed with peroxidase-conjugated antibody against rabbit immunoglobulin G (Dako). Lanes 1, commercial rabbit Pgm; lanes 2, *A. tumefaciens* wild-type extract; lanes 3, *A. tumefaciens* *pgm* mutant A5129 extract; lanes 4, *A. tumefaciens* *glgB* mutant A1120 extract. M, prestained molecular mass standards. Arrows on the right indicate the positions of Pgm proteins.

protein (58 kDa) was the same as that detected in wild-type extract; however, the amount of the protein with an apparent molecular mass of 66 kDa was severely reduced and barely detectable (Fig. 5B, lane 4). It can be observed that the antibody recognized a commercial rabbit Pgm (Fig. 5B, lane 1). These results indicate that in the *glgB* polar mutant A1120, Pgm protein was translated from the alternative transcript, which is expected to produce a protein 71 amino acids shorter than that translated from the polycistronic *glg* operon and could explain part of the remaining Pgm activity in the A1120 *glgB* mutant. The small amount of protein with a molecular mass of 66 kDa still detected in the *A. tumefaciens* A1120 mutant might be due to the presence of a cryptic promoter not detected by the primer extension experiment.

In order to confirm that the short Pgm protein is active *in vivo*, a complementation experiment with the dark-phenotype *A. tumefaciens* *pgm* mutant A5129 (35) and plasmid pCC15 was carried out. The recombinant plasmid pCC15 (Table 1) was constructed by cloning in plasmid pBBR1MCS-4 (19) a DNA fragment containing the *pgm* gene from ATG₁ (bp 7733 to bp 9377) as described in Materials and Methods. This construct does not contain the ribosome binding site upstream of ATG₁ but contains the postulated promoter region of *pgm2* upstream of ATG₂ (Fig. 4). pCC15 was electroporated into *A. tumefaciens* *pgm* mutant A5129 and plated on LB medium containing 100 μ g of carbenicillin per ml, 50 μ g of kanamycin per ml, and 0.02% Calcofluor (Sigma Chemical Co.). All the transformants obtained were Cb^r Km^r and bright under UV light, thus indicating that *pgm* function was restored and synthesis of exopolysaccharide had resumed. Since the DNA fragment was cloned in the opposite direction with respect to transcription of the endogenous promoter of the multiple cloning site of pBBR1MCS-4 (19), this result confirmed the presence of an alternative promoter between the two in-frame ATG codons (Fig. 4). Moreover, the experiment demonstrated that the short Pgm protein complements *in trans* the phenotype of the A5129 mutant. The possibility that recombination events were responsible for complementation was eliminated because 100% of the clones were Cb^r Km^r and bright.

DISCUSSION

The glycogen (*glg*) operon of *A. tumefaciens* was studied, and its complete sequence was determined. The operon comprises

five genes, *glgP*, *glgB*, *glgC*, *glgA*, and *pgm*, transcribed in the same direction as a single mRNA. A second promoter located downstream of *glgA* produces an alternative transcript of the *pgm* gene.

The overall organization of the operon in *A. tumefaciens* is different from that in *E. coli*, where it is formed by two operons, *glgB* and *glgCAP* (25); the *pgm* gene that is part of the *Agrobacterium glg* operon is not present in either of the two operons in *E. coli*.

The organization of the *glg* operon in *B. stearothermophilus* and *B. subtilis* (18, 34) is also different from that in *E. coli* or *A. tumefaciens*. In both bacilli, there is a single operon, *glgBC-DAP*, in which *glgD* and *glgC* encode the subunits of a heterotetrameric ADP-glucose pyrophosphorylase. Neither the *glgX* nor the *pgm* homologous gene is part of the *Bacillus* operon.

RT-PCRs carried out with total RNA extracted from the wild type and the *glgB* mutant, assays of *glg* operon enzymes in the wild type and in *glgP::Km^r* and *glgB::Km^r* polar mutants, β -galactosidase fusions, and primer extension experiments confirmed the presence of a *pgm* mRNA transcribed from an alternative promoter. This alternative promoter is located within the coding region of the *pgm* gene and produces a Pgm protein 71 amino acids shorter than the Pgm protein translated from the polycistronic *glgPBCapgm* mRNA. Downstream of this alternative transcription start, a second in-frame ATG codon preceded by a Shine-Dalgarno sequence was identified. The predicted amino acid sequence of the N-terminal region of the protein translated from the polycistronic mRNA is 77% identical to the N-terminal region of rat Pgm (35); the active site and the potential phosphorylated serine are located downstream of this region (35). These results suggest that the enzymatic activity and *pgm* mRNA observed to be present in *glgP::Km^r* and *glgB::Km^r* polar mutants may be explained by transcription and translation of the *pgm* gene starting at this alternative *pgm* promoter. Moreover, a recombinant plasmid containing a *pgm* gene starting with the second ATG start codon complemented in *trans* a Tn5 *A. tumefaciens* *pgm* mutant, thus indicating that the 71-amino-acid-shorter protein is active in vivo. This alternative promoter may be turned on under specific metabolic or environmental conditions. A similar situation was described for the bovine β (1-4) galactosyltransferase, in which two promoters produce long and short mRNAs. The short mRNA starts in a region between two in-frame ATG codons (31). The promoter of the short mRNA was found to be a mammary gland-specific promoter, while the long mRNA functions as a housekeeping promoter (15).

In other bacteria the *glg* operon is regulated at the level of transcription, under metabolic conditions that lead to the accumulation of glycogen. Since in *Agrobacterium* the Leloir pathway is absent (36), Pgm is absolutely required for the biosynthesis of UDP-glucose, the sugar donor used for the synthesis of structural cell wall polysaccharides, exopolysaccharides, and cyclic β (1-2) glucans. This makes reasonable the hypothesis that an alternative promoter may be required to ensure, under certain conditions, the synthesis of Pgm independent of *glg* operon regulation.

The structure of the alternative *pgm* promoter contains a motif that resembles other *Agrobacterium* plant-inducible promoters (11), which might imply that *pgm* is induced when the bacteria reach the plant environment. It was previously described that cyclic β (1-2) glucans are required for virulence (26, 39). Cyclic glucans are synthesized by an inner membrane β (1-2) glucan transferase encoded by the chromosomal gene denominated *chvB* (7, 39). The enzyme is constitutively expressed and uses the sugar donor UDP-glucose (39). Thus, the

supply of UDP-glucose must be guaranteed for the synthesis of cyclic glucans and for an effective infection. The presence of an alternative promoter that may be turned on when the bacterium reaches the plant environment might be required for the expression of *pgm* under nutrient-limiting conditions that may shut off the *glg* operon. Studies to investigate this possibility are in progress.

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