

Genes Coding for Phosphotransacetylase and Acetate Kinase in *Sinorhizobium meliloti* Are in an Operon That Is Inducible by Phosphate Stress and Controlled by PhoB

MICHAEL L. SUMMERS,[†] MICHAEL C. DENTON, AND TIMOTHY R. McDERMOTT*

Department of Land Resources and Environmental Sciences, Montana State University, Bozeman, Montana 59717

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Recent work in this laboratory has shown that the gene coding for acetate kinase (*ackA*) in *Sinorhizobium meliloti* is up-regulated in response to phosphate limitation. Characterization of the region surrounding *ackA* revealed that it is adjacent to *pta*, which codes for phosphotransacetylase, and that these two genes are part of an operon composed of at least two additional genes in the following order: an open reading frame (*orfA*), *pta*, *ackA*, and the partial sequence of a gene with an inferred peptide that has a high degree of homology to enoyl-ACP reductase (*fabI*). Experiments combining enzyme assays, a chromosomal *lacZ::ackA* transcriptional fusion, complementation analysis with cosmid subclones, and the creation of mutations in *pta* and *ackA* all indicated that the *orfA-pta-ackA-fabI* genes are cotranscribed in response to phosphate starvation. Primer extension was used to map the position of the phosphate starvation-inducible transcriptional start sites upstream of *orfA*. The start sites were found to be preceded by a sequence having similarity to PHO boxes from other phosphate-regulated genes in *S. meliloti* and to the consensus PHO box in *Escherichia coli*. Introduction of a *phoB* mutation in the wild-type strain eliminated elevated levels of acetate kinase and phosphotransacetylase activities in response to phosphate limitation and also eliminated the phosphate stress-induced up-regulation of the *ackA::lacZ* fusion. Mutations in either *ackA* alone or both *pta* and *ackA* did not affect the nodulation or nitrogen fixation phenotype of *S. meliloti*.

Through a complex set of biochemical communications, bacteria in the family *Rhizobiaceae* interact with specific legume hosts to produce root structures known as nodules. Numerous bacterial genes are involved in nodule formation and function. Two of the better-studied examples include the nodulation (*nod*) genes, which are expressed in response to isoflavones secreted by the plant (reviewed in references 14, 28, and 48), and the *dct* genes, which are required for uptake of dicarboxylic acids (malate and/or succinate), the primary sources of energy for the bacteria during symbiosis (reviewed in references 13, 33, and 51). In different rhizobia, two-component regulatory systems have been shown to be involved in the positive regulation of both of these activities (20, 21, 40). Two-component regulatory protein systems consist of a sensor histidine kinase which is paired with a cognate-response-regulatory protein. The sensor kinase detects a particular environmental or metabolic signal and transduces the signal to the cell via phosphorylation of the regulator protein (11, 50). When phosphorylated, the regulatory protein is then activated to increase the transcription of target genes under the control of the regulatory pair.

In addition to the sensor kinase, some response-regulatory proteins have been shown to be phosphorylated by other, less specific, secondary mechanisms involving low-molecular-weight phosphorylated compounds such as phosphoramidate, carbamylphosphate, and acetyl phosphate (19, 29–31, 55). Of these, the most extensively studied has been acetyl phosphate. Synthesis of acetyl phosphate is controlled directly by the en-

zymes acetate kinase and phosphotransacetylase (Fig. 1), which are encoded by the genes *ackA* and *pta*, respectively. Both reactions are reversible and, depending on the metabolic activities leading to the synthesis of their substrates, can influence the acetyl phosphate pool size. Factors thus far shown to influence the intracellular concentration of acetyl phosphate include the growth phase, carbon source, and temperature (30, 38, 56). Acetyl coenzyme A (acetyl-CoA) is a substrate for the Pta reaction and is a central metabolic intermediate tied directly to all anabolic processes. Fluctuation of acetyl-CoA levels in response to changing growth conditions could play an important role in determining acetyl phosphate concentrations and, thus, indirectly play a role in affecting gene expression via this less-specific phosphotransferase mechanism. Transcriptional control of *ackA* and *pta* would also be expected to be potentially important in this regard; however, comparatively little is known about the regulation of these genes (12).

The potential for acetyl phosphate to act as a signaling metabolite in the *Rhizobium*-legume symbiosis has been briefly examined for the *nod* and *dct* regulatory systems mentioned above. Loh et al. (27) recently reported that *Bradyrhizobium japonicum* NodW can be phosphorylated by acetyl phosphate in vitro, but Gu et al. (23) were unable to demonstrate this alternative phosphorylation mechanism for *Sinorhizobium meliloti* DctD. Preston et al. (37) and Smith et al. (46) examined the activities of both Pta and AckA in *B. japonicum* bacteroids and found that the levels of both enzymes increased in parallel with nodule nitrogen fixation (acetylene reduction) activity. This implied that both enzymes might contribute significantly to bacteroid carbon metabolism and, depending on the relative rates of each enzyme, might allow for the accumulation of acetyl phosphate to levels that could, in turn, influence gene expression.

In this study, we characterized a locus in *S. meliloti* that contains the genes coding for Pta and AckA. We also created

* Corresponding author. Mailing address: Department of Land Resources and Environmental Sciences, Montana State University, Bozeman, MT 59717. Phone: (406) 994-2190. Fax: (406) 994-3933. E-mail: timmder@gemini.oscs.montana.edu.

[†] Present address: Department of Biology, California State University—Northridge, Northridge, CA 91330-8303.

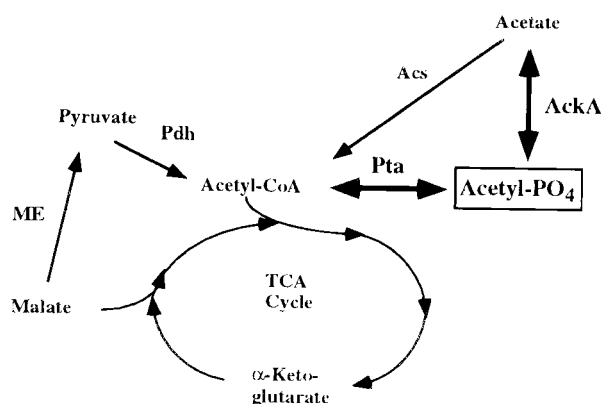


FIG. 1. The acetyl phosphate pathway in relation to the tricarboxylic acid cycle, which is a major metabolic pathway in *S. meliloti* bacteroids. The acetyl phosphate pathway is shown with thick boldfaced arrows. Abbreviations: TCA, tricarboxylic acid; ME, malic enzyme; Acs, acetyl-CoA synthase; AckA, acetate kinase; Pta, phosphotransacetylase; Pdh, pyruvate dehydrogenase.

pta and *ackA* mutants to establish metabolic blockades that would either impede the synthesis of acetyl phosphate or favor its accumulation. We used these mutants to assess the potential importance of acetyl phosphate acting as a signaling metabolite in the *S. meliloti*-alfalfa symbiosis. These mutations also allowed us to ask whether carbon metabolism via the Pta-AckA pathway is important to alfalfa bacteroids for normal symbiotic function.

MATERIALS AND METHODS

Strains and growth conditions. The strains of *S. meliloti* and *Escherichia coli* used in this study are shown in Table 1. *S. meliloti* wild-type strain 104A14 (47)

was the parent strain from which all mutants were derived. The isolation of the *S. meliloti ackA::Tn5B22* mutant strain *psi25* was performed as previously described (52), and in this study this strain has been renamed RmMSU4. Transposon Tn5B22 is a derivative of Tn5 (44) and carries a promoterless *lacZ* gene. When Tn5B22 inserts in the correct orientation relative to a nearby promoter, a *lacZ* transcription fusion construct that can be used to monitor gene expression is created. The genotypes and construction of other mutants are described below.

The basic medium used for culturing all strains was a minimal mannitol ammonium chloride (MMN) medium (47) that was modified to contain no added inorganic phosphate (P_i) salts in the basal medium but was buffered by 10 mM morpholinepropanesulfonic acid (MOPS; pH 7.0) and amended with various amounts of P_i . In some experiments, *S. meliloti* was cultured on YMB agar (47). *E. coli* DH5 α was used as a host strain for plasmid constructions, and *E. coli* S17-1 was used for conjugal transfer of plasmids to *S. meliloti*; both *E. coli* strains were cultured on Luria-Bertani medium (41). Ampicillin ($100 \mu\text{g} \cdot \text{ml}^{-1}$), gentamicin ($25 \mu\text{g} \cdot \text{ml}^{-1}$ for agar media; $15 \mu\text{g} \cdot \text{ml}^{-1}$ in broth cultures), streptomycin-spectinomycin (each at $200 \mu\text{g} \cdot \text{ml}^{-1}$), and/or 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal; $40 \text{ mg} \cdot \text{liter}^{-1}$ for agar media) was included as required.

Plasmids and mutagenesis. The plasmids used in this study are also shown in Table 1. DNA flanking the transposon cloned from RmMSU4 was used as a probe to identify a hybridizing colony in blots of a *S. meliloti* cosmid library, the construction of which was previously described (2). The library insert in the hybridizing cosmid p7C9 was subcloned in part or in full into pBluescript KS(+) and pCPP30 for sequencing and complementation experiments, respectively. The *S. meliloti phoB* gene from strain 104A14 was previously cloned on a 2.3-kb *HindIII* fragment (2), and for experiments in this study this fragment was subcloned into pUC19 and inactivated by insertion of an Ω cassette (18) into the unique *EcoRV* site located within the *phoB* coding region. This entire construct was linearized with *XbaI* and subcloned into pJQ200SK⁺ to create pMLS90. A *pta* mutation was similarly constructed by insertion of the Ω fragment into the *pta* gene. A 9-kb *SacI* fragment containing the *S. meliloti pta* gene was subcloned from p7C9 into pBluescript KS(+) which had been partially digested with *KpnI* and ligated to a Ω fragment released from pRL453 by *KpnI* digestion. A clone containing an Ω insertion within *pta* was identified by restriction mapping, linearized with *XbaI*, and then ligated to pJQ200SK⁺ to form pMLS101.

Both pMLS90 and pMLS101 were transformed into *E. coli* S17-1 for conjugation with *S. meliloti*. Isolation of *phoB::\Omega* and *pta::\Omega* allelic replacements in transconjugants was performed on MMN agar containing 5 mM P_i and amended with gentamicin, streptomycin, spectinomycin, and 10% (wt/vol) sucrose. Gene replacements were verified by Southern blotting with the wild-type *phoB* and *pta* genes as probes (2), by the loss of inducible alkaline phosphatase activity for the

TABLE 1. Strains of *S. meliloti* and *E. coli* and plasmids used in this study

Strain or plasmid	Relevant genotype or characteristic ^a	Reference or source
<i>S. meliloti</i> strains		
104A14	Wild type	47
RmMSU4	104A14 <i>ackA::Tn5B22</i>	52
RmMSU5	104A14 <i>pta::\Omega ackA::Tn5B22</i>	This study
RmMSU6	104A14 <i>pta::\Omega</i>	This study
RmMSU7	104A14 <i>phoB::\Omega ackA::Tn5B22</i>	This study
<i>E. coli</i> strains		
S17-1	Pro ⁻ Mob ⁺ ; donor strain in matings	43
DH5 α	Cloning host	41
Plasmids		
pUC19	Cloning vector; Amp ^r	58
pBluescript KS(+)	Cloning and sequencing vector; Amp ^r	Stratagene
pJQ200KS ⁺	<i>sacB sacR</i> Gent ^r Suc ^S	39
pCPP30	Broad-host-range plasmid; Gent ^r	D. Bauer, Cornell University
pRL453	pUC18/19 containing L.EHE1 and C.S3; Ω fragment	18
p7C9	Cosmid library clone containing the <i>S. meliloti orfA-pta-ackA-fabI</i> operon	This study
pMLS3	pCPP30 with 2.4-kb <i>XmaI-SacI</i> p7C9 fragment; Gent ^r	This study
pMLS4	pCPP30 with 3.2-kb <i>BamHI-SacI</i> p7C9 fragment; Gent ^r	This study
pMLS5	pCPP30 with 4.0-kb <i>BamHI</i> p7C9 fragment; Gent ^r	This study
pMLS6	pCPP30 with 8.8-kb <i>NotI</i> p7C9 fragment; Gent ^r	This study
pMLS7	pCPP30 with 5.0-kb <i>EcoRI-SacI</i> p7C9 fragment; Gent ^r Pta ⁺	This study
pMLS8	pCPP30 with 9-kb <i>SacI</i> p7C9 fragment; Gent ^r Pta ⁺	This study
pMLS90	pJQ200KS ⁺ ligated to linearized pUC19 containing the Ω -interrupted <i>phoB</i> in a 2.3-kb <i>HindIII</i> fragment	2; this study
pMLS101	pJQ200KS ⁺ containing the linearized 9-kb <i>SacI</i> p7C9 fragment ligated to pBluescript KS(+); <i>pta::\Omega</i>	This study

^a Amp^r, ampicillin resistant; Gent^r, gentamicin resistant; Suc^S, sucrose sensitive.

phoB mutant (2), and by the lack of P_i starvation-inducible acetate kinase and phosphotransacetylase activities in the different mutants (procedures described below).

Nucleic acid manipulations. The protocols of Sambrook et al. (41) were used for all manipulations of plasmid and chromosomal DNA. In previous work in which we sequenced the DNA flanking the Tn5B22 insert in RmMSU4, it was determined that the transposon was in the correct orientation for reporting *ackA* transcription (52). Briefly, total chromosomal DNA was harvested, digested with *Xma*I or *Sal*I, and then ligated into pBluescript KS(+). The ligated construct was transformed into *E. coli* DH5 α , and plasmids from transformants resistant to ampicillin and gentamicin were analyzed by restriction analysis to verify that each contained a single cloned fragment. Southern blotting was used to verify that the cloned fragment was identical to that in the genome from RmMSU4. Sequencing of the flanking DNA was done by the dideoxynucleotide method, using a kit purchased from United States Biochemical (Cleveland, Ohio). Primers 5'-AAC GACGGATCCATAAT-3' and 5'-CCATGTTAGGAGGTCACATGGAAGT CAG-3' were used to initiate sequencing from the *lacZ* and transposase termini, respectively (44). Large-scale sequencing of segments of p7C9 that contained the complementing *pta* and *ackA* genes was accomplished with an ABI 377 DNA sequencer (Perkin-Elmer, Norwalk, Conn.), using synthetic primers complementary to nucleotide sequences determined within the cloned fragments. Sequence homology searches were conducted by using the BLAST network service (3), and sequence alignments were done with the GAP program (15).

RNA isolation and primer extension. Total RNA was harvested from cells by the method described by Schmidt-Goff and Federspiel (42). Primer extension and end labeling of the oligonucleotide primer with [³²P]ATP (DuPont-NEN; 3,000 Ci mmol⁻¹, 10 mCi ml⁻¹) were performed on 20 μ g of strain 104A14 RNA as described by Ausubel et al. (4), using polynucleotide kinase and avian myeloblastosis virus reverse transcriptase from Promega (Madison, Wis.). The primer sequence was 5'-GAGCGGCAAGGCCGCAATCCCAATTAT-3'. Sequencing ladders employing the same primer were generated by the dideoxy termination method, using Sequenase II T7 polymerase (United States Biochemical) and Sequette (DuPont-NEN). The primer extension product with the corresponding sequencing ladder was separated on 6% polyacrylamide-7 M urea sequencing gels.

Phosphate stress induction experiments. For phosphate starvation experiments, cells were grown to mid-exponential phase in MMN broth containing 5 mM P_i and antibiotics as required, washed twice with MMN lacking P_i (25°C), resuspended in P_i -free MMN to an A_{595} of 0.2, and then divided into two cultures. No P_i was added to one of the cultures, while the other culture was amended to 5 mM P_i . Both cultures were incubated in a reciprocal shaking water bath at 30°C. After the desired incubation period, a culture sample was taken for measurement of Pta, AckA, or β -galactosidase activity.

Enzyme assays. β -Galactosidase activity was measured as previously described (52). For measuring phosphotransacetylase and acetate kinase activities, both P_i -grown and non- P_i -grown cells were washed and resuspended in a buffer that contained 50 mM Tris-HCl (pH 7.5) and 10 mM MgCl₂ and then were disrupted by sonication. The sonicate was centrifuged at 10,000 $\times g$ for 5 min to pellet debris, and then the resulting supernatant was removed and stored on ice for use in enzyme assays.

Determinations of phosphotransacetylase and acetate kinase activities were based on acetyl phosphate production or consumption and were measured by the hydroxamate assay (26). Phosphotransacetylase activity was assayed by the method described by Stadtman (49). Briefly, the 333- μ l assay mixture contained 10 mM Tris-HCl, 6 mM acetyl phosphate, 100 mM potassium chloride, 10 mM cysteine, 1 mM CoA, and 20 to 40 μ l of cell extract. After a 10-min incubation at 25°C, the reaction mixture was supplemented with sodium arsenate to 50 mM, incubated for an additional 45 min at 25°C, diluted 1:1 with 2 M neutralized NH₂OH-HCl, and incubated for a further 5 min at 25°C. The reaction was stopped by adding trichloroacetic acid to a final concentration of 5%. The final volume was brought to 1.5 ml with 2.5% FeCl₂ (dissolved in 2 N HCl) and centrifuged at 13,000 $\times g$ for 5 min to clear insoluble material, and then the absorbance was measured at 540 nm against a water blank. Acetate kinase activity was measured as described by Aceti and Ferry (1). In a total volume of 333 μ l, the assay mixture contained 145 mM Tris-HCl, 200 mM potassium acetate, 10 mM ATP, and 0.7 M neutralized NH₂OH-HCl; cell extract was added to initiate the reaction. After incubation at 25°C (15 to 30 min), trichloroacetic acid was added to a concentration of 5% to stop the reaction. The reaction mixture was then brought to a volume of 1 ml with 2.5% FeCl₂ (dissolved in 2 N HCl), incubated at 25°C for 15 min to allow for color development, and centrifuged at 13,000 $\times g$ for 5 min to clear insoluble material, and then the A_{540} was determined. Assays without added acetate and ATP were also conducted to correct for levels of acyl phosphates in cell extracts.

Plant growth and inoculation. Axenic alfalfa plants were cultured in Magenta growth boxes (Sigma, St. Louis, Mo.) that were described previously (32), using the sand-alumina plant culture technique (22) to control the P_i supply at levels that were P_i limiting and non- P_i limiting for the plants (2).

For each strain, the stability of the transposon and/or Ω cassette during symbiosis was assessed. Nodules were surface sterilized as described previously (2) and then crushed in a 0.85% (wt/vol) saline solution prior to being serially diluted; aliquots were then spread onto YMB agar. Sixty isolated colonies of each mutant were subcultured onto MMN and MMN plus antibiotic to determine

whether antibiotic resistance was retained. Southern blot analysis of chromosomal DNA from five nodule isolates for each strain was conducted to verify that the location of the transposon and/or Ω cassette had not changed.

Nucleotide sequence accession number. The nucleotide sequence of the *orfA-pta-ackA-fabI* gene sequence has been submitted to GenBank (accession no. AF095903).

RESULTS

Sequence analysis of the *S. meliloti ackA* locus. Using transposon Tn5B22 as a promoter probe, we previously identified *ackA* as one of several *S. meliloti* genes that are up-regulated in response to P_i stress (52). Identification of the interrupted *ackA* gene in mutant strain RmMSU4 was accomplished by cloning the transposon and flanking DNA from RmMSU4 (previously designated *psi25* [52]), sequencing the transposon-chromosome junctions, and then conducting homology searches of major databases (52). To more extensively characterize this locus, we used a fragment of DNA flanking the transposon to probe an *S. meliloti* cosmid library (2) and obtained cosmid p7C9, which contained *ackA*. Restriction mapping, Southern blot analysis, and enzyme assays following P_i starvation treatments confirmed that *ackA* was preceded by a suitable amount of DNA such that the transcriptional control elements for *ackA* could be studied (results not shown). A variety of hybridizing DNA fragments were subcloned from p7C9 into pBluescript and either partially or completely sequenced.

The results of this sequencing indicated the presence of three genes in addition to *ackA*. The first is a 2,199-bp open reading frame (ORF) capable of encoding a protein of 80.5 kDa. It does not have any homologs in the major protein or nucleotide databases and was designated *orfA*. It is preceded by a putative ribosome binding site and followed by a 182-bp region containing several stem-loops, but no factor-independent terminators were identified by computer analysis. This short segment was followed by a second ORF, 807 nucleotides in length and preceded by a ribosome binding site. This ORF is capable of being translated into a 28.5-kDa protein having an inferred amino acid sequence with 29% identity and 50% similarity to the phosphotransacetylase from *Clostridium acetobutylicum* (9). Phosphotransacetylases differ in size, and the identified gene is of the smaller variety that includes phosphotransacetylases from *C. acetobutylicum* (9) and *Methanosarcina thermophila* (25).

Following *pta* is an ORF spanning 1,179 bp that encodes an inferred 42.1-kDa peptide with 35% identity and 61% similarity to the acetate kinase from *C. acetobutylicum* (9). The predicted size, based on translation initiation occurring at the second methionine within the ORF, is supported by protein alignments with other inferred acetate kinases described in the databases. This initiation site would place the translational start site just 6 bp from the termination codon of *pta*.

Immediately downstream from *ackA* is an ORF coding for an inferred peptide with 50% identity and 70% similarity (based on partial sequence) to FabI, the enoyl-ACP reductase from *E. coli* (8). The putative *fabI* gene is preceded by a strong ribosome binding site in the small, 14-bp intergenic region. The sequenced region of the putative *fabI* sequence also exhibits similarly high levels of homology to *fabI* genes of several other organisms, which encode enzymes ranging in length from 262 to 278 amino acids, thus indicating that only approximately 20% of the corresponding *S. meliloti fabI* gene has been sequenced. The inferred peptide from the putative *S. meliloti fabI* gene identified here is most closely related to other enoyl-ACP reductases utilizing NADH as a substrate. The close association with *ackA* suggests the possibility of cotranscription

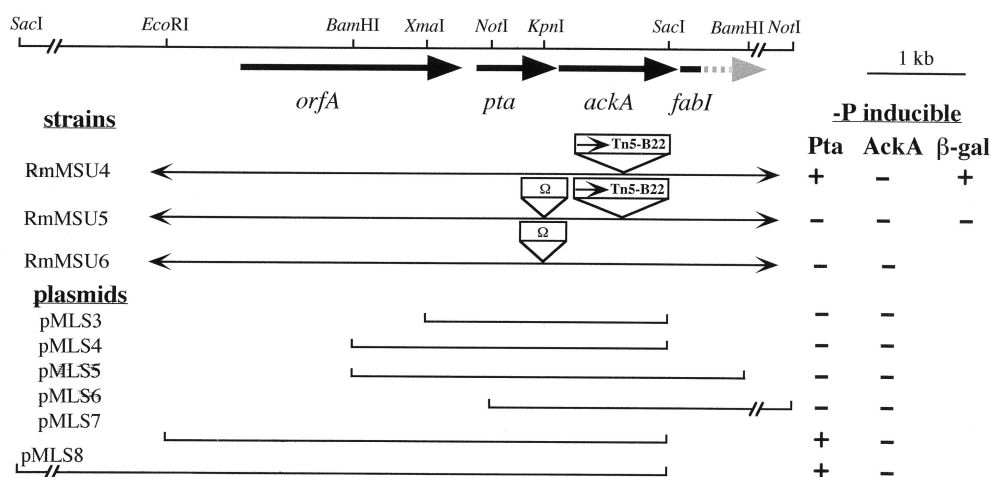


FIG. 2. Operon and complementation analysis of the *S. meliloti* *SacI-NotI* fragment containing the *orfA-pta-ackA-fabI* operon. An abbreviated *SacI-NotI* fragment is shown at the top, with thick boldfaced arrows delineating the boundaries of each gene shown. The positions of the restriction sites used in subcloning analysis are as shown. Below the fragment, mutant strain numbers with the location of Tn5B22 transposon or the Ω interposon in the *pta* and *ackA* genes are shown. At the bottom, plasmid numbers, along with their corresponding subclone inserts, are shown relative to the relevant restriction sites. At the right, the P_i starvation ($-P_i$) inducible activities of the Pta and AckA enzymes (+), or the lack thereof (-), as well as reporter enzyme levels (where relevant) are shown for each construct or strain. The inducibility of all plasmid-borne Pta and AckA activities was assessed in an RmMSU6 mutant background.

of *fabI* with upstream genes and is supported by the inability to detect independent transcription in primer extension analyses. Further evidence for cotranscription was supported by Northern analyses which showed the presence of an irresolute 7- to 10-kb transcript unique to phosphate-starved cells (data not shown).

Operon studies: mutational and complementation analysis of gene expression. The *orfA-pta-ackA-fabI* gene arrangement, the locations of Tn5B22 and the Ω cartridge in the mutant strains, and the different plasmid constructs are shown in Fig. 2. Enzyme activity levels observed under P_i -limiting and non- P_i -limiting conditions with different combinations of the mutant strains and plasmid constructs are presented in Table 2. Consistent with the sequence analysis of the Tn5B22 insertion in *ackA*, *S. meliloti* RmMSU4 lacked P_i starvation-inducible AckA activity. P_i starvation-inducible AckA activity could be restored to normal levels when this strain contained cosmid p7C9 (results not shown). However, p7C9 DNA fragments contained in pMLS3, pMLS4, pMLS7, or pMLS8 (Fig. 2) did not result in restoration of inducible AckA activity. For pMLS7 and pMLS8, this was apparently due to the absence of the C-terminal 45 amino acids of AckA beyond the *SacI* site (Fig. 2). When the complete AckA coding region was present in

pMLS6 but the upstream sequence was not (Fig. 2), there was a slightly increased basal level of AckA activity that lacked normal inducibility (Table 2) (similar results were obtained for pMLS5 [data not shown]). Strain RmMSU6, containing *pta* interrupted by the Ω cassette (Fig. 2), showed no P_i stress-inducible Pta activity (Table 2). Furthermore, the mutated *pta* in strain RmMSU6 also eliminated P_i stress-inducible AckA activity (Fig. 2; Table 2). RmMSU4 containing pMLS7 (data not shown) or pMLS8 (Table 2) displayed very high levels of Pta activity in response to P_i limitation; presumably this was due to a gene dosage effect. A *pta::\Omega* mutation was also introduced into RmMSU4 to create the *ackA pta* double-mutant strain RmMSU5 (Fig. 2), and as would be predicted, it had no P_i stress-inducible Pta or AckA activity (Table 2). The mutated *pta* in strain RmMSU5 also eliminated the P_i stress-inducible *ackA::lacZ* reporter activity (Fig. 2; also see Fig. 5 below). Together, these results indicate that *pta* and *ackA* are transcribed as an operon from a promoter located upstream of *pta*.

To obtain additional information to help identify the region containing the P_i stress-inducible promoter for the putative *orfA-pta-ackA-fabI* operon, cosmid subclones were analyzed for their ability to complement the loss of inducible Pta activity in RmMSU6. Neither pMLS3 nor pMLS4 (Fig. 2), containing

TABLE 2. Acetate kinase and phosphotransacetylase activities in wild-type and mutant *S. meliloti* strains

Strain	Genotype	Plasmid (relevant gene)	Phosphotransacetylase activity ^a		Acetate kinase activity ^a	
			+ P_i	- P_i	+ P_i	- P_i
104A14	Wild type		20 ± 10	100 ± 26	12 ± 3	134 ± 5
RmMSU4	<i>ackA::Tn5-B22</i>		21 ± 3	98 ± 16	7 ± 1	11 ± 1
RmMSU5	<i>pta::\Omega ackA::Tn5-B22</i>		35 ± 1	17 ± 2	11 ± 1	10 ± 2
RmMSU6	<i>pta::\Omega</i>		20 ± 10	17 ± 2	6 ± 1	11 ± 2
RmMSU7	<i>phoB::\Omega ackA::Tn5-B22</i>		21 ± 2	28 ± 3	9 ± 3	13 ± 1
RmMSU4	<i>ackA::Tn5-B22</i>	pMLS8 (<i>pta</i>)	43 ± 23	726 ± 107	8 ± 2	15 ± 3
RmMSU4	<i>ackA::Tn5-B22</i>	pMLS6 (<i>ackA</i>)	26 ± 7	70 ± 18	33 ± 1	47 ± 9
RmMSU6	<i>pta::\Omega</i>	pMLS8 (<i>pta</i>)	90 ± 22	716 ± 86	6 ± 1	10 ± 2
RmMSU6	<i>pta::\Omega</i>	pMLS6 (<i>ackA</i>)	27 ± 6	25 ± 3	18 ± 2	27 ± 5

^a Values are means ± standard errors for data from three to six separate experiments. Activities are expressed in nanomoles of acetyl phosphate consumed (Pta) or generated (AckA) per minute per milligram of protein.

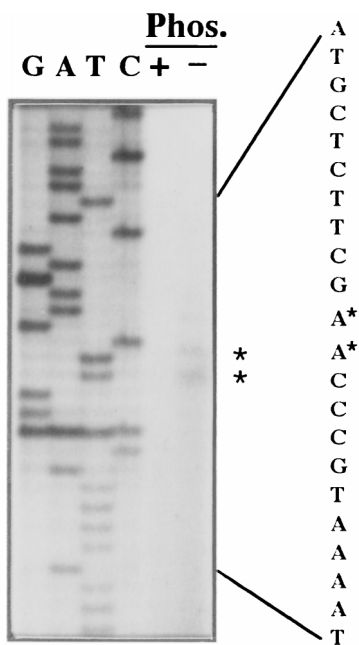


FIG. 3. Determination of the transcriptional start sites for the *S. meliloti orfA-pta-ackA-fabI* operon by primer extension. RNA was purified from cells of wild-type strain 104A14 after incubation under high-phosphate culture conditions (Phos. +) or under phosphate starvation conditions (Phos. -). Lanes G, A, T, and C show the sequence ladders generated with the same primer as that used in the extension reaction, with pMLS7 as a template. Relevant complementary DNA sequence is shown on the right, and the positions of the start sites are indicated with asterisks.

547 and 1,360 bp of DNA upstream of *pta*, respectively, was able to complement the Pta⁻ phenotype. However, pMLS7, containing all of *pta* and *orfA* and 793 bp of DNA upstream of *orfA*, was able to complement the Pta⁻ mutant, as did pMLS8, which contains a 9-kb *SacI* fragment. These results indicated that the promoter is located in the region upstream of *orfA*.

Promoter analysis and mutational confirmation of PhoB regulation. A P_i stress-inducible transcriptional start site was identified by primer extension analysis using a primer complementary to bp 671 to 697. The 73- to 74-nucleotide extension products identified two strong start sites, 171 or 172 bp upstream of the putative translational start predicted for OrfA (Fig. 3). Sequence 5' of the transcriptional start was found to have significant similarity to PHO box sequences identified for other *S. meliloti* P_i-sensitive genes (5) (GenBank accession no. M96263). The alignment of this region with other predicted

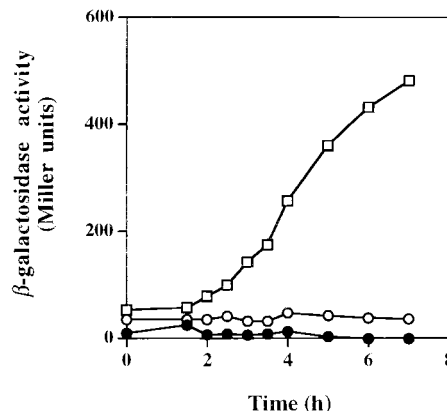


FIG. 5. Reporter enzyme activity of an *ackA::lacZ* transcriptional fusion construct in response to phosphate starvation. Cells of each strain were prepared and assayed as described in Materials and Methods. Reporter enzyme levels (in Miller units) for strains RmMSU4 (*ackA::Tn5B22*) (□), RmMSU5 (*pta::Ω ackA::Tn5B22*) (●), and RmMSU7 (*ackA::Tn5B22 phoB::Ω*) (○) are shown. Data points are the averages of data from duplicate cultures from one of three independent experiments documenting these responses. Reporter enzyme levels failed to increase when washed cells of all three strains were resuspended in high-phosphate medium (results not shown to simplify presentation).

P_i-regulated promoter regions allows the construction of an *S. meliloti* consensus PHO box (Fig. 4). The resulting consensus sequence shows similarities to that of *E. coli* (57). In addition to the PHO box, another feature of the promoter region is a less-understood, purine-rich direct repeat with the sequence 5'-AATAAAA-3'. The repeat is separated by 3 bp and is located in the leader sequence beginning 7 bp downstream from the transcriptional start site. An association of this direct repeat with any regulatory function has not yet been determined.

The sensitivity of this operon to P_i availability and the identification of a putative PHO box suggested that these genes are part of the Pho regulon in this organism and are under the control of the response-regulatory protein PhoB (6). To test this hypothesis, a *phoB* mutation was introduced into strain RmMSU4 to create strain RmMSU7. Following removal of P_i from the medium, strain RmMSU7 did not show inducible reporter activity during 7 h of P_i starvation (Fig. 5). Inducible β-galactosidase reporter enzyme activity was still not observed following overnight induction (results not shown). The *phoB* mutation had similar effects on Pta activity (Table 2); Pta activity in P_i-limited RmMSU7 cells did not increase beyond constitutive levels. The lack of reporter enzyme induction in strain RmMSU5 also demonstrated the polar effect of the Ω

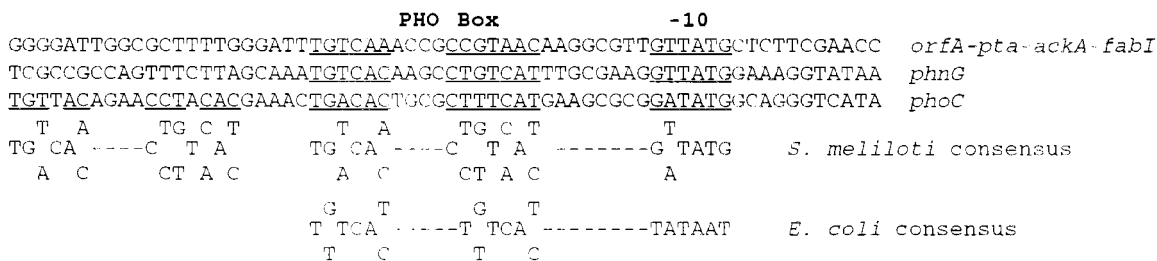


FIG. 4. Suggested structure of a consensus *S. meliloti* PHO box. The consensus nucleotide sequence is derived from a comparison of single PHO boxes identified in the promoter region of *phnG* (GenBank accession no. M96263) and the *orfA-pta-ackA-fabI* operon, as well as the two tandem PHO boxes reported for *phoC* (5). The *E. coli* consensus PHO box (57) is shown at the bottom for comparison. Nucleotides matching the *S. meliloti* consensus sequence for the -10 region and PHO box are underlined.

insert on the expression of the *ackA::Tn5B22* transcriptional fusion construct (Fig. 5) and, as with the results of experiments described above, suggested that *pta* and *ackA* are cotranscribed and are controlled by the same regulatory element in response to P_i starvation.

Symbiotic phenotype of *AckA*⁻ and *Pta*⁻ mutants. There were no apparent effects of either the single *ackA* mutation or the *pta ackA* double mutation on symbiosis. Dry-matter production of plants inoculated with either mutant was not significantly different from that of plants inoculated with the wild-type strain. When averaged across plants inoculated with the wild-type strain, RmMSU4, and RmMSU5, the mean shoot dry weights \pm the standard deviations were 11.3 ± 0.8 and 15.2 ± 0.4 mg \cdot plant⁻¹ for P_i -limited and non- P_i -limited plants, respectively. Uninoculated control plants averaged 5.5 ± 0.2 and 5.7 ± 0.3 mg \cdot plant⁻¹, respectively. The numbers of nodules per plant averaged 5.1 ± 0.3 and 7.9 ± 0.2 for P_i -limited and non- P_i -limited plants, respectively, and the nodule fresh weight per plant for P_i -limited plants was 4.8 ± 0.3 mg \cdot plant⁻¹, compared to 7.5 ± 0.3 mg \cdot plant⁻¹ for plants cultured with high P_i levels. As determined by analysis of variance, all differences in plant dry matter and nodulation between P_i -treated and non- P_i -treated inoculated plants were statistically significant.

DISCUSSION

In this study, the sequence, organization, and regulation of *S. meliloti* genes coding for phosphotransacetylase and acetate kinase activity were examined. These genes were determined to be located within an operon that includes an unidentified ORF and, based on partial sequence analysis, likely also contains *fabI*. The order of these genes in the operon was determined to be *orfA-pta-ackA-fabI*. A close physical association of *pta* with *ackA* and their cotranscription have also been observed in *C. acetobutylicum* (9), *E. coli* (24), and *M. thermophila* (45). Inclusion of the unknown *orfA* in the operon is unique to *S. meliloti*, and the future identification of OrfA's function may offer an important clue for determining the role of this operon in the physiology of the cell in general and in response to P_i limitation in particular.

This operon is sensitive to P_i levels in the medium, being up-regulated in response to P_i limitation. The location of the P_i -sensitive transcriptional start site upstream of *orfA* was confirmed by combining complementation experiments with mutation and primer extension analyses. Two strong adjacent transcriptional start sites were identified, and sequence analysis of the -35 region revealed the presence of a putative PHO box. In other bacteria, the PHO box serves as a target for the phosphorylated response-regulatory protein PhoB (see reference 57 for a review). When a *phoB* mutation was introduced into the various strains, increased levels of Pta and AckA enzyme activities and *ackA::lacZ* reporter activity in response to phosphate limitation were abolished. Taken together, the combined evidence strongly argues that *S. meliloti* genes coding for enzymes directly involved in the synthesis of acetyl phosphate are in an operon controlled by the Pho regulon in response to P_i availability.

Selective mutation of *pta* and *ackA* provided an opportunity to assess whether these particular enzymes are important for *S. meliloti* during symbiosis. Up-regulation of these genes could potentially influence *S. meliloti* symbiotic function at two different levels. First, the acetyl phosphate concentration could influence alternative signaling routes and thus affect gene expression during symbiosis. There is now overwhelming evidence showing that dicarboxylic acids are the primary energy

source for bacteroids (13, 33, 51), with a large proportion of the malate and succinate being routed to acetyl-CoA via a malic enzyme (Fig. 1) (16, 17) for metabolism through the tricarboxylic acid cycle (13, 33, 51). The acetyl-CoA pool size could therefore be substantial, with the flow of carbon through other pathways, such as the Pta-AckA pathway, being possible. Since *S. meliloti* bacteroid isolation protocols require a significant amount of time and incubation conditions can have significant effects on intracellular concentrations of acetyl-CoA (53), an important direct precursor of acetyl phosphate, reliable measurement of metabolites such as acetyl phosphate in bacteroids can be problematic. However, we reasoned that by creating metabolic blocks at Pta and/or AckA it should be possible to assess whether acetyl phosphate is potentially an important alternative signaling metabolite during symbiosis. If appreciable acetyl-CoA was channeled through this pathway in *S. meliloti* bacteroids, then inactivation of Pta would diminish acetyl phosphate synthesis, while a block at AckA should provide for an opportunity for acetyl phosphate pools to accumulate. If these mutations had either effect on acetyl phosphate pool size in bacteroids, there appeared to be no consequence for symbiosis, since neither the *ackA* mutant nor the *pta ackA* double mutant was different from the wild-type strain with respect to symbiotic phenotype.

A second level at which mutational analysis of *pta* and *ackA* could aid in our understanding of *S. meliloti* bacteroid physiology concerns the relative importance of acetate as an energy source for bacteroids or as a contributor to the acetyl-CoA pool. *B. japonicum* bacteroid AckA and Pta levels are correlated with nitrogenase activity during soybean nodule ontogeny (37, 46), and *B. japonicum* bacteroids are capable of using acetate as an energy source to support nitrogen fixation in vitro (36). Together, these reports suggest these enzymes may be important to carbon metabolism in soybean nodule bacteroids. By contrast, Miller et al. (34) reported that while isolated alfalfa nodule bacteroids could use succinate, fumarate, malate, or oxaloacetate to support nitrogenase activity, acetate was ineffective in this regard. This is not inconsistent with the symbiotic phenotype observed for the mutants studied in our experiments, for which the Nod⁺ Fix⁺ symbiotic phenotype of the *S. meliloti ackA* and *pta-ackA* mutants suggests that acetate metabolism is not a critical aspect of alfalfa bacteroid physiology. However, because of potential metabolic overlap between the Pta-AckA pathway and that of acetyl-CoA synthase (Acs) (Fig. 1), an *ackA-acs* double mutant would be required to completely assess this issue.

It is important to note that the *pta* mutants constructed in these experiments still retained some Pta activity, as demonstrated by the extract-dependent disappearance of acetyl phosphate. Likewise, based on the acetate- and ATP-dependent production of acetyl phosphate, AckA activity was judged to still be present in all of the mutants. While the levels of both apparent enzyme activities in these mutants were reduced by 80 to 90% (Table 2), the presence of acetyl phosphate-synthesizing and acetyl phosphate-consuming enzyme activities at least suggests that *S. meliloti* has other Pta and AckA enzymes and that these particular enzymes could be actively expressed during symbiosis. Functional reiteration is not an uncommon feature in bacteria in general, and in the context of the *S. meliloti*-alfalfa symbiosis, perhaps the best example is the occurrence of two distinct *S. meliloti* malic enzymes; the NAD-dependent malic enzyme is required for symbiosis, whereas the NADP-dependent malic enzyme is not (16, 17). Under the high-stringency conditions employed in the Southern blot analyses of the various mutants in this study, potential additional *pta* or *ackA* genes were not apparent (results not shown).

It is also important to note that the lack of a symbiotic phenotype associated with the mutants of these specific P_i stress-inducible enzymes is consistent with our earlier observations regarding the expression of P_i -sensitive genes in *S. meliloti* bacteroids. We have previously found that PhoB is not required for an effective symbiosis (2). Also, we have shown that several other genes subject to PhoB control in response to P_i limitation are also not required for symbiotic function and do not appear to be up-regulated in bacteroids, regardless of the P_i nutrition status of the host plant (52). Taken together, our data seem to suggest that *S. meliloti* bacteroids are not limited for P_i , that alfalfa nodule bacteroids are buffered from P_i deprivation when the host plant experiences P_i limitation, and that expression of genes under the control of the regulatory protein PhoB is not necessary for a normal symbiotic relationship with alfalfa. However, there are inconsistencies to this pattern. The P_i stress-inducible high-affinity P_i transport system encoded by *phoCDET* in *S. meliloti* has been reported to be required for normal symbiotic function (5, 10). The *phoCDET* operon is under the control of PhoB (6, 7), which also negatively regulates the recently identified *S. meliloti* pit low-affinity P_i transport system (6, 7). In cultured cells of *S. meliloti*, the Pit transporter is expressed under high-phosphate conditions and repressed under low-phosphate conditions (6, 7). In *E. coli*, most mutations that inhibit P_i transport through the high-affinity P_i transport system also result in constitutive expression of the Pho regulon (57). Mutations in *S. meliloti* *phoC* (the promoter-proximal gene in the *phoCDET* operon) have the same effect (6) and also result in the repression of the low-affinity Pit system (54). Spontaneous mutations that disconnect PhoB control of *pit* in the *phoC* mutant restore normal symbiotic function (10, 35). Therefore, it is possible that the symbiotic phenotype resulting from mutations in *S. meliloti* *phoCDET* is actually not due to the lack of P_i uptake through the P_i stress-inducible high-affinity P_i transport system per se but rather is due to mutations that suppress the normal symbiotic expression of Pit and that coincidentally eliminate the other primary mechanism of P_i uptake. Additional studies involving manipulation of the control of Pho regulon expression and P_i transport may provide important information to help resolve this issue.

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