Genetic Analysis of Mutations Affecting *pckA* Regulation in *Rhizobium* (*Sinorhizobium*) *meliloti*

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ABSTRACT

The enzyme phosphoenolpyruvate carboxykinase (Pck) catalyzes the first step in the gluconeogenic pathway in most organisms. We are examining the genetic regulation of the gene encoding Pck, *pckA*, in *Rhizobium* (now *Sinorhizobium*) *meliloti*. This bacterium forms N₂-fixing root nodules on alfalfa, and the major energy sources supplied to the bacteria within these nodules are C₄-dicarboxylic acids such as malate and succinate. *R. meliloti* cells growing in glucose minimal medium show very low *pckA* expression whereas addition of succinate to this medium results in a rapid induction of *pckA* transcription. We identified spontaneous mutations (*rpk*) that alter the regulation of *pckA* expression such that *pckA* is expressed in media containing the non-inducing carbon sources lactose and glucose. Genetic and phenotypic analysis allowed us to differentiate at least four *rpk* mutant classes that map to different locations on the *R. meliloti* chromosome. The wild-type locus corresponding to one of these *rpk* loci was cloned by complementation, and two Tn5 insertions within the insert DNA that no longer complemented the *rpk* mutation were identified. The nucleotide sequence of this region revealed that both Tn5 insertions lay within a gene encoding a protein homologous to the GalR/LacI family of transcriptional regulators that are involved in metabolism.

GLUCONEOGENESIS refers to the central metabolic pathway in which tricarboxylic acid (TCA) cycle intermediates such as citrate and malate are converted to glucose. In the glycolytic pathway, glucose is converted to pyruvate, and under aerobic conditions, the pyruvate is further oxidized via the TCA cycle. The regulation of the metabolic flux through the glycolytic *vs.* the gluconeogenic pathways plays an important role in central carbon metabolism. In many organisms, the first step of the gluconeogenic pathway is catalyzed by the enzyme phosphoenolpyruvate carboxykinase (Pck) (EC 4.1.1.49) which decarboxylates oxaloacetate (OAA) to phosphoenolpyruvate (PEP). Fructose biphosphatase, which converts fructose-1,6-bisphosphate to fructose-6-phosphate, is required for gluconeogenesis. All the other reactions in the gluconeogenic pathway are also employed in the glycolytic pathway.

Among prokaryotes, the regulation of the structural gene for Pck, *pckA*, has been most thoroughly examined in *Escherichia coli* where *pckA* expression is induced in cells grown on gluconeogenic substrates. When glucose is present in the culture medium, this expression is catabolite repressed. Expression of *pckA* was also shown to be stationary phase regulated in cells growing in LB, and this regulation was cAMP dependent (GOLDIE

1984). In addition, the regulatory protein FruR, also known as Cra (catabolite repressor/activator), regulates *pckA* expression (CHIN *et al.* 1987). Details regarding this precise regulatory mechanism remain to be resolved, and it is likely that FruR interacts with the other catabolite repressor protein (CRP) in regulating *pckA* (see SAIER and RAMSEIER 1996).

In this paper, we describe studies of the regulation of expression of the gene encoding Pck (*pckA*) in the N₂-fixing bacterium *Rhizobium* (now *Sinorhizobium*) *meliloti* that forms root nodules on alfalfa plants. During the past several years, interest in carbon metabolism in *Rhizobium* has centered on the carbon sources supplied by the host plant to the N₂-fixing bacteria (bacteroids) within root-nodules. It is now generally accepted that the C₄-dicarboxylic acids, malate and perhaps succinate, are the major carbon source(s) supplied to the bacteroid. These compounds are readily used by free-living bacteria that possess a gluconeogenic pathway for conversion of these compounds to hexose sugars. Enzyme assays have revealed that Pck is induced during gluconeogenesis, whereas fructose biphosphatase appeared to be constitutively expressed (MCKAY *et al.* 1985; SAROSO *et al.* 1986; FINAN *et al.* 1988, 1991). Mutants of *R. meliloti*, *R. leguminosarum* and *R. sp.* NGR234, which lack Pck activity, fail to grow with TCA cycle intermediates as sole carbon sources whereas these mutants grow like the wild-type strain on glycolytic carbon sources such as glucose or sucrose (MCKAY *et al.* 1985; FINAN *et al.* 1988; ØSTERÅS *et al.* 1991). We recently showed that mutations that increase pyruvate ortho-

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phosphate dikinase (Pdk) activity in *R. meliloti* effectively bypass the requirement for Pck in gluconeogenesis. The latter bypass pathway requires malic enzyme activity and is similar to an alternate gluconeogenic route employing malic enzyme and PEP synthase described in *E. coli* (COOPER and KORNBERG 1967; HANSEN and JUNI 1974; ØSTERÅS *et al.* 1997).

The structural *pckA* gene has been identified in *R. sp.* NGR234 and *R. meliloti* and encodes a Pck enzyme homologous to plant and microbial ATP-dependent Pcks (ØSTERÅS *et al.* 1991; 1995). The *R. meliloti pckA* transcription start site was determined and a σ^{70} -like promoter with -35 and -10 consensus sequences present in other *R. meliloti* promoters was identified. *R. meliloti pckA* was highly expressed in cells grown in minimal medium containing gluconeogenic substrates such as succinate, and this expression remained high in medium containing succinate plus glucose. Expression was very low in glucose-grown cells. As noted for *E. coli*, *pckA* expression in *R. meliloti* cells grown in complex LB medium was also found to be stationary phase regulated (ØSTERÅS *et al.* 1995).

The *R. meliloti pckA* gene appears to be regulated differently in the symbiotic bacteroid state than in free-living cells, as *R. meliloti* bacteroids contain no detectable Pck activity despite the fact that bacteroids are thought to be supplied with C₄-dicarboxylates by the plant host (FINAN *et al.* 1991). The role of *pckA* in the root nodule symbiosis is further complicated by the finding that *Rhizobium pckA* mutants are symbiotically defective on some host plants but behave like the wild-type strain on other host plants. This host-dependent phenotype suggests that the metabolites available to the bacteria during the nodulation process vary depending on the host plant (MCKAY *et al.* 1985; FINAN *et al.* 1991; ØSTERÅS *et al.* 1991).

Here we report the isolation and genetic characterization of *R. meliloti* mutants with altered *pckA* regulation in the free-living state and examine the effect of these mutations on symbiotic Pck repression. Last, we have cloned one of the regulatory loci and show that it encodes a protein homologous to the GalR/LacI family of transcriptional regulators.

MATERIALS AND METHODS

Bacterial strains, plasmids and media: Bacterial strains and plasmids are listed in Table 1. For growth, Luria broth (LB) was used as complex medium for *E. coli* and supplemented with 2.5 mM MgSO₄ and 2.5 mM CaCl₂ (LBmc) for *R. meliloti*. M9 was used as defined medium for all strains (MILLER 1972). The desired carbon source was filter sterilized and was added at a final concentration of 15 mM. When required, antibiotics were added at concentrations previously described (FINAN *et al.* 1986; ØSTERÅS *et al.* 1995). The expression of β -galactosidase in colonies was detected by plating on agar media containing 40 μ g/ml of X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactoside).

Genetic techniques: Bacterial matings, transductions, transposon mutagenesis, and transposon replacements were performed as previously described (FINAN *et al.* 1984, 1986, 1988;

DE VOS *et al.* 1986). Spontaneous mutants of RmG950 altered in *pckA* expression were obtained by plating 10⁹ cells from a culture grown in M9 glucose (to repress *pckA* expression) on M9 lactose agar plates. Colonies present on the plates after incubation at 28° for five days were purified and examined further. Tn5 insertions linked to the regulatory mutations (*rpk*) were isolated as previously described (ØRESNIK *et al.* 1994). A transducing phage lysate grown on a bank of random Tn5 insertions in the wild-type genome was used to transduce neomycin resistance (Nm^r) into the RmG950 *rpk* mutant strains. Transductants were selected on M9 glucose X-Gal containing 200 μ g/ml Nm and colonies in which Tn5 was linked to the wild-type *rpk* allele were identified as white colonies on these plates.

For genetic mapping of insertions in *R. meliloti*, we used a set of seven strains in which Tn5-*mob* mobilizes various regions of the chromosome (FINAN *et al.* 1988). The selected Tn5 insertions to be mapped (Ω 5345, Ω 5355, Ω 5356, Ω 5358) were first replaced by Tn5-233, which is a gentamycin-spectinomycin resistance (Gm^r-Sp^r) derivative Tn5, before being transduced into the seven mobilizing strains. After purification, the resulting transductants were crossed with Rm5000 in a triparental mating using an *E. coli* helper strain carrying pGM102 as the mobilizing plasmid. Rifampicin resistant (Rif^r)-Gm^r-Sp^r transconjugants were selected on LB containing Rf 25 μ g/ml, Gm 20 μ g/ml, and Sp 100 μ g/ml.

To construct strain RmH413, we first cloned the Ω interposon (Sp^r) (FREY and KRISCH 1985) into the *Eco*RI site of pTH189 (ØSTERÅS *et al.* 1995). The resulting plasmid, pTH273, was transferred into *R. meliloti* carrying the chromosomal *pckA6::lacZ* gene fusion and homogenotes (RmH413) in which the interposon (Sp^r) was recombined into the genome were selected as previously described (ØSTERÅS *et al.* 1995).

Cosmids clones restoring wild-type *pckA* expression in a *rpk-9* background were identified by transferring the *R. meliloti* pLAFR1 library (FRIEDMANN *et al.* 1982) into strain RmH286 and screening for white transconjugant colonies on M9 glucose supplemented with streptomycin (200 μ g/ml), tetracycline (2 μ g/ml) and X-Gal. Tn5 mutagenesis of plasmid pTH277 was performed as previously described (YAROSH *et al.* 1989).

DNA manipulations: Standard methods were used for plasmid DNA isolation, restriction analysis, agarose gel electrophoresis, Southern blotting, DNA ligation and transformation (SAMBROOK *et al.* 1989). *R. meliloti* genomic DNA was isolated as previously described (ØRESNIK *et al.* 1994). Hybridizations were performed at 68° with digoxigenin-labeled probe (DIG DNA Labelling and Detection Kit, Boehringer Mannheim).

DNA sequencing: The 2.5-kb *Eco*RI fragment containing *pckR* was cloned from pTH277 into pUC118, and the resulting plasmid pTH296 was used for subcloning and DNA sequencing. Single-stranded DNA was obtained by using host strain XL1Blue and helper phage M13K07 (VIEIRA and MESSING 1987), and its sequence determined using the -20 Universal primer. The two site-directed Tn5 insertions in *pckR* (n. 34 and 38) were subcloned as an *Eco*RI fragment from the pTH277-derivative plasmids into pUC118 followed by a *Bam*HI deletion to eliminate one of the IS50 of the transposon. The DNA sequence of the region flanking the insertion was determined by using a Tn5-specific primer (5'-TCACATG GAAGTCAGATCCT-3') (kindly provided by R. J. WATSON). DNA sequencing was performed on an ABI 373 Stretch automatic sequencer using the dye terminator chemistry and cycle sequencing. DNA and derived protein sequences were analyzed with the GDE (S. SMITH, unpublished results), BLAST (ALTSCHUL *et al.* 1990) and CLUSTALV (HIGGINS *et al.* 1992) software packages.

Biochemical techniques: Cell growth and the preparation of cell-free sonicated extracts were performed as described

TABLE 1
Bacterial strains and plasmids used in this study

Strains, plasmids or phages	Relevant characteristics	Reference
<i>Rhizobium meliloti</i>		
Rm1021	<i>R. meliloti</i> SU47, <i>str</i> -21	MEADE <i>et al.</i> (1982)
Rm5000	<i>R. meliloti</i> SU47, <i>rif</i> ⁵	FINAN <i>et al.</i> (1984)
Rm5065	Rm1021, <i>pck</i> -1::Tn5-132	FINAN <i>et al.</i> (1988)
RmG212	Rm1021, Lac ⁻	J. GLAZEBROOK
RmG263	RmG212, <i>pckA</i> 6::Tn3HoKm	This work
RmG950	RmG212, <i>pckA</i> 12::Tn3HoSp	This work
RmG980	Rm1021, Ω5315::Tn5 linked 95% to <i>pckA</i>	This work
RmH139 to H158	RmG950, <i>rpk</i> -1 to <i>rpk</i> -20 (see Table 2)	This work
RmH286	RmG212, <i>rpk</i> -9, Ω5345::Tn5	This work
RmH347	RCR2011, leu-53 ⁺ Ω601::Tn5-mob (-)	KLEIN <i>et al.</i> (1992)
RmH348	RCR2011, leu-53 ⁺ Ω602::Tn5-mob (+)	KLEIN <i>et al.</i> (1992)
RmH349	RCR2011, trp-33 ⁺ Ω611::Tn5-mob (+)	KLEIN <i>et al.</i> (1992)
RmH350	RCR2011, trp-33 ⁺ Ω612::Tn5-mob (-)	KLEIN <i>et al.</i> (1992)
RmH351	RCR2011, pyr-49 ⁺ Ω614::Tn5-mob (+)	KLEIN <i>et al.</i> (1992)
RmH352	RCR2011, pyr-49 ⁺ Ω615::Tn5-mob (-)	KLEIN <i>et al.</i> (1992)
RmH353	RCR2011, cys-11 ⁺ Ω637::Tn5-mob (+)	KLEIN <i>et al.</i> (1992)
RmH408	RmG212, <i>EcoRI</i> ::Ω (Sp ^r) in promoter of <i>pckA</i>	This work
RmH413	RmG263, <i>EcoRI</i> ::Ω (Sp ^r) in promoter of <i>pckA</i> 6::Tn3HoKm	This work
RmH443	Rm5000, Ω5345::Tn5-233	This work
RmH444	Rm5000, Ω5355::Tn5-233	This work
RmH445	Rm5000, Ω5356::Tn5-233	This work
RmH446	Rm5000, Ω5358::Tn5-233	This work
RmH464	RmG212, <i>rpk</i> -9, <i>pckA</i> 6::Tn3HoKm	This work
<i>Escherichia coli</i>		
DH5α	F ⁻ , <i>endA</i> 1, <i>hsdR</i> 17, <i>supE</i> 44, <i>thi</i> -1, <i>recA</i> 1, <i>gyrA</i> 96, <i>relA</i> 1Δ(<i>argF-lacZYA</i>)	GIBCO BRL
XL-1Blue	<i>supE</i> 44, <i>hsdR</i> 17, <i>endA</i> 1, <i>gyrA</i> 46, <i>relA</i> 1, <i>thi</i> , <i>recA</i> , [<i>F'</i> , <i>proAB</i> , <i>lacIqZΔM15</i> , Tn10(Tc ^r)]	BULLOCK <i>et al.</i> (1987)
MT607	<i>pro</i> -82, <i>thi</i> -1, <i>hsdR</i> 17, <i>supE</i> 44, <i>recA</i> 56	FINAN <i>et al.</i> (1986)
MT609	<i>thyA</i> 36, <i>polA</i> 1 Sp ^r , recipient for site-directed Tn5 mutagenesis	YAROSH <i>et al.</i> (1989)
MT614	MT607ΩTn5	YAROSH <i>et al.</i> (1989)
MT616	MT607 pRK600	FINAN <i>et al.</i> (1986)
MT620	MT607, Rif ^r	T. M. FINAN
Plasmids		
pLAFR1	IncP cosmid cloning vector, Tc ^r	FRIEDMAN <i>et al.</i> (1982)
pRK7813	IncP cosmid cloning vector, Tc ^r	JONES and GUTTERSON (1987)
pUC118/9	ColE1 <i>oriV</i> cloning vectors, Ap ^r	VIEIRA and MESSING (1987)
pRmT103	pLAFR1, <i>R. meliloti</i> cosmid clone with <i>pckA</i>	FINAN <i>et al.</i> (1988)
pTH87	pRK7813, 5-kb <i>EcoRI</i> fragment with <i>R. meliloti pckA</i>	ØSTERÅS <i>et al.</i> (1995)
pTH189	pRK7813, 5.3-kb <i>PstI</i> fragment with <i>R. meliloti pckA</i>	ØSTERÅS <i>et al.</i> (1995)
pTH273	pTH189, <i>EcoRI</i> ::Ω (Sp ^r) in the promoter of <i>pckA</i>	This work
pTH277	pLAFR1, <i>R. meliloti</i> cosmid clone restore wt <i>pckA</i> regulation	This work
pTH278	pLAFR1, <i>R. meliloti</i> cosmid clone restore wt <i>pckA</i> regulation	This work
pTH279	pLAFR1, <i>R. meliloti</i> cosmid clone restore wt <i>pckA</i> regulation	This work
pTH280	pLAFR1, <i>R. meliloti</i> cosmid clone restore wt <i>pckA</i> regulation	This work
pTH281	pLAFR1, <i>R. meliloti</i> cosmid clone restore wt <i>pckA</i> regulation	This work
pTH296	pUC118, 2.5-kb <i>EcoRI</i> fragment from pTH277 with <i>pckR</i>	This work
Phages		
φM12	<i>R. meliloti</i> transducing phage	FINAN <i>et al.</i> (1984)

previously (FINAN *et al.* 1988). Activities of malate dehydrogenase (MDH) (ENGLAND and SIEGAL 1969) and Pck (HANSEN *et al.* 1976) were determined as described in DRISCOLL and FINAN (1993) by following the change in NADH concentration at 340 nm on a Kontron Uvikon 930 spectrophotometer. β-galactosidase assays were performed at 30° as described in ØSTERÅS *et al.* (1995). Protein was measured using the Coomassie Blue Reagent (BioRad) as described in BRADFORD (1976) with bovine serum albumin as standard.

Plant tests and bacteroid isolation: Plant nodulation exper-

iments, plant shoot dry weight determinations and bacteroid isolation from harvested nodules were done following the procedures described in FINAN *et al.* (1991). Plants were grown in the absence of added nitrogen, and under these conditions, the plant dry weight gives an accurate estimate of the amount of N₂-fixed over the course of the experiment.

Nucleotide sequence accession number: The DNA sequence of the *R. meliloti* regulatory gene *pckR* has been submitted to GenBank/EMBL databank and assigned accession No. AF004316.

TABLE 2
Growth phenotype of mutant alleles affecting *pckA* expression

Allele	Initial strain <i>pckA::lacZ</i>	Growth on M9				Strain Pck ⁺ ^a	Growth on M9		Strain <i>pckA::lacZ</i> ^b	Growth on M9 lactose
		Lactose	Glucose	Glucose + Succinate	LB		Lactose	Glucose + Succinate		
Wild-type	RmG950	—	+	+	+	RmG980	+/-	+	RmG950	—
<i>rpk-1</i>	RmH139	+/-	+	+	+	RmH159	—	+	RmH249	+/-
<i>rpk-2</i>	RmH140	+	+	+	+	RmH160	—	+	RmH250	+
<i>rpk-3</i>	RmH141	+	+/-	+/-	+/-	RmH161	—	+/-	RmH251	+
<i>rpk-4</i>	RmH142	++	+	+	+	RmH162	—	+	RmH252	++
<i>rpk-5</i>	RmH143	++	+	+	+					
<i>rpk-6</i>	RmH144	+/-	+/-	+/-	+/-	RmH163	—	+/-	RmH253	+/-
<i>rpk-7</i>	RmH145	+	+/-	+/-	+/-	RmH164	—	+/-	RmH254	+
<i>rpk-8</i>	RmH146	+	+	+/-	+/-	RmH165	—	+/-	RmH255	+
<i>rpk-9</i>	RmH147	++	+	+	+	RmH166	—	+	RmH256	++
<i>rpk-10</i>	RmH148	+	+	+	+	RmH167	—	+	RmH257	+
<i>rpk-11</i>	RmH149	+	+/-	+/-	+/-	RmH168	—	+/-	RmH258	+
<i>rpk-12</i>	RmH150	+/-	+	+	+	RmH169	—	+	RmH259	+/-
<i>rpk-13</i>	RmH151	+/-	+	+	+	RmH170	—	+	RmH260	+/-
<i>rpk-14</i>	RmH152	+	+/-	+/-	+/-	RmH171	—	+/-	RmH261	+
<i>rpk-15</i>	RmH153	+/-	+	+	+	RmH172	—	+	RmH262	+/-
<i>rpk-16</i>	RmH154	+	+	+	+	RmH173	—	+	RmH263	+
<i>rpk-17</i>	RmH155	+	+	+	+	RmH174	—	+	RmH264	—
<i>rpk-18</i>	RmH156	+	+	—	+	RmH175	—	+	RmH265	+
<i>rpk-19</i>	RmH157	++	+	+	+					
<i>rpk-20</i>	RmH158	++	+	+	+	RmH176	—	+	RmH266	++

Growth of the strains was scored after 4 days relative to that of the corresponding wild-type strains RmG950 and RmG980 on M9 glucose for defined media and on LB for complex media; +, same growth as wild type; ++, faster growth; +/-, slow growth; —, no (or very poor) growth.

^a These strains were constructed by transducing the wild-type *pckA* gene from RmG980 (where it is linked to the Tn5 insertion Ω 5315 located 500 bp downstream of *pckA* (ØSTERÅS *et al.* 1995)) into the initial strains (RmH139–RmH158), selecting for Nm^r and loss of the Sp^r of the *pckA::Tn3HoSp* fusion.

^b These strains were constructed by transducing back the *pckA12::Tn3HoSp* fusion from RmG950 into the Pck⁺ *rpk*, Ω 5315::Tn5 strains (RmH159–RmH176), selecting for Sp^r and screening for loss of Nm^r.

RESULTS

Isolation of mutants affected in *pckA* expression: Transcription of the *pckA* gene of *R. meliloti* is repressed when compounds metabolized by the glycolytic pathway such as glucose or lactose are the only carbon sources available for growth (ØSTERÅS *et al.* 1995). To isolate mutants in which *pckA* was expressed under non-inducing conditions, we employed *R. meliloti* strain RmG950 which is Lac[–] and carries a Tn3HoSp insertion in the *pckA* gene such that the *lacZYA* genes within Tn3HoSp are regulated by the *pckA* promoter. When plated on M9 lactose, this strain failed to grow because neither of its lactose utilization genes are being expressed. RmG950 is Lac[–] due to a defect in the *R. meliloti* lactose utilization pathway. The fused-*lacZYA* genes are not expressed during growth on lactose as *pckA* is not induced in cells cultured with lactose as carbon source. RmG950 cells were spread on M9 lactose plates and after 5 days incubation at 28°, spontaneous Lac⁺ colonies were obtained. Twenty such isolates carrying putative regulatory mutations, named *rpk* (regulator *pck*), were purified and analyzed (Table 2). The aberrant β -galactosidase activity of these mutants was not limited to cells growing on M9 lactose, as all the

rpk strains also formed blue colonies on M9 glucose+X-Gal, where the parent strain RmG950 formed white colonies.

In addition to β -galactosidase expression, mutants carrying the *rpk* mutations *rpk-3*, *rpk-6*, *rpk-7*, *rpk-11* and *rpk-14* grew slowly on LB and M9 glucose, and this slow growth phenotype was observed both in the presence and absence of a functional *pckA* gene. We also observed that strain RmH156 (*rpk-18*) was unable to grow on M9 glucose plus succinate, although it was not affected for growth on LB or M9 glucose. This *rpk-18* phenotype was *pckA*-dependent as it was not observed in a Pck⁺ background (Table 2).

To verify that the β -galactosidase activity in the *rpk* mutants was directed from the *pckA::lacZ* fusion, we transduced the wild-type *pckA*⁺ gene via the tightly linked neomycin resistant Ω 5315::Tn5 insertion into each of the spontaneous *rpk* mutants using phage grown on RmG980 (*pckA*⁺ linked to Ω 5315::Tn5) (see Table 2, footnote a). Two of the mutants, RmH143 (*rpk-5*) and RmH157 (*rpk-19*), were found to be resistant to transduction with the *pckA*⁺ allele while markers located elsewhere in the genome could readily be transduced into these two strains (data not shown). No further

TABLE 3
Linkage of the different alleles as determined by transduction

Strain	Allele	Linkage to Tn5 insertion ^a						
		Ω5349	Ω5345	Ω5352	Ω5355	Ω5356	Ω5358	Ω5360
RmH139	<i>rpk-1</i>	—	0 (40)	—	0 (30)	0 (20)	—	—
RmH145	<i>rpk-7</i>	0 (20)	—	—	0 (1)	0 (93)	0 (40)	—
RmH150	<i>rpk-12</i>	0 (6)	—	—	0 (30)	0 (20)	—	—
RmH151	<i>rpk-13</i>	0 (20)	—	—	0 (30)	0 (20)	—	—
RmH147	<i>rpk-9</i>	56 (34)	78 (40)	88 (33)	—	—	—	—
RmH148	<i>rpk-10</i>	75 (6)	70 (40)	90 (30)	—	—	—	—
RmH153	<i>rpk-15</i>	62 (13)	80 (40)	80 (30)	—	—	—	—
RmH140	<i>rpk-2</i>	0 (10)	—	—	7 (30)	0 (20)	—	—
RmH142	<i>rpk-4</i>	0 (29)	—	—	7 (30)	0 (20)	—	—
RmH154	<i>rpk-16</i>	0 (19)	—	—	20 (30)	0 (20)	—	—
RmH158	<i>rpk-20</i>	0 (1)	—	—	20 (30)	0 (20)	—	—
RmH141	<i>rpk-3</i>	0 (20)	—	—	0 (96)	3 (300)	71 (139)	86 (408)
RmH144	<i>rpk-6</i>	0 (20)	—	—	0 (89)	3 (176)	73 (173)	84 (121)
RmH146	<i>rpk-8</i>	0 (26)	—	—	0 (1)	0 (41)	70 (305)	88 (200)
RmH149	<i>rpk-11</i>	0 (20)	—	—	0 (39)	3 (61)	71 (49)	91 (75)
RmH152	<i>rpk-14</i>	0 (6)	—	—	0 (79)	0 (11)	66 (29)	83 (35)
RmH156	<i>rpk-18</i>	0 (16)	—	—	0 (30)	40 (10)	—	—

^a Phage grown on donor strain containing the desired Ω::Tn5 insertions were used to transduce Nm^r into the recipient strains RmH139–RmH158. Transductants selected on LB + Nm were screened for expression of the *pckA::lacZ* fusion on M9 Glucose + X-Gal. Linkage is given as percentage of white colonies on M9 Glucose + X-Gal. The number of transductants tested is given in parenthesis.

investigations were carried out with these strains. All 18 of the remaining Pck⁺ transductants failed to grow on M9 lactose plates, showing that expression of the *pckA::lacZ* fusion was responsible for the Lac⁺ phenotype of the original *rpk*, *pckA::Tn3HoSp* mutants (Table 2). In an additional transduction, the original *pckA::lacZ* gene fusion was reintroduced into the 18 Pck⁺ *rpk* Ω5315::Tn5 strains (RmH159–RmH176) (Table 2, footnote b). All of the resulting transductants except RmH264 (*rpk-17*) grew on M9 lactose plates, indicating that 17 of the 18 *rpk* mutations were not linked to *pckA*.

Mutations affecting *pckA* expression map to different loci: Tn5 insertions Ω5355, Ω5358, Ω5345, Ω5349, Ω5352, and Ω5360 linked in transduction to the *rpk-2*, *rpk-3*, *rpk-4*, *rpk-10*, *rpk-11*, and *rpk-18* alleles respectively, plus two insertions Ω5345 and Ω5349 linked to *rpk-9* were isolated as described in the MATERIALS AND METHODS. To determine whether several *rpk* mutations occurred in the same gene region, lysates from strains carrying these insertions linked to the wild-type *rpk* allele were used to transduce the neomycin resistance into the other *rpk* mutants. Transductants were screened on M9 glucose+X-Gal to score the *pckA::lacZ* expression phenotype. As shown in Table 3, only four of the 17 *rpk* mutants, RmH139 (*rpk-1*), RmH145 (*rpk-7*), RmH150 (*rpk-12*) and RmH151 (*rpk-13*) were not linked to any of the Tn5 insertions. The remaining *rpk* alleles clustered into four groups; group I comprised *rpk-9*, *rpk-10* and *rpk-15*; group II: *rpk-2*, *rpk-4*, *rpk-16* and *rpk-20*, group III: *rpk-3*, *rpk-6*, *rpk-8*, *rpk-11* and *rpk-14*; and group IV contained only *rpk-18*. Group II could be divided in two distinct subgroups as the cotransduc-

tional frequency was different between *rpk-2/rpk-4* (7% linkage to Ω5355, IIa) and *rpk-16/rpk-20* (20% linkage to Ω5355, IIb). Insertion Ω5356 (linked to *rpk-18*, Group IV) showed weak linkage to some mutations of group III (3% linkage to *rpk-3*, *rpk-6* and *rpk-11*). Other insertions linked to groups III and IV (Ω5357, Ω5358, Ω5359) were transduced into mutants of group III and IV; the cotransfer linkage frequency determined from the resulting cotransduction frequencies confirmed that the two mutated regions are located approximately 140 kb apart (Figure 1).

Chromosomal location of *rpk* mutations: To map the *rpk* mutations on the *R. meliloti* chromosome, the Nm^r Tn5 insertions Ω5345, Ω5355, Ω5358, Ω5356 that are linked to the four *rpk* mutant groups were replaced with Tn5-233, which encodes gentamycin and spectinomycin resistance (Gm^r-Sp^r). The replaced insertions were then transduced into seven Tn5-*mob* mapping strains each of which conjugally transfers large regions of the chromosome (see Figure 1, MATERIALS AND METHODS) (FINAN *et al.* 1988; KLEIN *et al.* 1992). The resulting strains were then crossed with a rifampicin resistant recipient and relative frequency of Gm^r-Sp^r transfer was determined (Table 4). The results showed that insertions linked to groups I and II were located in the same region of the chromosome, between *leu-53* and *trp-33*. Also, as expected, insertions linked to groups III and IV were located together, and these mapped between *cys-11* and *leu-53* (Table 4, Figure 1). No *rpk* mutations mapped close to the *pckA* gene (Figure 1).

Pck activity in glucose-grown cells of the *rpk* mutants: The altered *pckA* expression phenotype of the

FIGURE 1.—Genetic map of *R. meliloti* SU47 chromosome showing the location of the *rpk* loci. Location of *cys-11*, *pyr-49*, *trp-33*, *leu-53* and *pckA* are from FINAN *et al.* (1988) and KLEIN *et al.* (1992). Arrows with insertion numbers on the chromosome map indicate positions of the Tn5-*mob* insertions. The arrowhead is the origin of transfer, and the arrow tail is the direction of transfer. The roman numbers inside the chromosome indicate the mutant group to which the *rpk* allele belongs. The enlarged chromosome region shows the Tn5 insertions (Ω5356–Ω5359) linked to *rpk-3* and *rpk-18* with the frequencies of transductional linkage on the arrows.

mutants was identified with a transcriptional *pckA::lacZ* fusion. To measure the effect of the *rpk* mutations on expression of the wild-type *pckA* gene, Pck and malate dehydrogenase (as a control) enzyme activities were measured in crude extracts from M9 glucose-grown strains harboring the *rpk* mutations together with the wild-type *pckA* gene (Table 5). Several mutants, RmH159 (*rpk-1*), RmH165 (*rpk-8*), RmH168 (*rpk-11*),

RmH170 (*rpk-13*) and RmH173 (*rpk-16*) showed very low Pck activity ($2\text{--}4\text{ nmol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$ protein) that was marginally higher than the wild-type RmG980 ($1\text{ nmol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$ protein). For the other mutants, higher Pck activity was detected ($>8\text{ nmol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$ protein). Two strains, RmH166 (*rpk-9*) and RmH167 (*rpk-10*), had a more than threefold higher Pck activity than any other mutant, reaching levels ob-

TABLE 4
Conjugal mapping of the regulatory mutation groups

Donor marker	Mutant group	No. of recombinants per plate						
		Ω601 (–)	Ω602 (+)	Ω611 (+)	Ω612 (–)	Ω614 (+)	Ω615 (–)	Ω637 (+)
Ω5345::Tn5-233	I	3	90	2	130	0	1	1
Ω5355::Tn5-233	II	2	100	1	51	6	2	6
Ω5358::Tn5-233	III	302	0	1	2	1	1	150
Ω5356::Tn5-233	IV	236	2	1	4	0	0	259

The conjugal matings done to generate the above data were performed as described in MATERIALS AND METHODS. The donor strains were derivative of the RCR2011 Tn5-*mob* mapping strains carrying one of the four Ω::Tn5-233 marker. The mating was done overnight with Rm5000 (Rif^r) in the presence of the mobilizing plasmid pGMI102. Transconjugants were selected on LB + Rif + Gm + Sp.

TABLE 5

PEP carboxykinase and malate dehydrogenase activity in glucose-grown cells and bacteroids

Strain	Relevant characteristics	Group	Symbiotic phenotype ^a	MDH ^b		PCK ^b	
				Glucose	Bacteroid ^c	Glucose	Bacteroid
RmG980	wild type ($\Omega 5315::Tn5$)		100	573 $\pm$ 51	1453 $\pm$ 92	1 $\pm$ 1	0
RmH166	<i>rpk-9</i> ($\Omega 5315::Tn5$)	I	97	749 $\pm$ 18	1654 $\pm$ 67	105 $\pm$ 4	13 $\pm$ 1
RmH167	<i>rpk-10</i> ($\Omega 5315::Tn5$)	I	74	727 $\pm$ 10	1618 $\pm$ 48	63 $\pm$ 2	10 $\pm$ 1
RmH172	<i>rpk-15</i> ($\Omega 5315::Tn5$)	I	64	604 $\pm$ 46	1349 $\pm$ 16	9 $\pm$ 1	6 $\pm$ 1
RmH160	<i>rpk-2</i> ($\Omega 5315::Tn5$)	II	86	778 $\pm$ 3	1521 $\pm$ 49	8 $\pm$ <1	2 $\pm$ 1
RmH162	<i>rpk-4</i> ($\Omega 5315::Tn5$)	II	57	628 $\pm$ 13	1407 $\pm$ 10	18 $\pm$ 2	2 $\pm$ <1
RmH173	<i>rpk-16</i> ($\Omega 5315::Tn5$)	II	70	973 $\pm$ 15	1535 $\pm$ 14	2 $\pm$ 2	1 $\pm$ 1
RmH176	<i>rpk-20</i> ($\Omega 5315::Tn5$)	II	48	722 $\pm$ 21	ND ^d	16 $\pm$ 1	ND
RmH161	<i>rpk-3</i> ($\Omega 5315::Tn5$)	III	20	696 $\pm$ 2	1348 $\pm$ 17	19 $\pm$ 1	2 $\pm$ 1
RmH163	<i>rpk-6</i> ($\Omega 5315::Tn5$)	III	28	647 $\pm$ 10	1275 $\pm$ 36	17 $\pm$ 2	3 $\pm$ 1
RmH165	<i>rpk-8</i> ($\Omega 5315::Tn5$)	III	18	604 $\pm$ 15	1727 $\pm$ 39	4 $\pm$ 1	2 $\pm$ <1
RmH168	<i>rpk-11</i> ($\Omega 5315::Tn5$)	III	16	512 $\pm$ 11	ND	4 $\pm$ 3	ND
RmH171	<i>rpk-14</i> ($\Omega 5315::Tn5$)	III	28	635 $\pm$ 12	ND	15 $\pm$ 1	ND
RmH175	<i>rpk-18</i> ($\Omega 5315::Tn5$)	IV	71	568 $\pm$ 24	1501 $\pm$ 55	23 $\pm$ 3	5 $\pm$ 2
RmH159	<i>rpk-1</i> ($\Omega 5315::Tn5$)		59	518 $\pm$ 25	1383 $\pm$ 30	4 $\pm$ 3	3 $\pm$ <1
RmH164	<i>rpk-7</i> ($\Omega 5315::Tn5$)		23	628 $\pm$ 18	1590 $\pm$ 43	22 $\pm$ 2	3 $\pm$ 1
RmH169	<i>rpk-12</i> ($\Omega 5315::Tn5$)		32	673 $\pm$ 42	1135 $\pm$ 29	10 $\pm$ 2	3 $\pm$ 1
RmH170	<i>rpk-13</i> ($\Omega 5315::Tn5$)		89	889 $\pm$ 49	1740 $\pm$ 30	2 $\pm$ 2	2 $\pm$ <1

^a Symbiotic N₂-fixation phenotype was measured as shoot dry weight of plants 33 days after inoculation. Values represent the percent dry weight of wild-type RmG980 (100% = 87 mg/plant; uninoculated control = 13%).

^b Enzyme-specific activity is given in nmol per min per mg of protein $\pm$ standard error of the mean.

^c Bacteroids were prepared from plants 33 days after inoculation.

^d ND = not determined.

served in the wild-type strain grown under induced conditions (~ 100 nmol $\cdot$ min⁻¹ $\cdot$ mg⁻¹ protein).

Symbiotic phenotype and bacteroid Pck activities of *rpk* mutants: As wild-type bacteroids lack Pck activity, we previously suggested that the regulation of Pck synthesis may play an important role in the control of bacteroid proliferation in alfalfa nodules (FINAN *et al.* 1991). As several *rpk* mutations clearly expressed *pckA* under non-inducing free-living conditions, we determined the symbiotic phenotype of these and the other *rpk* mutants. All of the mutants formed root nodules on alfalfa, and the dry weight of the plants revealed that the slow-growing strains of group III and RmH164 were strongly reduced in their nitrogen fixation abilities compared with the isogenic wild-type strain RmG980 (Table 5). Pck and malate dehydrogenase (as a control) activities were measured in extracts of bacteroids isolated from nodules (Table 5). Only bacteroids of group I (RmH166, RmH167, RmH172) and IV (RmH175) mutants showed high Pck activity (5–13 nmol $\cdot$ min⁻¹ $\cdot$ mg⁻¹ protein) compared with wild type. These levels were reduced compared with the free-living bacteria and were $\sim 10\%$ of the levels seen for succinate grown wild-type cells. We note that the increased malate dehydrogenase activity of bacteroids compared with free-living cells has been previously observed (FINAN *et al.* 1991).

Isolation of the *rpk* group I wild-type gene: As group I mutants showed highest *pckA* expression on M9 glucose and in bacteroids, we decided to identify and char-

acterize the locus where these mutations were located. The mutant strain RmH286 (*rpk-9*, *pckA::lacZ*) forms blue colonies on M9 glucose + X-Gal and recombinant plasmids able to restore wild-type *pckA* regulation (white colonies) were isolated from the pLAFR1 clone bank as described in MATERIALS AND METHODS. Ten complementing plasmids were further analyzed by agarose gel electrophoresis of *EcoRI*-restricted plasmid DNA. The results revealed that the DNA inserts of these plasmids were overlapping, but only a 2.5-kb fragment was common to all plasmids. A restriction map of the region spanned by five of the cosmid clones (pTH277–pTH281) is shown in Figure 2. To locate the region necessary for restoration of wild-type *pckA* regulation, 12 Tn5 insertions spanning the insert DNA of pTH277 were isolated. These plasmids were transferred into RmH147 and the plasmids carrying insertions 34 and 38 were unable to restore glucose-repressed *pckA* expression (data not shown and Table 6). Both of these insertions were located within the 2.5-kb *EcoRI* fragment, confirming the position of the *rpk-9* locus. (Figure 2).

To identify the gene complementing the group I mutants, we determined the complete DNA sequence of a 1250-bp *PstI-EcoRI* fragment where the Tn5 insertions n. 34 and 38 mapped (Figure 2). One major open reading frame of 341 residues, named *pckR*, was identified in this region with an ATG start codon at position 133 preceded by a ribosome binding site, AACGG, located 6 bp upstream. A GenBank search with the PckR protein

FIGURE 2.—(A) Physical and genetic map of the DNA region containing the *R. meliloti pckR* gene. The *EcoRI* restriction map of the genome region containing *pckR* is shown with the size and position of the fragments present in the complementing cosmids pTH277–pTH281. A complete restriction map for *Bam*HI, *Eco*RI and *Hind*III of pTH279 is shown under the genome fragment. The arrowheads represents the location of Tn5 insertions that do (filled) or do not (open) complement *pckA* regulation. The position of the *pckR* gene is shown below the 2.5-kb *Eco*RI fragment subcloned in pTH296 and the shaded box represents the sequenced region. Restriction sites: B, *Bam*HI; C, *clal*; E, *Eco*RI; H, *Hind*III; P, *Pst*I; Sm, *Sma*I; Sp, *Sph*I and X, *Xho*I (B) Physical map of the *R. meliloti pckA* promoter region with the position of the transcription start site (+1), the –10/–35 consensus sequences and the location of the *Eco*RI site where the Ω interposon was inserted. The shaded box indicates the region reported to be the promoter (ØSTERÅS *et al.* 1995).

revealed strong homology to regulators of the GalR-LacI family. The strongest overall similarities were observed with *E. coli* CytR (30.3% identity, GenBank accession No. P06964), PurR (28.4% identity, GenBank accession No. P15039), MalI (28.1% identity, GenBank accession No. P18811), GalR (27.9% identity, GenBank accession No. P03024) and RbsR (27.5% identity, GenBank accession No. P25551). As previously shown in the GalR-LacI family, the most conserved sequence is the N-terminal region that contains the DNA-binding domain (WEICKERT and ADHYA 1992). The two non-complementing Tn5 insertions were subcloned into pUC118 and mapped by sequencing the flanking DNA regions. It could then be confirmed that they were located within the *pckR* coding region, after amino acid 291 for insertion n. 34 and after amino acid 218 for insertion n. 38. The finding that the *pckR* gene encodes a DNA-binding regulatory protein raised the question of its interaction with the *pckA* promoter.

A truncated *pckA* promoter is constitutively expressed: In experiments directed toward defining the *pckA* promoter region, we employed plasmids pTH86 and pTH87, which carry the complete *pckA* region as a 5-kb *Eco*RI fragment extending from an *Eco*RI site conveniently located 62 bp upstream of the *pckA* transcriptional start site to an *Eco*RI site located 3 kb downstream of the *pckA* gene (see Figure 1 in ØSTERÅS *et al.* 1995). When *R. meliloti* transconjugants carrying these plasmids were grown in M9 glucose and assayed for Pck activity, we detected very high activity levels with values $>400 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ protein. These data suggested to us that the *Eco*RI site either bisected or removed a *cis* acting operator-like sequence in the *pckA* promoter. To investigate this possibility further, we constructed *R. meliloti* strain RmH413, which carries an Ω interposon (Sp^r) inserted in the *Eco*RI site in the promoter region of the chromosomal *pckA::lacZ* (*pckA6::Tn3HoKm*) gene fusion. A comparison of *pckA* expression was performed among three strains, all of which contain the wild-type *pckA* gene on the low copy number cosmid clone pRmT103. Strain RmG295 also contains the chromosomal *pckA::lacZ* fusion (*pckA6::Tn*

TABLE 6

Expression of the *pck12::Tn3HoSp* fusion in a *rpk-9* background and complementation by pTH277

Strain	Characteristics	β -galactosidase activity (Miller units) ^a in cells grown on		
		LB	M9 glucose	M9 glucose + succinate
RmG950	RmG212, <i>pckA12::Tn3HoSp</i>	81 $\pm$ 5	30 $\pm$ 6	447 $\pm$ 9
RmH147	RmG950, <i>rpk-9</i>	2081 $\pm$ 67	145 $\pm$ 4	729 $\pm$ 35
RmH420	RmH147, pTH277 (Rpk ⁺)	265 $\pm$ 6	27 $\pm$ 2	485 $\pm$ 2
RmH439	RmH147, pTH277 <i>rpk::Tn5</i> n.34	2072 $\pm$ 54	141 $\pm$ 10	788 $\pm$ 26
RmH440	RmH147, pTH277 <i>rpk::Tn5</i> n.38	1902 $\pm$ 44	192 $\pm$ 51	872 $\pm$ 15

^a Each value represents the mean for triplicate assays $\pm$ the standard error of the mean.

TABLE 7
MDH, PCK and β -galactosidase activities

Strain	Characteristics	MDH ^a		PCK ^a (plasmid <i>pckA</i>)		β -galactosidase ^a (<i>pck-6::Tn3HoKm</i>)	
		LB	M9 glucose	LB	M9 glucose	LB	M9 glucose
RmG295	RmG212, <i>pckA6::Tn3HoKm</i> , pRmT103	633 $\pm$ 32	773 $\pm$ 31	100 $\pm$ 3	25 $\pm$ 3	34 $\pm$ 4	18 $\pm$ 2
RmH465	RmG212, $\Omega::pckA6::Tn3HoKm$, pRmT103	616 $\pm$ 11	694 $\pm$ 3	151 $\pm$ 2	24 $\pm$ 1	2808 $\pm$ 128	2453 $\pm$ 132
RmH466	RmG212, <i>pckA6::Tn3HoKm</i> , <i>rpk-9</i> , pRmT103	853 $\pm$ 9	941 $\pm$ 12	1552 $\pm$ 26	489 $\pm$ 40	4419 $\pm$ 108	1855 $\pm$ 79

Cell extracts were obtained by sonication of LB- or M9 Glucose-grown cultures. RmG295, RmH465 and RmH466 have the *pckA6::Tn3HoKm* fusion recombined in the chromosome. The wild-type *pckA* gene is present on the pLAFR1-derived plasmid pRmT103. In addition, RmH465 contains the Ω interposon in the *EcoRI* site of the chromosomal *pckA6::Tn3HoKm* fusion promoter and RmH466 contains the regulatory mutant allele *rpk-9*.

^a Enzyme specific activity is given in nmol per min per mg of protein as the mean for triplicate assays $\pm$ the standard error of the mean.

3HoKm), strain RmH465 carries the same fusion with the Ω insertion in the promoter region, and RmH466 is the same as RmG295 except it carries the *rpk-9* allele in its genome. The results presented in Table 7 clearly indicate that the Ω insertion (RmH465) results in strong constitutive expression of the downstream *pckA::lacZ* fusion, β -galactosidase activity, (*cis* effect) without affecting the normal expression of the plasmid *pckA* gene, which is preceded by a complete promoter. The presence of the *rpk-9* mutant allele resulted in *trans* expression of both the chromosomal *pckA::lacZ* fusion and the plasmid-located *pckA* gene (RmH466).

DISCUSSION

The *rpk* mutations resulted in the expression of the *pckA* gene under conditions where it would not normally be expressed. These mutations mapped to at least five distinct loci on the *R. meliloti* chromosome, indicating that the expression of *pckA* is under control, at least partially, of several regulatory systems (Figure 1). Given the importance of Pck in the gluconeogenic pathway, one might expect that several factors could control *pckA* gene expression in addition to regulation by available carbon sources. *R. meliloti* and *E. coli* *pckA* expression has previously been observed to be subject to stationary phase regulation (GOLDIE 1984; ØSTERÅS *et al.* 1995). As *pckA* expression is likely to be responsive to the levels of cellular metabolites, it is also likely that mutations in metabolic pathways could alter the levels of cellular metabolites and thus appear as *rpk* mutations. Group III and IV mutants showed a pleiotropic phenotype with either reduced growth on all media (group III) or the inability to use glucose as a carbon source in the presence of succinate (group IV). One of the mutants not linked to one of the four groups, RmH145 (*rpk-7*), has a similar phenotype to group III. Thus the *pckA* expression phenotype in these mutants might not be a direct effect of the *rpk* mutations but rather be a side effect of the growth alteration.

Except for two of the group I *rpk* mutants, most of

the other strains showed <20% of the Pck activity that is present in cells growing under inducing conditions (Table 5). This is consistent with what one would predict for a locus that is subject to a multi-regulatory control.

The symbiotic phenotype of the mutants as deduced from a comparison of the dry weights of plants inoculated with RmG980 *vs.* the two *rpk* mutants, RmH166 and RmH167, suggests that the presence of a low level PCK activity in bacteroids has a minimal effect on symbiotic nitrogen fixation (Table 5). We had previously suggested that the absence of Pck activity in wild-type bacteroids might be a regulatory mechanism to control their growth within the nodule (FINAN *et al.* 1991). The above data are not supportive of this hypothesis, although it is possible that the presence of higher levels of Pck activity in bacteroids might have more significant consequences on the symbiosis.

Many of the mutant strains that grew poorly as free-living cells were strongly affected in their ability to fix N₂. We assume that the symbiotic defects in these mutants result from their overall poor physiological status rather than from a slight increase in Pck activity. Overall the Pck activity levels of *rpk* mutants obtained in glucose-grown free-living cells were higher than the activities detected in bacteroids. This is consistent with the idea that the regulation of gluconeogenesis is different during symbiosis than in free-living cells.

The finding that the *pckR*, which complements the group I mutant *rpk-9*, encodes a protein homologous to the well-characterized GalR-LacI family of prokaryotic regulatory proteins allows us to make several predictions regarding how PckR might control transcription from the *pckA* promoter (WEICKERT and ADHYA 1992; NGUYEN and SAIER 1995). Members of the GalR-LacI family of repressor/activator proteins regulate transcription of target genes through the binding of a dimer to an operator sequence with dyad symmetry, and their activity is modulated by the binding of an effector to the protein. These regulators control transcription of genes involved in carbon metabolism, especially carbo-

hydrate and nucleoside transport and utilization. It is likely that PckR binds to an operator-like sequence in the *pckA* promoter and that the binding may be modulated by an unknown effector molecule. We have presented evidence that the *pckA* promoter contains at least one region that decreases *pckA* expression (Table 7, Figure 2B), and examination of the DNA sequence upstream of the *EcoRI* site of the *pckA* promoter (ØSTERÅS *et al.* 1995) reveals that one region at -76 (5'-TTAAATCGATTAAT-3') fits well with the left half-site consensus for GalR/LacI palindromic binding sites (5'-NNNAANC-3') (WEICKERT and ADHYA 1992).

It was believed that GalR-LacI proteins repressed transcription in the absence of the effector molecules, but recent studies have revealed that they can also activate gene expression (WEICKERT and ADHYA 1992; RAMSEIER *et al.* 1995). A number of the members of this family are also known to interact with other transcriptional activators. For example in promoters that are regulated by the CytR repressor protein, cAMP-CRP acts as an activator of transcription as well as a corepressor for CytR (VALENTIN-HANSEN *et al.* 1996). It is thus very possible that PckR may interact with another regulator that also interacts with the *pckA* promoter. In the case of *E. coli*, the FruR protein, which is also a member of the GalR-LacI family, interacts with cAMP-CRP in regulating transcription from the *pckA* promoter (see SAIER and RAMSEIER 1996). We are currently examining this and other possibilities in studies directed toward understanding the role of PckR in regulating transcription of *pckA*.

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