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Molecular and Expression Analysis of the *Rhizobium meliloti* Phosphoenolpyruvate Carboxykinase (*pckA*) Gene

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The *pckA* gene of *Rhizobium meliloti*, encoding phosphoenolpyruvate carboxykinase, was isolated from a genomic cosmid library by complementation of the succinate growth phenotype of a Pck$^-$ mutant. The gene region was mapped by subcloning and Tn*5* insertion mutagenesis. The DNA sequence for a 2-kb region containing the structural gene and its promoter was determined. The *pckA* gene encodes a 536-amino-acid protein that shows homology with other ATP-dependent Pck enzymes. The promoter was identified following primer extension analysis and is similar to σ^{70}-like promoters. Expression analysis with a *pckA*::*lacZ* gene fusion indicated that the *pckA* gene was strongly induced at the onset of stationary phase in complex medium. When defined carbon sources were tested, the expression level of the *pckA* gene was found to be high when cells were grown in minimal media with succinate or arabinose as the sole carbon source but almost absent when glucose, sucrose, or glycerol was the sole carbon source. Glucose and sucrose were not found to strongly repress *pckA* induction by succinate.

Phosphoenolpyruvate carboxykinase (EC 4.1.1.49) (Pck) catalyzes the decarboxylation and phosphorylation of oxaloacetate to phosphoenolpyruvate. This reaction is the first step in the gluconeogenic pathway in which tricarboxylic acid (TCA) cycle intermediates are converted to hexose sugars. With the exceptions of phosphoenolpyruvate carboxykinase and fructose bisphosphatase, all of the enzymes employed in the gluconeogenic pathway are also used in the glycolytic pathway.

With N_2-fixing root nodules induced by bacteria such as the alfalfa symbiont *Rhizobium meliloti*, there is much evidence to suggest that the plant supplies the C_4-dicarboxylic acids succinate and malate to the N_2-fixing bacteria (bacteroids) within nodules (5, 9, 17, 42, 47, 51). Pck is required for growth and metabolism of C_4-dicarboxylates and other TCA cycle intermediates in free-living cells of *R. meliloti*, *Rhizobium leguminosarum*, and *Rhizobium* sp. strain NGR234. The symbiotic importance of Pck during nodule infection and N_2 fixation requires clarification. While Pck$^-$ mutants of *R. meliloti* show a reduced level of nitrogen fixation, Pck activity is not detected in wild-type bacteroids (15). *R. leguminosarum* Pck$^-$ mutants have no apparent symbiotic phenotype, yet Pck activity was detected at low levels in wild-type bacteroids (33). A Pck$^-$ mutant of *Rhizobium* sp. strain NGR234, a broad-host-range fast-growing rhizobium, exhibited a host-dependent symbiotic phenotype. N_2 fixation was reduced to 60 and 20% of the wild-type level in *Leucaena leucocephala* and *Macroptilium atropurpureum*, respectively, whereas on *Vigna unguiculata*, this mutant induced completely Fix$^-$ nodules (39). The structural analysis of these nodules showed defects in the nodule development process and the occurrence of early senescence of bacteroids. These results suggest that the importance of Pck in symbiosis is dependent on the plant host metabolites available to the bacteria during the infection process and bacteroid differentiation.

The structural gene for phosphoenolpyruvate carboxykinase (*pckA*) has been cloned and sequenced in two prokaryotes, *Escherichia coli* and *Rhizobium* sp. strain NGR234 (35, 39). The gene encodes an ATP-dependent enzyme that is homologous to ATP-dependent Pck enzymes from the eukaryotic microorganisms yeasts and trypanosomes and from plants but shares little similarity in primary structure to the GTP-dependent Pck enzymes of animals. We are interested in studying the *pckA* gene of *R. meliloti* as (i) Pck is a central metabolic enzyme, and the role of Pck in symbiotic N_2 fixation requires clarification; (ii) other than that derived from *E. coli*, there is little information available on how the *pckA* gene is regulated; and (iii) although the *pckA* gene from *Rhizobium* sp. strain NGR234 has been identified and sequenced, genetic analysis in NGR234 is difficult, unlike the case with the genetically amiable strain *R. meliloti* SU47.

Here we report the identification, nucleotide sequence, transcriptional start site, and regulation of the *pckA* gene of *R. meliloti*. We find that the *pckA* gene is subject to a stationary phase and carbon source levels of regulation and the *pckA* promoter is similar to σ^{70}-like promoters.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The strains and plasmids used in this study are listed in Table 1. *Rhizobium* strains and *E. coli* were grown in complex media (Luria broth [LB], supplemented with 1 mM $MgSO_4$ and 0.25 mM $CaCl_2$ for *Rhizobium* strains [LBmc]) or defined M9 media (36). Sterilized carbon sources (15 mM) were added to the autoclaved M9 mineral salts solution. Thus, M9-succinate refers to M9 mineral salts containing 15 mM succinate. Antibiotic concentrations were as previously described (13, 14). Indicator plates for β-galactosidase expression contained X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) at a concentration of 40 μg/ml.

Genetic techniques and transposon mutagenesis. Bacterial matings and *R. meliloti* øM12 transductions were performed as previously described (14, 16). Tn*5* insertions into the recombinant plasmids pTH84 and pTH85 were isolated by site-directed Tn*5* mutagenesis, followed by mobilization of these plasmids from *E. coli* MT614 into *E. coli* MT609, as previously described (51). The Tn*5* insertions were then characterized by restriction analysis of plasmid DNA. Tn*3*HoHo1-derivative insertions (20) in pRmT103 were isolated by following the procedure described by Stachel et al. (45). Only insertions in the *pckA* gene were characterized. These were identified following mobilization of the mutated plasmids into Rm5065 and screening of transconjugant colonies that appeared blue on LB–X-Gal agar for their ability to grow on M9-succinate. Tn*3*HoHo1-derivative and Tn*5* site-directed mutations were recombined from plasmids onto the *Rhizobium* chromosome as described previously (11).

DNA manipulations. For plasmid DNA isolation, restriction digestion, agarose

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TABLE 1. Bacterial strains and plasmids used in this study

Strain, plasmid, or phage	Relevant characteristic(s)	Reference or source
Strains		
R. meliloti SU47		
Rm1021	*str-21*	34
Rm5000	*rif-5*	13
Rm5065	Same as Rm1021 but with *pck-1*::Tn*5*-132	16
Rm5234	Same as Rm1021 but with *pck-2*::Tn*5*-VB32	16
Rm5439	Same as Rm1021 but with *pck-1*::Tn*V*	16
RmG212	Same as Rm1021 but Lac$^-$	Jane Glazebrook
RmG319	Same as RmG212 but with pF94	This work
RmH189	Same as RmG212 but with *ntrA74*::Tn*5*-233, pF94	This work
RmH190	Same as RmG212 but with *dctB18*::Tn*5*-233, pF94	This work
RmH191	Same as RmG212 but with *dctD16*::Tn*5*-233, pF94	This work
RmH192	Same as RmG212 but with *dctA14*::Tn*5*-233, pF94	This work
E. coli		
DH5α	F$^-$ *endA1 hsdR17 supE44 thi-1 recA1 gyrA96 relA1* Δ(*argF-lacZYA*)	Bethesda Research Laboratories
XL-1Blue	*supE44 hsdR17 endA1 gyrA46 relA1 thi recA* [F′ *proAB lacI*^{q} *Z*ΔM15 Tn*10*(Tc^r)]	10
MT607	*pro-82 thi-1 hsdR17 supE44 recA56*	14
MT609	*thyA36 polA1* Sp^r, recipient for Tn*5* mutagenesis of IncP plasmids	48
MT614	MT607ΩTn*5*	51
MT616	MT607(pRK600)	14
F86	DH5α(pSShe, pHoSp)	20
F87	DH5α(pSShe, pHoKm)	20
Plasmids		
pLAFR1	IncP cosmid cloning vector, Tc^r	19
pRmT103	pLAFR1, Pck$^+$	16
pRK7813	IncP1 cosmid cloning vector, Tc^r	29
pUC118	ColE1 *oriV* cloning vector, Ap^r	49
pTH84	Same as pRK7813 but with 7.5-kb *Bam*HI fragment containing *pckA*	This work
pTH85	Same as pRK7813 but with 7.5-kb *Bam*HI fragment containing *pckA*	This work
pTH86	Same as pRK7813 but with 5-kb *Eco*RI fragment containing *pckA*	This work
pTH87	Same as pRK7813 but with 5-kb *Eco*RI fragment containing *pckA*	This work
pTH137	Same as pUC118 but with 3.2-kb *Sma*I fragment with partial *pckA*	This work
pTH189	Same as pRK7813 but with 5-kb *Pst*I fragment containing *pckA*	This work
pTH190	Same as pRK7813 but with 5-kb *Pst*I fragment containing *pckA*	This work
pTH195	Same as pUC118 but with 1.6-kb *Sst*I fragment containing *pckA*	This work
pTH196	Same as pUC118 but with 1.6-kb *Sst*I fragment containing *pckA*	This work
pTH224	Same as pUC118 but with 3.2-kb *Sma*I fragment with partial *pckA*	This work
pF93	Same as pRmT103 but with *pckA5*::Tn*3*HoKm	This work
pF94	Same as pRmT103 but with *pckA6*::Tn*3*HoKm	This work
pF95	Same as pRmT103 but with *pckA7*::Tn*3*HoKm	This work
pF96	Same as pRmT103 but with *pckA8*::Tn*3*HoKm	This work
pG3	Same as pRmT103 but with *pckA9*::Tn*3*HoSp	This work
pG4	Same as pRmT103 but with *pckA10*::Tn*3*HoSp	This work
pG5	Same as pRmT103 but with *pckA11*::Tn*3*HoSp	This work
pG6	Same as pRmT103 but with *pckA12*::Tn*3*HoSp	This work
Phages		
M13K07	M13 derivative, helper phage	49
ΦM12	*R. meliloti* transducing phage	14

and polyacrylamide gel electrophoresis, Southern transfer, ligations, and transformation, standard methods were used as described previously (43). *Rhizobium* genomic DNA was isolated as described previously (34). Hybridizations were performed with either ^{32}P- or digoxigenin (Boehringer Mannheim)-labelled DNA probes. Unbound probe was removed by washing the filters twice at room temperature for 15 min with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate (SDS) and twice for 15 min at 65°C with 0.1× SSC–0.1% SDS.

DNA sequencing. The 1.6-kb *Sst*I and 3.5-kb *Sma*I fragments containing *pckA* were subcloned from pTH84 into pUC118 in both orientations. A series of unidirectional nested deletions was isolated for each plasmid by exonuclease III treatment followed by S1 nuclease digestion as described by Sambrook et al. (43). Single-stranded DNA was obtained by using host strain XL-1Blue (Stratagene) and helper phage M13K07 (49). The universal −20 primer was employed for the annealing procedure. DNA sequencing was performed by dideoxy chain termination (44) according to the protocol of United States Biochemicals for the Sequenase 2.0 enzyme, by using [α-^{35}S]ATP (NEN/DuPont) and 7-deaza dGTP (Pharmacia). Both strands of DNA were sequenced. DNA and derived protein sequences were analyzed with the PC/Gene (Intelligenetics), Blast (2), CLUSTALV (27), and PHYLIP (12) software packages.

RNA extraction. RNA was extracted from wild-type Rm1021 cultures as previously described (23), with the following modifications. After the first ethanol precipitation, the nucleic acids were resuspended in 100 μl of diethyl pyrocarbonate (DEPC)-treated H_2O and 0.3 ml of 3 M Na-acetate (pH 7) was added. After incubation overnight at −20°C, the RNA was precipitated by centrifugation (13,000 × *g*, 20 min, 4°C). The RNA was again resuspended in 100 μl of

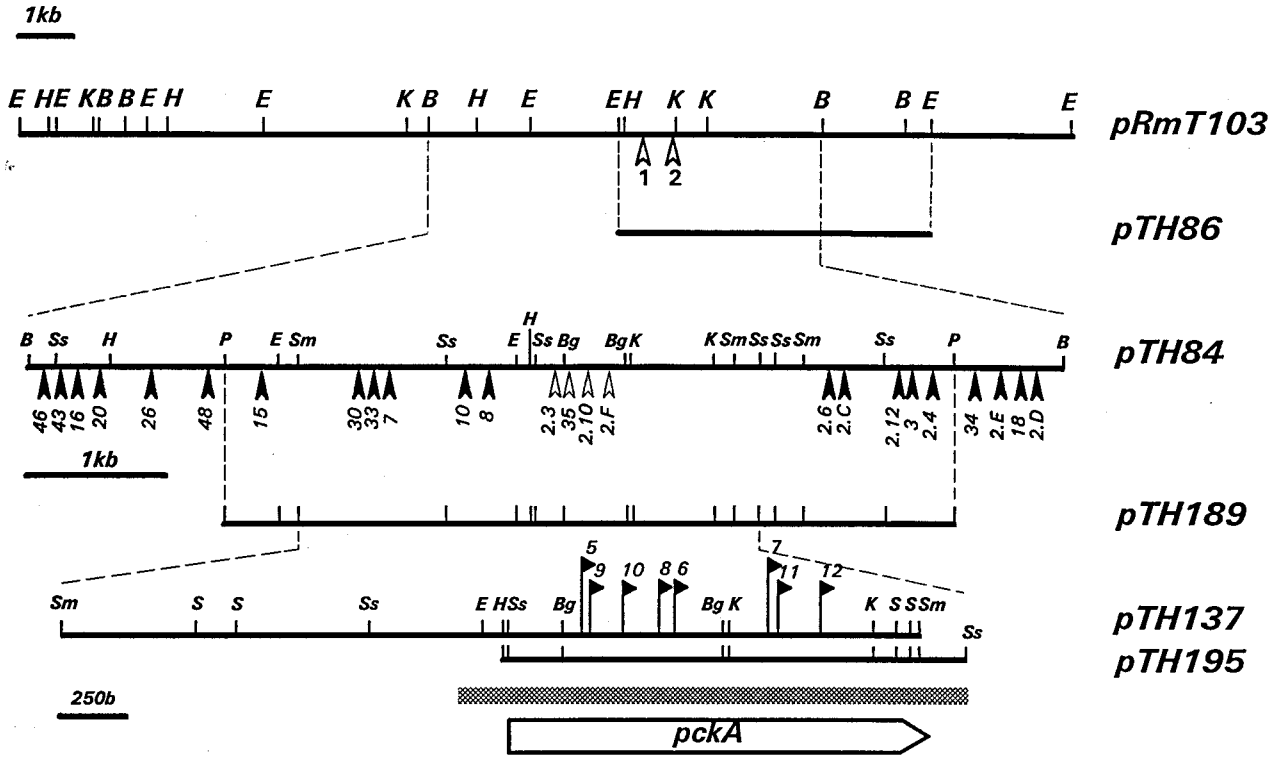

FIG. 1. Physical and genetic map of the pRmT103 cosmid and derived subclones containing the *pckA* gene of *R. meliloti*. Open and filled arrowheads on pTH84 indicate Tn*5* insertions that do and do not, respectively, abolish the ability of the plasmid to complement Pck$^-$ mutants. The open arrowheads on pRmT103 represent the locations of the genomic Tn*5* insertions previously isolated (16), with the number of the allele. The flags on pTH137 indicate the positions of the Tn*3*HoHo1-derivative insertions in *pckA*. The direction that the flag points indicates the orientation of the *lacZ* fusion. The allele numbers are shown above the flags. The shaded box indicates the sequenced region, with the position and orientation of the *pckA* gene below it. Restriction sites shown are *Bam*HI (B), *Bgl*II (Bg), *Eco*RI (E), *Hin*dIII (H), *Kpn*I (K), *Pst*I (P), *Sal*I (S), *Sma*I (Sm), and *Sst*I (Ss).

DEPC-treated H_2O, and the precipitation was repeated. Following a further ethanol precipitation, the pellet was resuspended in 50 to 200 μl of H_2O. The purity and quality of the RNA were checked by electrophoresis through a 1.2% agarose gel with TAE running buffer (43). If necessary, contaminating DNA was removed by treatment with 60 U of RNase-free DNase (Boehringer-Mannheim) per ml for 30 min at 37°C in the presence of 400 U of RNase inhibitor (RNAguard; Pharmacia) per ml. This was followed by a phenol-chloroform-isoamyl alcohol (25:24:1) extraction and ethanol precipitation of the RNA.

Primer extension. To identify the 5′ end of the *pckA* mRNA, a specific oligonucleotide (5′-GTCCGATAGAGGGATTGCGGCTGCCGAGCT-3′) complementary to the beginning of the coding region of the gene was synthesized. Labelling of the primer was performed by end labelling with [γ-^{33}P]ATP (NEN/DuPont) and T4 polynucleotide kinase (BioLabs) at 37°C for 30 min. After inactivation by heat (5 min at 65°C), the labelled primer was purified on a 6% denaturing polyacrylamide gel. The primer (5×10^4 cpm) was hybridized to 5 to 30 μg of total RNA overnight at 30°C, in a 30-μl volume. The primer was extended with 25 U of avian myeloblastosis virus reverse transcriptase at 42°C for 90 min as described by Ausubel et al. (6). One-half of the extension product was loaded onto a 6% denaturing polyacrylamide gel beside a sequencing reaction mixture obtained with the same primer, used as a reference.

Enzyme assays. For standard β-galactosidase assays, 5 ml of defined media with 15 mM the required carbon source was inoculated with 20 μl of an overnight culture. The resulting culture was incubated with shaking overnight at 30°C to an optical density at 600 nm of between 0.3 and 1.0. For kinetic analysis, 300 ml of LB or defined media in a 1-liter Erlenmeyer flask was inoculated with 1.5 ml of an overnight M9-glucose culture (A_{675} of <1.0). The cultures were incubated with shaking, and samples (5 ml) were taken every 2 h. The A_{675} was measured, and the samples were then kept on ice prior to measuring of the β-galactosidase activity as previously described (36). Miller units (36) were calculated as $1{,}000[OD_{420}/(\text{volume of culture added in ml}) (\text{reaction time in minutes}) (OD_{600}$ of the culture)].

Preparation of cell extract and procedures followed to assay for Pck activity were as previously described (16).

Nucleotide sequence accession number. The DNA sequence of the *R. meliloti pckA* gene has been submitted to the GenBank/EMBL data bank and assigned accession number U15199.

RESULTS

Identification and subcloning of the *pckA* locus. In previous work, we identified *R. meliloti* Rm5065 (*pck-1*) and Rm5234 (*pck-2*). These mutants lacked Pck activity and grew very slowly on minimal medium with succinate or other TCA cycle intermediates as the sole carbon source (16). These mutants showed no growth defect when supplied with glucose or other sugars as the sole carbon source. A plasmid clone, pRmT103, which complemented the succinate growth phenotype of *pck-1* and *pck-2* mutants was isolated from a pLAFR1 clone bank containing wild-type *R. meliloti* DNA (16, 19). A restriction map of pRmT103 for the enzymes *Eco*RI, *Bam*HI, *Hin*dIII, and *Kpn*I was subsequently constructed (Fig. 1). Southern hybridization with labelled pRmT103 DNA to genomic DNA from Rm1021, Rm5065, and Rm5234 showed that both *pck-1* and *pck-2* insertions were located within a 6-kb *Eco*RI fragment present in pRmT103 (Fig. 1). The 6-kb *Eco*RI fragment was subcloned into pRK7813 in both orientations (pTH86 and pTH87), as was an overlapping 7.5-kb *Bam*HI fragment (pTH84 and pTH85). These constructs clearly complemented the Pck$^-$ mutants for growth on succinate; however, we note that growth was not fully restored to wild-type levels. The latter growth effect may be caused by a high-level Pck activity which results from these plasmids (38a). This is currently being investigated and will not be further discussed in this communication.

To identify the *pck* gene region more precisely, we carried out saturation Tn*5* mutagenesis of the 7.5-kb *Bam*HI fragment

cloned in pTH84 and pTH85. Of 24 insertions within this fragment, 4 failed to complement the succinate growth phenotype of Rm5065. Physical mapping of these insertions (2.3, 2.10, 2.F, and 35; Fig. 1) together with the other 20 insertions within the 7.5-kb fragment revealed that the *pck* gene was located within a region of 2.5 kb. Recombination of the four noncomplementing Tn*5* insertions into the wild-type genome resulted in strains with a succinate growth phenotype similar to that of Rm5065. In addition, cell extracts prepared from these recombinants were found to lack Pck activity. Conversely, strains obtained by recombining Tn*5* insertions which flank the 2.5-kb *pck* region (insertions 2.6, 2.C, 3, and 8) showed no succinate growth phenotype. As the Tn*5* mutagenesis experiment revealed that the entire *pck* gene was present on a 5-kb *Pst*I fragment, this fragment was subcloned from pTH84 into pRK7813 in both orientations (pTH189 and pTH190). These plasmids showed the same succinate complementation phenotype as did pTH84 when transferred into Rm5065.

Nucleotide sequence of the *pckA* gene. In order to identify the *pckA* gene, the complete DNA sequence of the 1.6-kb *Sst*I fragment was determined (Fig. 1). Preliminary comparison analysis of this sequence to the *pckA* gene of *Rhizobium* sp. strain NGR234 indicated that the 5′ end of the gene was not present on this fragment. The 3.5-kb *Sma*I fragment (pTH137) was used to complete the nucleotide sequence of the 5′ end of the gene and of the promoter region (Fig. 2). Analysis of the possible translation frames of the complete 1,976-bp fragment, delimited by restriction sites *Sau*3AI and *Sst*I, identified one major open reading frame of 536 residues with an ATG start codon at position 206. The ATG was preceded by a potential ribosome binding site, AAGGA, located 9 bp upstream (Fig. 2). An inverted repeat present downstream of the stop codon (TAA), and centered at position 1855, showed a G+C-rich stem-loop structure followed by at least four T residues, the characteristics of a *rho*-independent terminator (41).

The *pckA* gene of *R. meliloti* has a G+C content (63%) similar to that of other genes from this species. A strong bias for G and C in the third position of the codons (84.9%) was observed for the coding region, as expected for G+C-rich organisms (37). An alignment of the *pckA* coding DNA sequences of *R. meliloti* and *Rhizobium* sp. strain NGR234 showed a high degree of conservation (87%) between the two genes. Some mismatches in the *Rhizobium* sp. strain NGR234 sequence, were found, and several were corrected after verification. Of the 209 substitutions occurring within the gene, 151 occurred in the third position of the codons and the majority of these were silent (84%). The conservation at the nucleotide level between the two species was not limited to the coding region, as the promoter regions up to 158 bp from the translation start site showed a high degree of similarity (87%) (see Fig. 4B). The same was also true for the 3′ end, where the *rho*-independent terminator is conserved.

Analysis of the encoded protein sequence. The primary structure of ATP-dependent *pckA* genes from five organisms, *Saccharomyces cerevisiae* (46), *Trypanosoma brucei* (40), *Trypanosoma cruzi* (31), *E. coli* (35), and *Rhizobium* sp. strain NGR234 (39), have been characterized, and their encoded proteins were shown to share significant amino acid sequence similarity. The predicted protein translated by the *R. meliloti pckA* gene showed strong homology to these sequences (Fig. 3A). A very high degree of identity is observed between the two *Rhizobium* species, but these did not show greater homology to the *E. coli* protein than to the eukaryotic proteins. Phylogenic analysis by different methods (neighbor-joining, parsimony, and maximum likelihood) was performed, both with the complete sequence of the protein and with a more conserved central region (residues 106 to 434) (see Materials and Methods). The results obtained by the different methods were consistent with each other and separated the prokaryotic from the eukaryotic sequences (Fig. 3B). The Pck protein could be divided into three regions by sequence homology (39). The central region (amino acids 189 to 402) was highly conserved both in length and sequence between the different species (Fig. 3A), while more variability was present in the N- and C-terminal regions. The phosphate-binding (G--G-GKT) and adenine-binding (LLLL-D) consensus sites of ATP-dependent proteins (residues 233 to 243 and 345 to 351, respectively) were identified in highly conserved regions (26). A divalent or transition metal ion binding (G---EG) site consensus sequence (residues 265 to 271) could also be determined by sequence homology (31).

Identification of the transcription start site. In order to identify the *pckA* promoter, the transcriptional start site of the *pckA* gene was determined by primer extension with a 30-mer oligonucleotide complementary to the 5′ end of the coding region. Template RNA was extracted from Rm1021 grown to early log and late log-early stationary phases in LBmc (optical density at 675 nm = 0.3 and 2.0, respectively). Total RNA was also extracted from late-log-phase LBmc-grown RmG319 cells. RmG319 contains the plasmid-borne *pckA6*::*lacZ* fusion and shows a higher level of expression than does the single-copy chromosomal *pckA6*::*lacZ* gene.

Two major extension products were observed at the same positions in all three cases (Fig. 4A). The 3′ end finished with T or C (longer) residues corresponding to G and A at positions 165 and 166, respectively, on the noncoding strand (Fig. 4B). The amount of product, as shown by the intensity of the bands, reflects the amount of *pckA* mRNA present and differed among the three RNAs. Only a weak signal was detected with RNA extracted from wild-type Rm1021 cells harvested during early logarithmic growth in LBmc (data not shown). An extension product was readily detected with RNA from RmG319 cells (Fig. 4A).

Gene expression depends on growth phase and carbon source. Tn*3*HoHo1 and its Nm^r and Sp^r derivatives generate transcriptional and translational fusions to the *lacZ* gene (20, 45). Eight independent Tn*3*HoHo1-derivative insertions in pRmT103, which failed to complement the succinate-negative growth phenotype of Rm5065 and formed blue colonies on LB–X-Gal agar medium, were isolated. Restriction analysis of these plasmids with *Bam*HI and *Eco*RI showed that the insertions were all located within the *pckA* gene and were oriented such that *lacZ* would be transcribed together with the truncated *pckA* (Fig. 1; flags indicate the orientation and position of Tn*3*HoHo1-derivative insertions 5 through 12). The eight insertions were recombined into the *R. meliloti* genome, and the resulting eight recombinants lacked Pck enzyme activity and failed to grow on succinate minimal medium.

To determine whether *pckA* expression was dependent on growth phase, as has been reported for *E. coli* (21), we measured β-galactosidase activity over the complete growth cycle of RmG319 cells growing in LBmc, M9-glucose, and M9-succinate (Fig. 5). RmG319 is a Lac^- wild-type derivative carrying the plasmid-borne *pckA6*::Tn*3*HoKm insertion. When cells were grown in LBmc (Fig. 5A), *pckA* was expressed during the log phase at a constant level of 1,000 Miller units. At the onset of the stationary phase, an abrupt 10-fold induction was observed. In glucose-grown RmG319 cells (Fig. 5B), a very low level of expression was detected throughout all stages of cell growth. In succinate-grown cells, the induction was observed very early and increased steadily during the log phase; no further increase in expression in the stationary phase was ob-

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GATCCGGTTTCCCCGCAATTGGCCTCGCAGGTGGGTTTCCGGAAGCTGCGATTCAACCGATTAAAAAATGCATATTTTTCTTTAAATCGA    90
                                                                                ▼▼
TTAATATACTGAATTCATTATCCTTTTAGAATTGGCTATCTTGTCTTGGGTCAGCCTTGCCGGTATGTTCCGACGAAATTCAACGCCAAT  180
                                        ------------------------------
GGCCAAACACGAAGGAAGCTTGACCATGGATGAGCTCGGCAGCCGCAATCCCTCTATCGGACTGGAATCGATCGGATTTTCCGACCTGTC  270
                          MetAspGluLeuGlySerArgAsnProSerIleGlyLeuGluSerIleGlyPheSerAspLeuSer   22

GGTGGTCCGCTACAACTTCGAGGCGGCGCAGCTTTACGAAGAAGCCCTGGCCCGCGGTGAAGCCGAATTGACGGCCCATGGCGCGCTTTG  360
 ValValArgTyrAsnPheGluAlaAlaGlnLeuTyrGluGluAlaLeuAlaArgGlyGluAlaGluLeuThrAlaHisGlyAlaLeuCys   52

CGCCCGCACCGGCCAGCACACGGGCCGCTCGCCCAAGGACAAGTATGTCGTGCGCGACGCCAACACGGCCGACCAGATCTGGTGGGACAA  450
 AlaArgThrGlyGlnHisThrGlyArgSerProLysAspLysTyrValValArgAspAlaAsnThrAlaAspGlnIleTrpTrpAspAsn   82

CAACAGCGCGATTTCGCCGGAGCATTTCGAGGTTCTTCGTCGGGACATGCTCGCGCATGCAAAGGGCATGTCGCTGTATGTCCAGGACCT  540
 AsnSerAlaIleSerProGluHisPheGluValLeuArgArgAspMetLeuAlaHisAlaLysGlyMetSerLeuTyrValGlnAspLeu  112

CGTCGGCGGTGCCGACCCGGAGAACGCGCTGCCGACGCGCGTCGTTACCGAGTTCGCCTGGCACTCGCTGTTCATCCGCAATCTGTTGAT  630
 ValGlyGlyAlaAspProGluAsnAlaLeuProThrArgValValThrGluPheAlaTrpHisSerLeuPheIleArgAsnLeuLeuIle  142

CCGCCCGGAGCGCGAGGCGCTTCCGTCTTTTCAGCCGAAGCTCACGATCATCGACCTGCCGAGCTTCAAGGCCGATCCCGTACGCCACGG  720
 ArgProGluArgGluAlaLeuProSerPheGlnProLysLeuThrIleIleAspLeuProSerPheLysAlaAspProValArgHisGly  172

CTGCCGCAGCGAGACGGTCATCGCCTGCGACCTGACCAACGGCCTCGTGCTGATCGGCGGTACTTCCTATGCGGGCGAGATGAAGAAGTC  810
 CysArgSerGluThrValIleAlaCysAspLeuThrAsnGlyLeuValLeuIleGlyGlyThrSerTyrAlaGlyGluMetLysLysSer  202
                                                   1.
GGTTTTCACCGTTCTCAATTACCTGCTGCCCGAGAAGTCGGTGATGCCGATGCACTGCTCGGCCAATGTCGGGCCCGCCGGCGACACGGC  900
 ValPheThrValLeuAsnTyrLeuLeuProGluLysSerValMetProMetHisCysSerAlaAsnValGlyProAlaGlyAspThrAla  232

GATCTTCTTCGGCCTCTCGGGCACCGGCAAGACGACGCTCTCCGCCGATCCGAACCGTACCCTGATCGGTGACGACGAGCACGGCTGGAG  990
 IlePhePheGlyLeuSerGlyThrGlyLysThrThrLeuSerAlaAspProAsnArgThrLeuIleGlyAspAspGluHisGlyTrpSer  262
          2.
CGAAAAGGGCGTCTTCAATTTCGAGGGCGGCTGCTATGCCAAGGCGATCCGCCTTTCGGAAGCGGCCGAGCCCGAGATCTTTGCGACGAC 1080
 GluLysGlyValPheAsnPheGluGlyGlyCysTyrAlaLysAlaIleArgLeuSerGluAlaAlaGluProGluIlePheAlaThrThr  292
    3.
GCGGCGCTTCGGTACCGTGATGGAGAACGTCGTCCTCGACGAGCGGCGCCTTCCTGACTTCGACGACGGCTCGCTGACGGAGAACACCCG 1170
 ArgArgPheGlyThrValMetGluAsnValValLeuAspGluArgArgLeuProAspPheAspAspGlySerLeuThrGluAsnThrArg  322

CTGCGCCTATCCGCTGCACTTCATTCCGAACGCCAGCAAGACCGGCACGGCGCCGCAGCCGCGCACGATCATCATGCTGACGGCGGACGC 1260
 CysAlaTyrProLeuHisPheIleProAsnAlaSerLysThrGlyThrAlaProGlnProArgThrIleIleMetLeuThrAlaAspAla  352
                                                                    4.
CTTCGGGGTTCTGCCGCCGATCGCCAAGCTGACGCCGGAACAGGCCATGTATCACTTCCTCTCCGGCTACACCGCCAAGGTTGCCGGCAC 1350
 PheGlyValLeuProProIleAlaLysLeuThrProGluGlnAlaMetTyrHisPheLeuSerGlyTyrThrAlaLysValAlaGlyThr  382

CGAAAAGGGCGTGACGGAGCCGGAGGCGACTTTCTCGACCTGCTTCGGCGCACCCTTCATGCCGCGCCATCCGTCCGAATACGGCAATCT 1440
 GluLysGlyValThrGluProGluAlaThrPheSerThrCysPheGlyAlaProPheMetProArgHisProSerGluTyrGlyAsnLeu  412

TCTCAAGGACCTCATCGCCAGGAACGGCGTCACCTGCTGGCTCGTCAACACCGGCTGGACCGGCGGCGCCTTTGGCACGGGAAGCCGCAT 1530
 LeuLysAspLeuIleAlaArgAsnGlyValThrCysTrpLeuValAsnThrGlyTrpThrGlyGlyAlaPheGlyThrGlySerArgMet  442

GCCGATCAAGGTGACGCGCGCGCTTCTTTCGGCAGCGCTCGACGGCTCGCTGAACAACGCCTCGTTCCGCACGGATGCGAATTTCGGCTT 1620
 ProIleLysValThrArgAlaLeuLeuSerAlaAlaLeuAspGlySerLeuAsnAsnAlaSerPheArgThrAspAlaAsnPheGlyPhe  472

CGCGGTGCCGGTGTCGGTACCGGGCGTCGAGGCCGGCATTCTCGACCCGCGCTCGACCTGGGCGGATGGCGTGGCTTATGACACCCAGGC 1710
 AlaValProValSerValProGlyValGluAlaGlyIleLeuAspProArgSerThrTrpAlaAspGlyValAlaTyrAspThrGlnAla  502

ACGGCGCCTCGTCGACATGTTCATTGCCAACTTCGCCAAGTTCGAGCGCCATGTCGACGGCAGCGTGCGCGACGCAGCCCCGGGCGCGAG 1800
 ArgArgLeuValAspMetPheIleAlaAsnPheAlaLysPheGluArgHisValAspGlySerValArgAspAlaAlaProGlyAlaArg  532

GGTGGCCGCCGAATAAACCGGTCTCTTGCCATGATTCACGTGAAACCCGGCTGCCTGCGGCCGGGTTTTGCATTTGCGGCTGGTTTTCAG 1890
 ValAlaAlaGlu---   536

GCATGCGATAGAACGGCCGAGGAAGAACCATGGCAAGCGAACCGCTTTACATCAACGAAAACATCGTGATCGCCGGATGGGAGCTC 1976
```

FIG. 2. Nucleotide and deduced amino acid sequences of the 1,976-bp fragment containing the *R. meliloti pckA* gene. A putative ribosome binding site (nucleotides 193 to 197) is underlined, as is a potential *rho*-independent terminator (nucleotides 1845 to 1865). The probable −35 and −10 regions are overlined, and the transcription start sites are indicated by vertical arrowheads. Putative functional sites are shown in boldface, with the consensus residues underlined and numbered (1, Pck-specific domain; 2 and 4, ATP-binding sites; 3, covalent metal ion binding site). The position of the primer used for primer extension is indicated by a dotted line.

served. The final level of expression was about one-third of that seen with LBmc stationary-phase cells. When both glucose and succinate were present in the culture media (Fig. 5C), *pckA* expression was induced but the highest level of expression reached only one-half that observed with succinate alone. Addition of succinate to log-phase cells growing in glucose minimal medium resulted in the induction of *pck* expression, but again the level of expression reached was only one-half that observed with succinate alone (Fig. 5C).

The regulation of *pckA* gene expression was also tested by growing RmG319 cells in minimal media with several other carbon sources, metabolized via diverse pathways, individually and in combination (Table 2). Cells grown in arabinose, which is metabolized via the TCA cycle in *R. meliloti* (32), had a level

A

% amino acid identity

	R.meliloti	*R.sp.* NGR234	*E.coli*	*S.cerevisiae*	*T.brucei*	*T.cruzi*
R.meliloti	----	90.7	41.8	44.2	44.1	42.6
R.sp. NGR234	93.5	----	42.1	44.3	43	42.2
E.coli	54.1	55.2	----	40.5	36.6	36.2
S.cerevisiae	59.1	58.1	53.7	----	50.8	50
T.brucei	57.4	55.9	47.8	63.7	----	84.3
T. cruzi	53.8	53	47.4	63.1	89.6	----

B

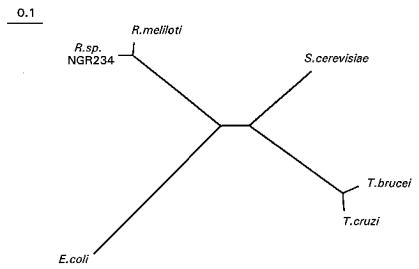

FIG. 3. Amino acid sequence comparison of six known ATP-dependent Pck proteins. (A) The upper right and lower left parts of the table indicate percent identities of the whole protein and a central region (residues 189 to 402 in *R. meliloti*), respectively, between the two species compared. (B) Unrooted tree obtained by neighbor-joining analysis with the PHYLIP software package (12) of the conserved central region of the proteins (residues 106 to 434 in *R. meliloti*). The lengths of the branches reflect the distances of divergence.

of *pckA* expression similar to that of cells grown in succinate as the sole carbon source. Cells grown with sucrose, glycerol, or glucose as the sole carbon source showed undetectable expression of *pckA*. Expression of *pckA* in cells grown on glucose or sucrose in combination with succinate was twofold lower than that in cells grown on succinate alone. Arabinose-induced *pckA* expression was repressed more than 10-fold by glucose but was still significantly greater than that in glucose-grown cells. Cells grown on glycerol plus succinate had a level of expression further reduced twofold from the level observed with cells grown on glucose plus succinate. In addition, glycerol-plus-arabinose-grown cells showed one-quarter the level of expression in cells grown on arabinose alone.

R. meliloti has an inducible transport system specific for C_4-dicarboxylates (Dct) and expression of this transport system is dependent on an alternative sigma factor (σ^{54}) encoded by *rpoN* (51). To determine if the *pckA* induction by succinate is linked to this system, defined mutations in the *dct* genes and in *rpoN* were transduced into RmG319. The resulting RmG319 derivatives were grown with glucose plus succinate or arabinose as sole carbon sources and assayed for β-galactosidase activity (Table 3). The results showed that the presence of the Dct$^-$ mutations abolished succinate-dependent *pckA* expression. However, the induction of *pckA* expression by arabinose occurred normally (Table 3). The latter result presumably reflects the fact that arabinose is assimilated independently of the Dct system.

DISCUSSION

We have identified the *R. meliloti pckA* gene region, determined the transcriptional start site, and determined some basic characteristics of *pckA* expression. Our results show that in the previously characterized *R. meliloti* Pck$^-$ mutants, the *pck-1* and *pck-2* alleles resulted from transposon insertions in the *pckA* structural gene rather than in a regulatory gene (15, 16). It would appear likely that transcription of the *pckA* gene terminates at the *rho*-independent termination sequence im-

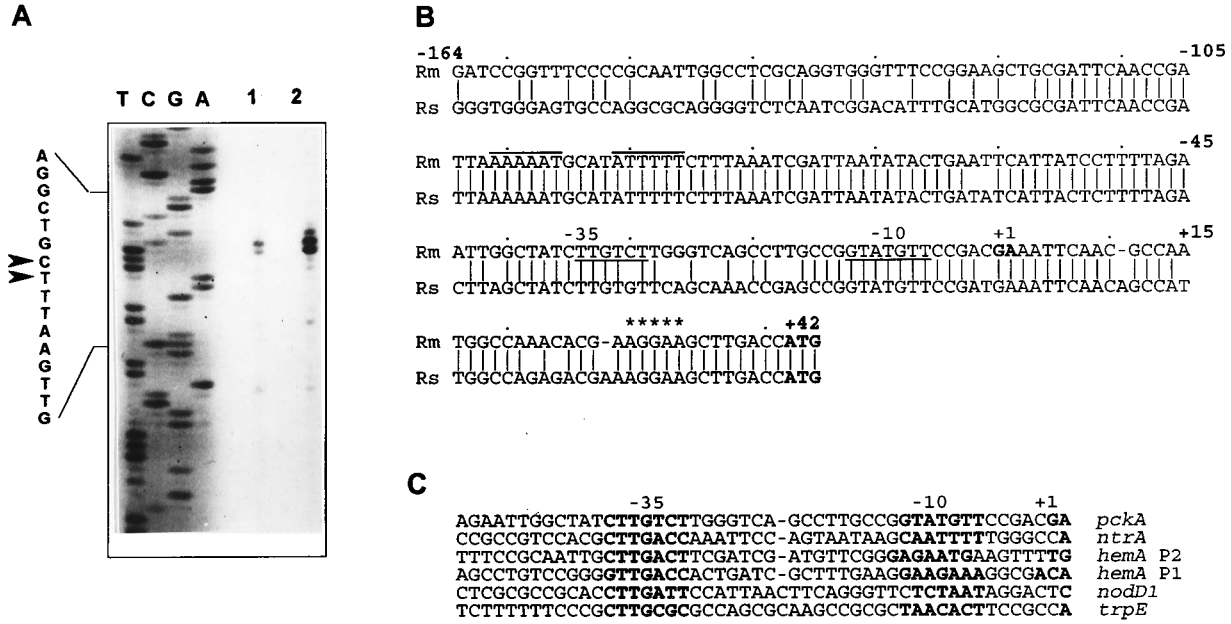

FIG. 4. Promoter analysis of the *R. meliloti pckA* gene. (A) Determination of the transcription start site of *pckA*. Primer extension reactions were performed as described in Materials and Methods. The RNA used was from Rm1021 grown to late log phase in LBmc (lane 1, 30 μg of RNA). Lane 2 shows the product obtained with LB-grown RmG319 RNA (10 μg). The products of the sequencing reactions, using the same primer, are shown on the left of the extension products. The relevant sequence is shown to the left of the gel, and the positions of the major extension products are indicated by arrowheads. (B) Alignment of the *R. meliloti* (Rm) and *Rhizobium* sp. strain NGR234 (Rs) promoter region DNA sequences. The ATG start codon is shown in boldface, as are the two major transcription start sites identified in *R. meliloti*. The −35 and −10 regions deduced from the locations of the start sites are underlined. A conserved hairpin loop is overlined. The putative ribosome binding site is indicated by asterisks. (C) Alignment of identified σ^{70}-like promoters from *R. meliloti* with emphasis on the −35, −10, and +1 regions (shown in boldface).

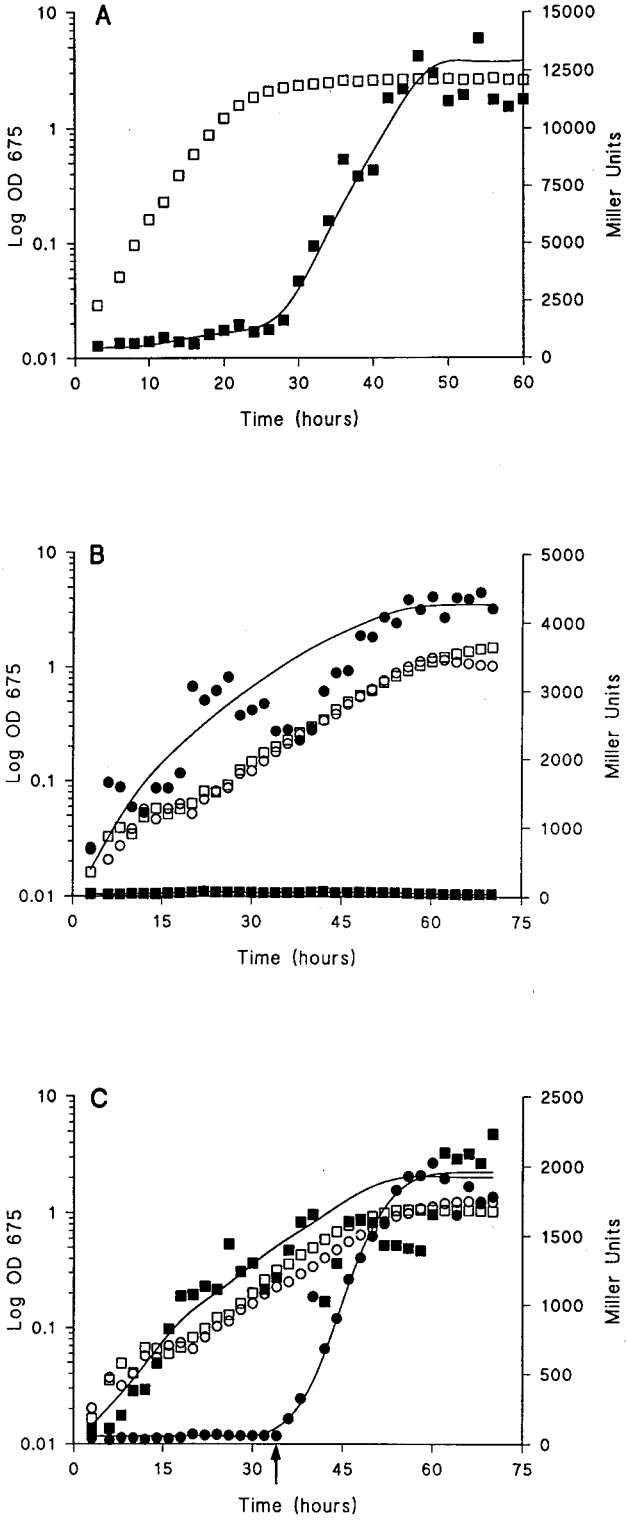

FIG. 5. Relation between growth and *pckA*::*lacZ* fusion expression in different media. Shaded symbols indicate β-galactosidase activity, and open symbols represent the culture density. (A) Growth and β-galactosidase activity observed for RmG319 grown in LB media. (B) Growth and β-galactosidase activity observed for RmG319 grown in M9 containing glucose (□, ■) or succinate (○, ●). (C) Growth and β-galactosidase activity observed for RmG319 grown in M9 containing glucose and succinate (□, ■) or in M9 containing glucose with succinate added at 34 h (arrowhead) (○, ●).

TABLE 2. Effect of growth on different carbon sources on *pckA*::*lacZ* gene expression

Carbon source (15 mM each)	β-Galactosidase activity (Miller units)[a]
Sucrose	73 ± 2
Glucose	35 ± 1
Glycerol	60 ± 4
Succinate	3,871 ± 221
Arabinose	4,095 ± 241
Sucrose + succinate	2,205 ± 20
Glucose + succinate	2,028 ± 210
Glycerol + succinate	1,182 ± 16
Glycerol + arabinose	899 ± 42
Glucose + arabinose	298 ± 11

[a] Each value represents the mean for triplicate assays ± the standard error, expressed as Miller units, with RmG319.

mediately 3′ to the gene; thus, the reduced symbiotic phenotype (15) of the *pck-1* and *pck-2* mutants appears to be a direct consequence of the loss of Pck activity rather than an effect of the insertions on downstream genes.

The DNA sequence of *R. meliloti pckA* was highly homologous (87%) to the *pckA* gene of *Rhizobium* sp. strain NGR234. Such homology has been observed in previous studies in which the sequences of genes from the two species were compared, like those of *rpoN* (79.7%) and *dctA* (86.9%) (47, 48), and is in agreement with recent phylogenetic analysis by 16S rRNA sequence comparison that showed *Rhizobium* sp. strain NGR234, *Rhizobium fredii*, and *R. meliloti* to be very closely related (28, 50). The *pckA* nucleotide sequence conservation in the two *Rhizobium* species not only was limited to the coding region, but also involved the promoter (Fig. 4B) and termination regions.

The transcription start site was found by primer extension to be 42 bp upstream of the start codon ATG in *R. meliloti*. This start site was preceded by potential −10 (TATGTT) and −35 (TTGTCT) sequences which are separated by 17 bp. This promoter structure suggests that *pckA* expression requires a sigma factor analogous to the *E. coli* σ^{70}. A vegetative sigma factor with a molecular mass of 93 kDa, significantly larger than *E. coli* σ^{70}, has previously been identified in *R. meliloti* (38). However, it is not certain that the *pckA* gene is expressed by the vegetative sigma factor, as several alternative sigma factors recently identified in some bacteria, like *rpoS*, can recognize promoters with a closely related −35/−10 structure (25). We compared the promoter regions of four *R. meliloti* genes (*hemA*, *ntrA*, *nodD1*, and *trpE*) whose transcriptional start sites have been determined (1, 7, 18, 30). The *hemA* gene was determined to have two promoters (P1 and P2) (30). Sequence alignment of these promoters together with that of

TABLE 3. Effect of different Dct⁻ backgrounds on the induction of *pckA*::*lacZ* expression by succinate and arabinose

Strain	Relevant characteristics	β-Galactosidase activity (Miller units)[a] in medium containing:	
		Glucose + succinate	Arabinose
RmG319	Wild type carrying pF94	1,864 ± 81	3,086 ± 276
RmH189	*rpoN74*::Tn*5*-233(pF94)	46 ± 1	2,763 ± 67
RmH190	*dctB18*::Tn*5*-233(pF94)	47 ± 2	3,110 ± 321
RmH191	*dctD16*::Tn*5*-233(pF94)	44 ± 1	3,517 ± 223
RmH192	*dctA14*::Tn*5*-233(pF94)	51 ± 1	4,144 ± 150

[a] Each value represents the mean for triplicate assays ± the standard error.

pckA (Fig. 4C) demonstrated that the −35 region was well conserved between the six promoters (TTGacc) and showed a high degree of similarity to the *E. coli* −35 consensus (TTGaca) (24). The −10 sequence was more divergent between the different genes, and so no consensus could be derived. Comparison of only six promoters was not enough to draw a general conclusion on σ^{70}-like promoters in *R. meliloti* other than the fact that, unlike in *E. coli*, the −35 consensus seemed more important than the −10 region. The conservation of the consensus sequences in *Rhizobium* sp. strain NGR234 suggested a similar promoter. The two species were almost identical (95%) in the region further upstream (bp −40 to −120). This region has a high AT content (75%) and a hairpin loop centered at position −95 (Fig. 4B), and it may be required for the regulation of *pckA* expression. If this interpretation is correct, a similar regulation of *pckA* expression in *Rhizobium* sp. strain NGR234 would be expected.

The protein encoded by *pckA* was very similar to the other ATP-binding-type Pck proteins characterized. Phylogenetic analysis by several independent methods separated the prokaryotic proteins from those of the lower eukaryotes but showed divergence within the two groups (Fig. 3B). Analysis of the protein revealed conserved regions, putatively essential to enzyme function. These include, in addition to the potential ATP-binding sites and a divalent metal ion binding site, a region that is also present in GTP-dependent Pck enzyme but whose function is unknown (31). Biochemical analysis of the yeast Pck enzyme has identified two reactive cysteines (Cys-364 and Cys-457), present in the GTP-dependent Pcks, whose chemical alteration resulted in enzyme inactivation (3). These two residues are not conserved among the ATP-dependent Pcks and biochemical analysis of the *E. coli* Pck did not identify any reactive cysteine (8), which would suggest that these cysteines are not required per se for the enzyme function.

There appear to be two types of regulation of *pckA* in *R. meliloti* (Fig. 5; Tables 2 and 3). A growth phase effect was observed in LBmc-grown cells, as the expression was induced 10-fold at the onset of the stationary phase (Fig. 5A). Although this induction was not observed to occur in cells grown in minimal medium (Fig. 5B), it should be noted that these cells stopped growing at a much lower cell density. The *E. coli pckA* gene was also observed to be induced (100-fold) in stationary-phase cells (21); however, the mechanism by which this regulation occurs remains to be determined. It is possible that *rpoS*, which is required for expression of many stationary-phase genes, is also involved in the stationary-phase regulation of *pckA* (25).

The expression of *pckA* was also dependent on the carbon source present in the growth media (Table 2; Fig. 5). Gluconeogenic carbon sources like succinate and arabinose induced *pckA* expression, whereas no expression was observed to occur in cells growing on glycolytic carbon sources like glucose and sucrose. Glucose or sucrose in succinate minimal media reduced the level of *pckA* expression by 50%; however, this reduction differs from the absence of detectable Pck activity observed when sucrose was added to phosphate-limited chemostat cultures of *R. leguminosarum* growing with fumarate as the carbon source (33). It is possible that the difference in results reflects the different culture conditions employed; it is also worth noting that we monitored *pckA*::*lacZ* expression, whereas McKay and colleagues assayed Pck activity. The difference in these results is worth pursuing because of the implications of these results for the physiological status of the bacteroid.

The lack of *R. meliloti pckA* expression in cells grown on glycerol contrasts with what is observed with *E. coli*, where growth on glycerol results in high-level *pckA* expression (21). Growth on glycerol requires gluconeogenesis from glyceraldehyde-3-phosphate and dihydroxyacetone phosphate to hexose and hexose phosphates and thus does not require Pck (16, 22). In *E. coli pckA* is subject to catabolite repression by glucose; hence, the expression in glycerol-grown cells may well result from derepression of the *pckA* gene. Moreover, in *R. meliloti*, unlike *E. coli*, succinate catabolism results in a catabolite repression of lactose and mannose utilization (4). The lack of *pckA* expression in *R. meliloti* cells grown on glycerol is consistent with the apparent inability of glucose or sucrose to repress succinate-dependent *pckA* expression (Table 2; Fig. 5C). Interpretation of the two-carbon-source data in Table 2 is complicated by the possibility that utilization of one substrate could repress utilization of the other. Hence, the apparent glucose-mediated repression of the arabinose-dependent *pckA* expression may be an indirect consequence of repression of the arabinose transport system or metabolism. While these possibilities remain to be resolved, we have shown that *pckA* expression occurs independently of the regulatory genes *dctB* and *dctD* and the *rpoN* gene encoding σ^{54}, and that (as expected) succinate-mediated induction of *pckA* expression requires succinate transport (Table 3). We have begun to characterize mutants which express *pckA* in glucose minimal medium, and through the analysis of these mutants we hope to identify genes directly involved in *pckA* gene expression.

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