PCK GENE REGULATION IN *S. MELILOTI*

PHOSPHOENOLPYRUVATE CARBOXYKINASE (PCK) GENE REGULATION IN *SINORHIZOBIUM MELILOTI*

By

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ABSTRACT

Phosphoenolpyruvate carboxykinase (Pck) catalyzes the first step of gluconeogenesis, and the gene which encodes this enzyme (*pck*A) is transcriptionally regulated. High *pck*A expression is observed in succinate-grown cells, while little expression is observed in glucose-grown cells. *pckA* regulatory mutants have previously been isolated (Osteras *et al.* 1997) and *pck*R, a gene encoding a LacI-GalR DNA-binding transcriptional regulator, has been implicated in the regulation of *pck*A transcription.

Here we show that *pck*R insertion mutations result in a dramatic decrease in *pck*A expression even in succinate-grown cells. We demonstrate that the previously identified *rpk*-9 mutation is tightly linked to *pck*R. The *rpk*-9 mutation results in constitutive *pck*A expression, and we show that plasmids carrying the *pck*R gene complement the *rpk*-9 mutation in glucose-grown cells.

A putative LacI-GalR operator binding site has been identified in the *pck*A promoter, however no evidence of an interaction between this site and the *pck*R gene product could be found.

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ABBREVIATIONS

Φ	phage
ΩSp	spectinomycin resistant interposon
A	absorbance
ADP	adenosine diphosphate
Ap	ampicillin
ATP	adenosine triphosphate
bp	base pairs
BSA	bovine serum albumin
DIG	digoxigenin-dUTP
DME	diphosphopyridine nucleotide dependent malic enzyme
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
EMSA	electrophoretic mobility shift assay
Gm	gentamycin
LB	Luria broth media
LBmc	Luria broth with calcium and magnesium
M9	minimal salts media
mA	milliamps
MDH	malate dehydrogenase
Nm	neomycin
OAA	oxaloacetate
ORF	open reading frame
Pck	phosphoenolpyruvate carboxykinase
PCR	polymerase chain reaction
PEP	phosphoenolpyruvate carboxykinase
POD	pyruvate orthophosphate dikinase
^{R}	resistant/resistance
Rf	rifampicin
Sp	spectinomycin
Taq	*Thermus aquaticus* DNA polymerase
Tc	tetracycline
TCA	tricarboxylic acid
TME	triphosphopyridine nucleotide dependent malic enzyme
V	volts
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

CHAPTER 1: INTRODUCTION

Root nodule bacteria

Members of the genera *Rhizobium*, *Sinorhizobium*, *Bradyrhizobium*, and *Azorhizobium*, all form N_2-fixing nodules on the roots of leguminous plant hosts. The relationship between plant and bacteria is mutually beneficial, or symbiotic. The metabolic needs of the bacteria are supplied by the plant, and in exchange, the bacteria provides nitrogen to the plant. In their symbiotic or bacteroid state, the bacteria break the triple bond of atmospheric dinitrogen (N_2), converting it into ammonia (NH_3) in an energy-requiring reaction catalyzed by the nitrogenase enzyme. The bacteroid provides the plant with a usable form of nitrogen thus reducing the agricultural need for nitrogen, which must otherwise be industrially produced as nitrogen fertilizer through the expensive Haber-Bauch process.

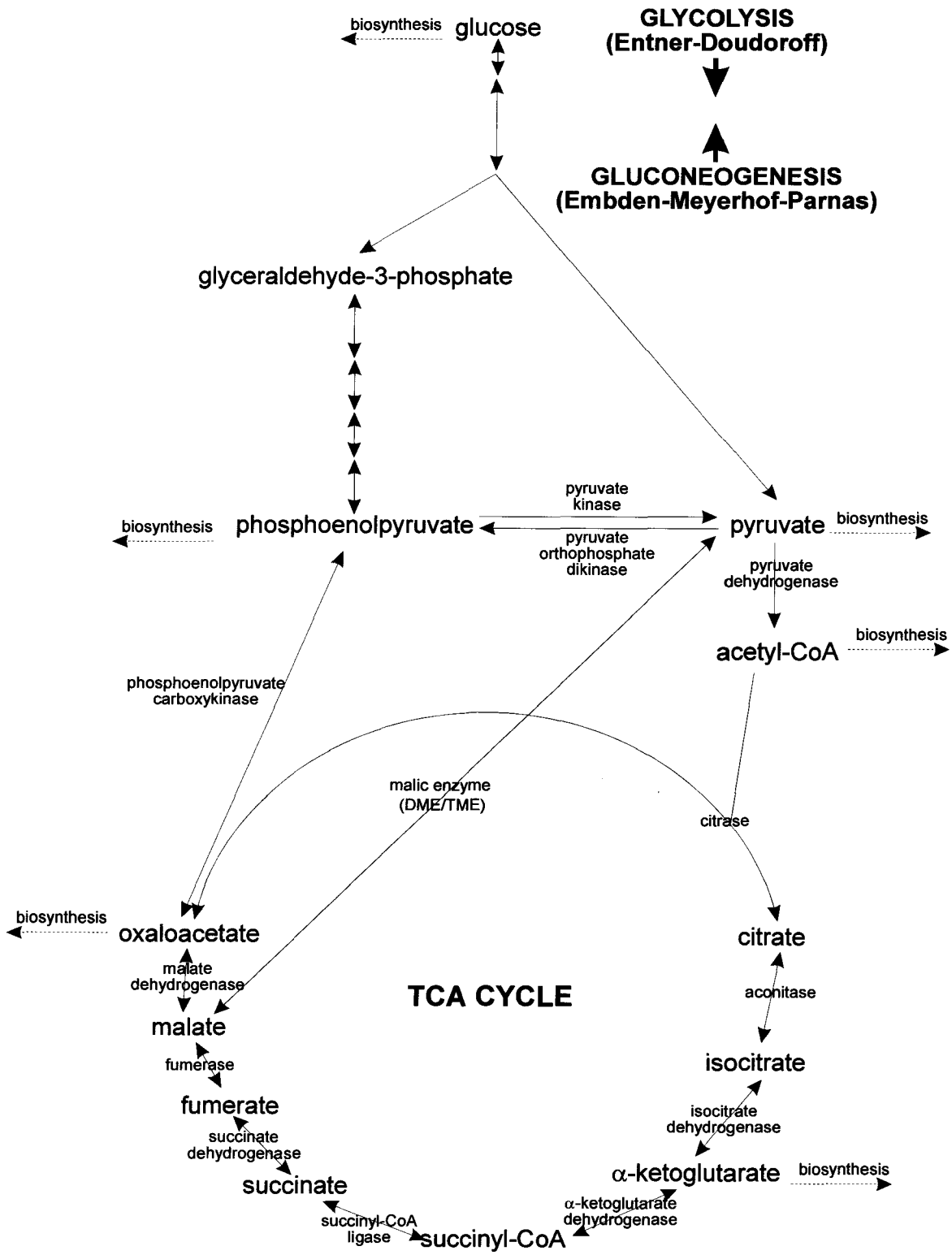
Carbon metabolism

Root nodule bacteria use the Entner-Doudoroff pathway for glycolysis, as evidenced by activation of enzymes involved in this pathway during growth on glucose and not succinate (Arwas *et al.* 1986; Finan *et al.* 1988; Ronson *et al.* 1981; Stowers 1985). Pyruvate is fed from glycolysis into the tricarboxylic acid (TCA) cycle diagrammed in Figure 1. Pyruvate is converted into acetyl-CoA by

pyruvate dehydrogenase. Acetyl-CoA combines with oxaloacetate (OAA) from the TCA cycle to form citrate, and the cycle from citrate to OAA proceeds generating carbon dioxide, ATP, and reduced cofactor. The reduced cofactor can be fed directly into the electron transport chain for further generation of ATP. Intermediates can be fed out of the TCA cycle and into various biosynthetic pathways as cellular needs require. A source of pyruvate, or the ability to regenerate pyruvate, is key to a functional TCA cycle.

Evidence suggests that C_4-dicarboxylic acids, intermediates in the TCA cycle, are the form of carbon that the bacteroid is supplied with from the plant. First, C_4-dicarboxylic acid transport mutants (Dct) form nodules that are incapable of fixing atmospheric nitrogen (Finan *et al.* 1983; Ronson *et al.* 1981; Watson *et al.* 1988; Yarosh *et al.* 1989). Second, nitrogenase activity is stimulated by the presence of C_4-dicarboxylic acids (Stowers 1985). C_4-dicarboxylic acids, such as malate, fumarate and succinate, are directly metabolized via the TCA cycle, unlike glucose which enters from glycolysis as pyruvate. Without a supply of pyruvate from glycolysis, the cell must produce pyruvate via other mechanisms.

Figure 1: The tricarboxylic acid (TCA) cycle



Malate can be converted to pyruvate by malic enzyme. Two malic enzymes have been found in *Sinorhizobium meliloti*—DME, which requires NAD^+ as a cofactor, and TME, which requires $NADP^+$ as a cofactor (Driscoll and Finan 1993). Another mechanism of generating pyruvate is via the action of phosphoenolpyruvate carboxykinase (Pck) and pyruvate kinase (Pyk). However, no Pck activity is detected in *S. meliloti* bacteroids (Finan *et al.* 1991). In the free-living state, Pck activity allows DME/TME mutants to grow on succinate (Driscoll and Finan 1993). DME mutants form nodules that are incapable of fixing atmospheric nitrogen (Fix^-) indicating that, in the bacteroid, DME is the pathway used for synthesis of pyruvate from TCA cycle intermediates. TME mutants produce wild type nodules on alfalfa plants (Driscoll and Finan 1996). The *dme* gene is constitutively expressed in both free-living cells and bacteroids, while the *tme* gene is repressed in bacteroids (Driscoll and Finan 1997).

In addition to pyruvate regeneration, Pck plays an important role in gluconeogenesis in free-living cells growing on C_4-dicarboxylic acids. Pck converts OAA, a component of the TCA cycle, into phosphoenolpyruvate (PEP), which is then channeled into gluconeogenesis. Gluconeogenesis produces glucose for biosynthetic purposes in actively growing cells. The lack of detectable Pck activity in *S. meliloti* bacteroids suggests that these cells are not undergoing gluconeogenesis via this mechanism (Finan *et al.* 1991).

Phosphoenolpyruvate carboxykinase (Pck) and its regulation

Pck catalyzes the following reversible decarboxylation reaction:

$$OAA + ATP \rightleftharpoons PEP + CO_2 + ADP$$

The *pck*A gene, coding for Pck, from *S. meliloti* and *Rhizobium* NGR234 has been identified and sequenced (Osteras *et al.* 1991; Osteras *et al.* 1995). The rhizobial Pck employs ATP as a cofactor, and similar ATP-dependant Pck enzymes have been found in *Escherichia coli* (Medina *et al.* 1990), *Trypanosoma brucei* (Linss *et al.* 1993), yeast (Stucka *et al.* 1988) and plants such as the grass, *Urochloa panicoides* (Finnegan and Burnell 1995), and cucumber (Kim and Smith 1994). The Pck enzyme found in animals requires GTP as a cofactor, and bears no similarity, beyond function, to the ATP-hydrolyzing Pck.

The *pck*A gene has been extensively studied in *E. coli* where its expression is known to be controlled by several different regulatory factors as outlined below.

Catabolite Repression

Measurements of β-galactosidase activity from *pck*A transcriptional fusions with *lac*Z demonstrated that *pck*A expression is induced by gluconeogenic carbon sources, such as glycerol and succinate, and upon entry into stationary phase (Goldie 1984). Addition of glucose to complex media causes a reduction in *pck*A expression, and expression can be restored by addition of cAMP (Goldie and Sanwal 1980b). The carbon source effect, but not the growth phase effect, is subject to catabolite repression in *E. coli* since maximum *pck*A expression was

observed in stationary phase even in the presence of cAMP (Goldie and Sanwal 1980b). Catabolite repression operates such that when levels of glucose are high, levels of cyclic AMP (cAMP) are low, and vice versa (as reviewed by Kolb *et al.* 1993). cAMP, in a complex with CRP (catabolite repressor protein), can either activate or repress the transcription of an operon (as reviewed by Kolb *et al.* 1993). A strain that is lacking cAMP (Δ*cya*) has very low level expression of *pck*A, indicating that cAMP-CRP is required for the expression of *pck*A (Goldie 1984). A potential cAMP-CRP binding site is found in the promoter of *pck*A in *E. coli* at –90 bp relative to the transcriptional start site (Medina *et al.* 1990).

Cra Activation

The transcription of *pck*A is positively regulated by Cra, or catabolite repressor/activator (also known as FruR) in *E. coli* and *S. typhimurium*. In a *S. typhimurium* Cra insertion mutant, Pck activity is reduced five-fold compared to wild type during growth on complex media (Chin *et al.* 1989). Cra is a member of the LacI-GalR family of transcriptional regulators. Cra binds upstream of the promoter of *pck*A, likely promoting transcription by looping the DNA and allowing for contact with the transcriptional initiation complex (Ramseier *et al.* 1995; Saier and Ramseier 1996). However in the presence of fructose-1-phosphate, a glycolytic intermediate, Cra disassociates from the operator binding site, leading to a decrease in the transcription of the *pck*A gene (Ramseier *et al.* 1993). The presence of a glycolytic carbon source therefore turns off the transcription of *pck*A, whose product is required for growth on gluconeogenic carbon sources.

Csr Repression

Pck activity in *E. coli* is also regulated by the pleiotrophic regulator encoded by *csr*A, carbon source regulator. The *csr*A gene product affects the expression of structural genes in gluconeogenesis and glycogen synthesis, and is believed to control a yet unidentified adaptive cellular response. This response is related to the stationary phase response, and has effects on motility and adherence (Romeo *et al.* 1993; Romeo 1998). *pck*A is expressed two-fold higher in a *csr*A mutant, than in wild type, but stationary phase induction is still observed (Romeo *et al.* 1993). Additionally, *csr*A negatively regulates phosphoglucomutase, fructose-1,6-bisphosphatase and phosphoenolpyruvate synthetase, while positively regulating glucose-6-phosphate isomerase, triose-phosphate isomerase and enolase (Sabnis *et al.* 1995). Recently it has been found that CsrA has a 5'-to-3' mRNA degradative ability that is antagonized by CsrB, a copurifying RNA molecule (Liu *et al.* 1995). It is unclear however whether mRNA stability accounts for the effect that CsrA has on *pck*A expression.

Activation by Metal Ions

Pck requires metal ions (Mg^{2+}, Ca^{2+} or Mn^{2+}) for activity as evidenced by inactivity following EDTA (a metal ion chelator) treatment (Goldie and Sanwal 1980a). In saturating (40 mM) amounts of $MgCl_2$, 1 mM of calcium or manganese activates Pck *in vitro* (Goldie and Sanwal 1980a). Pck has a calcium binding site, as evidenced by desensitization to calcium activation by trypsin

treatment (Goldie and Sanwal 1980a), sequencing and crystallization (Matte *et al.* 1996; Medina *et al.* 1990).

Pck of *Sinorhizobium meliloti*

Free-living *S. meliloti* requires a functional *pck*A gene in order to grow on succinate as sole carbon source (Finan *et al.* 1988). During growth on glucose, there is essentially no detectable expression of *pck*A, whereas growth on succinate strongly induces the expression of *pck*A (Osteras *et al.* 1995). When glucose and succinate are present in equal amounts in the culture media, *pck*A expression is about half that observed with succinate alone (Osteras *et al.* 1995). As in *E. coli*, the *pck*A gene of *S. meliloti* is stationary phase regulated. During growth on complex media, expression of *pck*A is constant during log growth and induced 10-fold upon entry into stationary phase (Osteras *et al.* 1995).

In the bacteroid, where the C_4-dicarboxylic acids succinate and malate are the major source of carbon, there is no detectable Pck activity (Finan *et al.* 1991). Yet, plants inoculated with *S. meliloti pck*A mutant strains have 60% of wild type shoot dry weight (Finan *et al.* 1991). *pck*A mutations in *Rhizobium* NGR234 caused a variable phenotype depending on the plant host (Osteras *et al.* 1991). On *Vigna unguiculata*, *pck*A mutant strains formed ineffective nodules (Fix^-), while on *Leucaena leucocephala* and *Macroptilium atroptilium* the mutant bacteroids had 60% and 15% of wild type nitrogenase activity, respectively (Osteras *et al.* 1991). Pck mutants in *Rhizobium leguminosarum* form nodules

that were comparable to wild type (McKay *et al.* 1985). These host dependant phenotypes may be explained by the different carbon sources available to the bacteroid during symbiosis (Osteras *et al.* 1991).

The fact that there is no detectable Pck activity in *S. meliloti* bacteroids, suggests there is a regulatory mechanism for turning off the expression of *pck*A in the bacteroid despite the fact that gluconeogenic carbon sources appear to be the only available form of carbon.

The *pck*A genes of *E. coli*, *S. meliloti* and *Rhizobium* NGR 234 have been shown to have a σ^{70}-like promoter (Osteras *et al.* 1995). Characteristically these promoters contain consensus –10 and –35 regions. In *S. meliloti*, it has been found that a truncated *pck*A promoter is constitutively expressed (Osteras *et al.* 1997). When an ΩSp interposon was inserted at the *Eco*RI site 62 bp upstream of the *pck*A transcriptional start site, high constitutive expression of the *pck*A gene was observed. This suggests that the Ω insertion at the *Eco*RI site either bisected or removed a *cis* acting negative operator sequence from the *pck*A promoter (Osteras *et al.* 1997).

Spontaneous *pck*A regulatory mutants

In order to identify genes involved in the carbon source regulation of *pck*A, spontaneous mutants were isolated that expressed *pck*A during growth on glycolytic carbon sources (Osteras *et al.* 1997). A strain with *pck*A fused to *lac*ZYA (*pck*A12::Tn3HoHo1) in a Lac$^-$ background was employed to isolate

these spontaneous mutants. Since *pck*A is not expressed during growth on glycolytic carbon sources, such as glucose and lactose, this strain is unable to grow on lactose. Spontaneous mutants able to grow on lactose were isolated, and named *rpk* mutants for regulator of *pck* (Osteras *et al.* 1997). Several of these mutants, when used to inoculate alfalfa plants, produced bacteroids that, unlike the wild type, had Pck activity. The mutants were mapped to four linkage groups (I, II, III, IV). Group I, consisting of mutant alleles *rpk*-9, *rpk*-10 and *rpk*-15, was of particular interest because these mutants showed the highest Pck activity in the bacteroid, and the highest expression of *pck*A when grown as free-living cells (Osteras *et al.* 1997).

Ten cosmids were isolated from a *S. meliloti* clone bank that complemented the *rpk*-9 mutant phenotype and restored wild type levels of *pck*A expression during growth in glucose (Osteras *et al.* 1997). The complementing cosmids contained a common 2.5 kb *Eco*RI fragment. One of these cosmids, pTH277, was mutagenized with Tn*5*, and insertions that were no longer able to complement the *rpk*-9 phenotype were isolated. These insertions were found to be located within a gene bearing strong homology to the LacI-GalR family of transcriptional regulators (Osteras *et al.* 1997; Chapter 3, this thesis).

LacI-GalR-type transcriptional regulators

The LacI-GalR family of transcriptional regulators is a large family of DNA-binding proteins. Thus far, members of this family have only been identified from

prokaryotes. Characteristically, they have a N-terminal helix-turn-helix (H-T-H) domain, which is involved in DNA binding, and a C-terminal effector binding domain (as reviewed by Weickert and Adhya 1992; as reviewed by Nguyen and Saier 1995). These proteins function by binding an operator site in the promoter being regulated. Most members of this family act to repress transcription (as reviewed by Weickert and Adhya 1992). Binding of the effector molecule to the LacI-GalR-type protein generally reduces the affinity of the protein for the operator, thereby releasing repression and allowing transcription to occur. However, the LacI-GalR family is a very diverse group of proteins, and there are almost as many exceptions to the latter general scheme as there are those that are typical. Four well-studied members of this family will be discussed—LacI, PurR, CytR and Cra/FruR.

LacI

LacI (sometimes called LacR) has been extensively studied and is the paradigm of a negative regulator. Recently, the 3-dimensional structure of LacI has been determined, providing a physical model for the interaction of this protein with the operator and effector (Lewis *et al.* 1996; Retallack and Friedman 1995). In the absence of inducer, LacI binds to a high affinity operator site nine base pairs past the transcriptional start site, overlapping the binding site for RNA polymerase. From this position, LacI prevents the transcription of the *lac*ZYA genes which encode genes involved in lactose utilization. In the presence of the inducer lactose, the affinity of LacI for the operator is reduced. When LacI is not

bound to the operator, transcription occurs. Therefore, in the absence of lactose the cell prevents the unnecessary transcription of genes required for its metabolism.

Generally, members of the LacI-GalR family act as homodimers, but there are several members that act as homotetramers including LacI and Cra/FruR (as reviewed by Weickert and Adhya 1992) (Cortay *et al.* 1994). The ability of LacI to form tetramers allows this protein to bind to two operator sites concurrently, causing the DNA between the operator sites to loop. The binding of LacI to operator sequences has been extensively studied using electrophoretic mobility shift assays (Fried and Crothers 1981).

PurR

PurR is known to repress transcription of 14 different operons involved in purine and pyrimidine biosynthesis and salvage in *E. coli* (Schumacher *et al.* 1994). PurR is 341 amino acids in length, typical for members of the LacI-GalR family, but unlike most of this family, PurR is autoregulated (Meng *et al.* 1990; Rolfes and Zalkin 1990) (as reviewed by Weickert and Adhya 1992). As well, to bind to the operator PurR must first be cooperatively bound to its co-repressor.

The 3-dimensional structure of PurR, bound to both co-repressors—hypoxanthine and guanine—and the *purF* operator has been determined revealing a great deal of information about this protein, including confirmation of the presence of the N-terminal DNA-binding motif (Schumacher *et al.* 1994; Schumacher *et al.* 1997). The 3-dimensional structure revealed that the H-T-H

motif contacts the DNA in the major groove, while a "hinge" helix contacts the minor groove. Three helices (I, II, III) form a globular subdomain that is connected to the C-terminal co-repressor binding domain by the hinge helix (Schumacher *et al.* 1994). One particular residue in the hinge helix believed to be structurally and functionally significant is the leucine residue at position 54. Side chains of Leu-54 and Leu-54' from the two monomers of PurR intercalate between the central guanine and cytidine of the operator sequence. These residues are responsible for prying open the minor groove allowing unwinding and kinking of the DNA towards the major groove and away from PurR.

All members of the LacI-GalR family examined to date, except for CytR, have a leucine corresponding to Leu54 of PurR (Schumacher *et al.* 1994), suggesting that the interaction with the minor groove which causes kinking of the DNA is conserved. The alanine in position 51 in PurR is also conserved among all members, except CytR which substitutes a glycine at this position. This residue is structurally restricted because it directly touches the DNA backbone.

The C-terminal domain of PurR consists of a complex series of α-helices and β-strands. There is structural similarity in the C-terminal domain to bacterial periplasmic binding proteins, particularly the ribose binding protein. These bacterial periplasmic binding proteins are involved in the transport of metabolites across the cell membrane, however in contrast to the LacI-GalR family, they act as monomers.

CytR

CytR is the 341 amino acid *E. coli* cytidine repressor, which regulates the expression of genes involved in nucleoside and deoxynucleoside uptake and metabolism. CytR acts as a repressor in all known natural promoters studied to date (Mollegaard *et al.* 1993). However in recombinant promoters CytR was found to activate transcription in co-operation with cAMP-CRP by forming a large polymerase initiation complex stretching over 100 bp upstream of the transcriptional start site (Rasmussen *et al.* 1996).

CytR requires the cooperative binding of CRP to bind efficiently to the operator (Pedersen and Valentin-Hansen 1997). The binding of CRP to its binding site, and protein-protein interactions between CytR and CRP increase the affinity of CytR for its binding site. When CytR is bound to its operator, it overlaps with the RNA polymerase binding site and prevents transcription. However, if the CytR operator is located upstream of the RNA polymerase binding site, CytR can act as an activator (Rasmussen *et al.* 1996).

A molecular cause for the loose interaction of CytR with DNA may be the lack of a leucine residue corresponding to the Leu54 of PurR (Schumacher *et al.* 1994). The requirement for synergistic binding of corepressor in the interaction of CytR with the operator may lead to versatility in operator recognition causing CytR operators to be highly degenerate (Valentin-Hansen *et al.* 1996). As well, the dimers of CytR possess an ability to bind to operators with varying distances

between them, likely due to CytRs flexibility (Pedersen and Valentin-Hansen 1997).

Cra/FruR

Cra, or catabolite repressor/activator, is the 334 amino acid *E. coli* and *S. typhimurium* regulatory protein that was initially identified as being the fructose repressor (FruR) (Chin *et al.* 1987; Saier and Ramseier 1996; Scarabel *et al.* 1995). Cra binds to the operator as a homotetramer like LacI (Cortay *et al.* 1994; Weickert and Adhya 1992).

Cra affects the expression of many genes involved in metabolism, including phosphoenolpyruvate synthase, Pck, isocitrate dehydrogenase, malate synthase, isocitrate lyase, fructose catabolic enzymes, and phosphotransferase system (Ramseier *et al.* 1993; Ramseier 1996). The role of Cra appears to be to activate genes whose products are involved in oxidative and gluconeogenic metabolism, and to repress genes whose products are involved in fermentative metabolism. Thus, Cra functions opposite to catabolite repression. Those operons that are activated by Cra are subject to catabolite repression, while those operons that are repressed by Cra are subject to catabolite activation.

The operator binding site is the same for operons repressed and activated by Cra (Ramseier *et al.* 1995). The effector molecule, frucose-1-phosphate, reduces the affinity of the protein for the operator DNA and displaces Cra, regardless of whether Cra is acting as a repressor or as an activator (Ramseier *et al.* 1993).

This work

In this work, the regulation of *pck*A expression in the two metabolically distinct states—free-living and bacteroid—of *S. meliloti* was examined.

The complete sequence and analysis of the *pck*R gene, encoding the LacI-GalR-type regulator that complemented the *rpk*-9 mutation (Osteras *et al.* 1997) was determined. The phenotype of *pck*R insertion mutants and the spontaneous group I *rpk* mutants was examined. Mutation of *pck*R lead to low-level expression of *pck*A. In contrast, the *rpk*-9 mutation causes high-level expression of *pck*A during growth in glucose. The *pck*R insertions and the group I *rpk* mutations were found to be linked in transduction.

The expression of *pck*A in *S. meliloti* bacteroids from alfalfa root nodules indicated that Pck levels in the bacteroid are controlled at the level of transcription of *pck*A (Finan *et al.* 1991; Osteras *et al.* 1997).

Electrophoretic mobility shift assays (EMSA) were employed to look for interaction between the *pck*A promoter and the *pck*R gene product. No evidence of such an interaction was found, however there appear to be several unidentified proteins which do bind to the *pck*A promoter.

CHAPTER 2: MATERIALS AND METHODS

Bacterial strains and growth conditions

S. meliloti was incubated at 30°C while *E. coli* cells were incubated at 37°C. *E. coli* was routinely grown in Luria broth (LB) media (10 g/L bacto tryptone, 5 g/L yeast extract, 5 g/L NaCl, with the addition of 4 ml/L (liquid medium) or 1 ml/L (solid medium) of 1 N NaOH (Miller 1972). The complex medium used for the growth of *S. melilcti* was LBmc with 2.5 mM $MgSO_4$ and 2.5 mM $CaCl_2$ added to liquid media after sterilization. Solid media was prepared with the addition of 15 g/L Difco Bacto Agar. The defined media used for the growth of *S. meliloti* was M9 salts media (Gibco-BRL). A carbon source (glucose or succinate), $MgSO_4$, $CaCl_2$ and biotin were added to final concentrations of 15 mM, 2.5 mM, 1.25 mM and 1 μg/ml respectively after sterilization.

Antibiotics were added at the following concentrations to solid media for growth of *E. coli*: kanamycin monosulfate (Km), 20 μg/ml; gentamycin sulfate (Gm), 5 μg/ml; Na-Ampicillin (Ap), 100 μg/ml; spectinomycin dihydrochloride (Sp), 100 μg/ml; tetracycline hydrochloride (Tc), 10 μg/ml; chloramphenicol (Cm),

20 μg/ml. Antibiotics were added at the following concentrations to solid media for growth of *S. meliloti*: streptomycin sulfate (Sm), 200 μg/ml; neomycin sulfate (Nm), 200 μg/ml; rifampicin (Rf), 20 μg/ml; spectinomycin dihydrochloride (Sp), 100 to 300 μg/ml; tetracycline hydrochloride (Tc), 5 μg/ml. In liquid media, antibiotics were added to half the indicated concentration.

For growth in liquid M9, an overnight starter culture in LBmc with the appropriate antibiotics was used to inoculate M9 containing the desired carbon source by first washing the culture in 0.85% NaCl. Tubes (5 ml) of M9 were grown for 2 days, while flasks (300 ml) were grown for 3 days.

For experiments in which the expression of *pck*A was measured from a plasmid *pck*A::*lac*Z fusion over a time course, starter cultures of the strains were grown in LBmc containing Tc (2 μg/ml) and subcultured into starter cultures of M9 glucose media. The time course began when an aliquot from the M9 glucose culture was added to a 300 ml flask containing either M9 glucose, M9 succinate or LBmc. The flasks were grown with aeration for 90 hours (M9) or 60 hours (LBmc). Samples were removed approximately every 5 hours and stored at –20°C until all samples were collected. The OD_{600} and β-galactosidase activity of each sample was determined.

Strains of *S. meliloti* and *E. coli*, and plasmids used and constructed in this study are listed in Table 1.

Table 1: *S. meliloti* and *E. coli* strains and plasmids used in this study

***Sinorhizobium meliloti* strains**

Strain	Genotype	Reference
Rm1021	SU47, *str-21*	Meade *et al.* 1982
Rm5000	SU47, *rif-5*	Finan *et al.* 1984
RmG212	Rm1021, Lac$^-$	Jane Glazebrook
RmG263	RmG212, *pck*A6::Tn3HoHo1Km	Osteras *et al.* 1997
RmG319	RmG212/pF94	Osteras *et al.* 1995
RmG950	RmG212, *pck*A12::Tn3HoHo1Sp	Osteras *et al.* 1997
RmH147	RmG950, *rpk*-9	Osteras *et al.* 1997
RmH166	RmH147, *pck*A Ω5315::Tn*5*	Osteras *et al.* 1997
RmH286	RmG212, *rpk*-9, Ω5345::Tn*5*	Osteras *et al.* 1997
RmH443	Rm5000, Ω5345::Tn5-233	Osteras *et al.* 1997
RmH464	RmH147, *pck*A6::Tn3HoHo1Km	Osteras *et al.* 1997
RmH466	RmH464/pRmT103	Osteras *et al.* 1997
RmH853	RmG212, *pck*R34::Tn*5*	This study
RmH854	RmG212, *pck*R38::Tn*5*	This study
RmH857	RmH853/pF94	This study
RmH858	RmH854/pF94	This study
RmH903	Rm5000, *pck*R34::Tn*5*	This study
RmH904	Rm5000, *pck*R38::Tn*5*	This study
RmH905	RmH147/pTH446	This study
RmH906	RmH148/pTH446	This study
RmH907	RmH153/pTH446	This study

RmH908	RmH166/pTH446	This study
RmH914	RmH286/pF94	This study
RmH925	Rm5000, *pck*R34::Tn*5*-233	This study
RmH926	Rm5000, *pck*R38::Tn*5*-233	This study
RmH928	RmG263, *pck*R34::Tn*5*-233	This study
RmH929	RmG263, *pck*R38::Tn*5*-233	This study
RmH962	RmG263, *pck*R::ΩSp	This study
RmH964	RmH928/pRmT103	This study
RmH965	RmH929/pRmT103	This study
RmH971	RmH962/pRmT103	This study
RmK118	Rm5000, *pck*R::ΩSp/R751-pGM2	This study
RmK124	RmG212, *pck*R::ΩSp	This study
RmK136	RmG950/pRmT103	This study
RmK141	RmK124/pF94	This study

***Escherichia coli* strains**

Strain	Genotype	Reference
DH5α	F^-, *end*A1, *hsd*R17 (r_k^-, m_k^-), *sup*E44, *thi*-1, *rec*A1, *gyr*A96, *rel*A1, Δ(*arg*F-*lac*ZYA) U169, ϕ80d*lac*Z, ΔM15	BRL Inc.
MT607	MM294A *rec*A-56	Finan *et al.* 1986
MT616	MT607/pRK2013 *npt*::Tn*9*	Finan *et al.* 1986

Plasmids

Plasmids	Genotype	Reference
pET21a(+)	protein overexpression vector	Novagen
pF94	pRmT103, *pck*A6::Tn3HoHo1Km	Osteras *et al.* 1995
pG6	pRmT103, *pck*A12::Tn3HoHo1Sp	Osteras *et al.* 1995
pHP45Ω	pBR322 derivative carrying the Sm^r/Sp^r Ω interposon	Prentki and Krisch 1984
pLAFR1	broad host range cosmid vector, Tc^R	Friedman *et al.* 1982
pRK600	pRK2013 *npt*::Tn9, Cm^R	Finan *et al.* 1986
pRK7813	broad host range cloning vector, Tc^R	Jones and Gutterson 1987
pRmT103	pLAFR1clone complementing *pck*A mutants	Finan *et al.* 1988
pTH137	3.2 kb *Sma*I fragment with partial *pck*A in pUC118	Osteras *et al.* 1995
pTH277	pLAFR1 cosmid restoring normal *pck*A regulation in RmH147	Osteras *et al.* 1997
pTH296	2.5 kb *Eco*RI fragment from pTH277 into pUC118	Osteras *et al.* 1997
pTH402	0.6 kb *Pst*I fragment from pTH296 into pUC118	This study
pTH403	1.7 kb *Hind*III fragment from pTH296 into pUC118	This study
pTH404	1.7 kb *Hind*III fragment from pTH296 into pUC118 (opposite orientation to pTH403)	This study
pTH405	0.9 kb *Hind*III fragment from pTH296 in pUC118	This study
pTH406	0.9 kb *Hind*III fragment from pTH296 in pUC118 (opposite orientation to pTH405)	This study
pTH419	*Cfr*91 deletion of pTH296	This study
pTH420	*Pst*I (P3) deletion of pTH296	This study
pTH421	0.7 kb *Cfr*91/*Hind*III fragment from pTH296 into pUC119	This study
pTH446	2.5 kb *Eco*RI fragment from pTH296 into pRK7813	This study
pTH447	2.5 kb *Eco*RI fragment from pTH296 into pRK7813 (opposite orientation to pTH446)	This study
pTH475	ΩSp cloned into the 5' *Hind*III site of pTH296	This study

pTH476	*Eco*RI fragment from pTH475 into pRK7813	This study
pTH531	89 bp PCR product from the *pck*A promoter in pUC118	This study
pUC118/9	ColE1 *oriV* cloning vectors, Ap^r	Vieira and Messing 1987
R751-pGM2	IncP1	Jacoby *et al.* 1976

Genetic techniques

Bacterial matings

Plasmids were transferred from *E. coli* to *S. meliloti* by combining 0.5 ml of an overnight culture of both donor and recipient strains with 0.5 ml of MT616 which carries the plasmid pRK600 (Cm^R derivative of pRK2013), and provides the transfer functions *in trans*. Alternatively, if the plasmid being transferred was a pRK2013 derivative, addition of pRK600 was not necessary. This mix was centrifuged (3 minutes at 13,000 rpm) and resuspended in approximately 50 μl of LB, spotted on an LB plate and left overnight at 30°C. The mating spot was resuspended in 1 ml of 0.85% NaCl and dilutions were plated on appropriate media for selection of *S. meliloti* recipient and the plasmid.

Transduction

Transductions were performed as described by Finan *et al.* (1984) using ΦM12. Phage lysates were prepared by adding 50 μl of phage (approximately 10^{9-10} PFU/ml) to an actively growing (OD_{600} approximately 0.4) *S. meliloti* culture in LBmc media, and incubating overnight at 30°C with aeration. Lysates were sterilized with $CHCl_3$ and centrifuged to remove cell debris.

For transductions, equal quantities of *S. meliloti* culture of approximately an OD_{600} of 0.4 and diluted phage were mixed to approximately a multiplicity of infection of 0.5, and incubated at 30°C for 20 minutes. Phage was removed by two washes in 0.85% NaCl and the resulting cell pellet was resuspended in a

volume of 0.85% NaCl equal to the original volume of bacterial culture used. 100 µl aliquots of the cells was plated on LB selecting for the antibiotic of the marker being transduced and incubated at 30°C for 3 to 5 days. Resulting transductants were purified at least three times.

Transposon replacements

The replacement of Tn*5* with Tn*5*-233 in an Rm5000 background was accomplished via homologous recombination between the flanking IS50 elements as previously described by Charles (1990).

Homogenetization

Gene fragments in pRK7813 containing the ΩSp interposon were recombined into the chromosome of Rm5000 using the Gm resistant IncP1 plasmid (R751-pGM2) as previously described by Charles *et al.* (1991). Colonies were subsequently screened for Tc sensitivity to indicate the loss of the pRK7813 plasmid. Insertions were transduced into RmG212, and confirmed by Southern blotting.

DNA manipulations

Small scale and large scale preparations of plasmid DNA were prepared by alkaline lysis (Sambrook *et al.* 1989). Large scale preparations of DNA were further purified using the LiCl, PEG/NaCl precipitation procedure. Restriction endonucleases and other DNA-modifying enzymes were used according to

manufacturer's recommendations. To visualize, retrieve, or purify DNA fragments, 0.8% to 2% agarose (GibcoBRL) gels were electrophoresed with Tris-acetate-EDTA (TAE) running buffer. For visualization of fragments less than 500 bp, 2.5% to 4.5% NuSeive GTG agarose (FMC BioProducts) gels were used. If necessary, bands were excised and eluted using either the Geneclean II kit (BIO101) or the QIAEX II Gel Extraction kit (QIAGEN). Alternatively, a "freeze and squeeze" protocol was used, involving addition of phenol to the crushed gel slice and freezing at –70°C, followed by a series of phenol/chloroform extractions and an ethanol precipitation. Ligations were carried out overnight at room temperature. Plasmids were transformed into DH5α cells which were made competent using the modified calcium chloride procedure (Ausubel *et al.* 1989).

Polyacrylamide gel electrophoresis

Polyacrylamide gels were also used to visualize small DNA fragments where retrieval of DNA from the gel was not necessary. 8 to 20% acrylamide gels were made from a concentrated 30% acrylamide, 1% N,N'-methylenebisacrylamide filtered stock as described by Sambrook *et al.* (1989). Tris-borate-EDTA (TBE) was used as the running buffer for electrophoresis. The gel was cast with 15.5 x 19 cm plates with 1.5 mm spacers.

Southern blotting

Total genomic DNA for use in Southern blotting was prepared in a procedure adapted from Meade *et al.* (1982) as described by Oresnik (1990).

The procedure for Southern blotting, and DIG detection was as described by Boehringer Mannheim (Boehringer Mannheim, The DIG System User's Guide for Filter Hybridization, 1995). Probes for Southern blotting were labeled overnight with digoxigenin-dUTP (DIG) (Boehringer Mannheim) according to manufacturers instructions. DNA fragments were separated by agarose gel electrophoresis and transferred overnight to nylon membrane (ICN Biomedicals) by capillary transfer. Membranes were hybridized with DIG-labeled probe at 65°C, washed and developed as recommended by the manufacturer, and as described by Charles *et al.* (1991).

Polymerase Chain Reaction

Primers for PCR were synthesized (Mobix Central Facility) and resuspended in $T_{10}E_1$ to 100 pmoles/μl. All PCR reactions were performed in a Perkin Elmer *GeneAmp* PCR System 2400. A master mix containing 1X buffer (Perkin Elmer/MBI Fermentas), ddH_2O, 200 μM each dNTP, 1 μM each primer, and 2.5 units Taq (Perkin Elmer/MBI Fermentas) was prepared and added to PCR tubes containing 5 μL of template DNA (< 1ng plasmid or 50 ng genomic). $MgCl_2$ was added and the tubes were mixed prior to PCR amplification. The optimal $MgCl_2$ concentration (1 to 5 mM) was empirically determined for each primer set. The final reaction volume was 100 μL. 25 to 30 cycles of amplification were used with 30 seconds each of melting (94°C), annealing and extension (72°C) unless otherwise stated. The final step was 7 minutes at the

extension temperature. The annealing temperature was between 55°C and 65°C (5°C below the lowest Tm of a primer pair). Reactions were checked by electrophoresing 5 to 10 µl of the reaction on an agarose gel. For use in subsequent cloning procedures, the remaining reaction volume was ethanol precipitated (Sambrook *et al.* 1989). For sequencing, the remaining reaction volume was ethanol precipitated and gel purified.

DNA sequencing and analysis

DNA sequencing was performed on an ABI 373 Stretch automatic sequencer using the dye terminator chemistry and cycle sequencing (Mobix Central Facility). The standard M13-forward (5'-GTAAAACGACGGCCAGT-3') and M13-reverse (5'-AACAGCTATGACCATG-3') primers were used for sequencing unless otherwise stated.

Contiguous sequences were aligned using Genetic Data Environment version 2.0 (S. Smith, unpublished results). ClustalW (Thompson *et al.* 1994) was used to align amino acid sequences.

Biochemical techniques

Cell extract preparation

Cells were washed twice in 20 mM Tris pH 7.8, 1 mM $MgCl_2$, and resuspended in approximately 4 ml per gram wet pellet weight of Buffer 1 (20 mM Tris pH 8.4, 1 mM $MgCl_2$, 10 mM KCl, 20% glycerol, 1 mM DTT). Samples

were sonicated in the Heat Systems Model XL2020 instrument equipped with a Cup Horn. For *S. meliloti*, approximately 2 hours of sonication time were required for lysis. Extracts were centrifuged at 5000 rpm for 15 minutes and subsequently at 13,000 rpm for 15 minutes to pellet cells and cell debris. Extracts were stored on ice or at 4 °C throughout all steps of sonication, and stored at –20°C.

Alternatively, cell extracts were prepared by French Press. A small French Press cell (maximum volume approximately 7 ml) was used with a hydraulic press. For *S. meliloti*, approximately four passes through the French Press cell at a maximum of 600 psi were required to completely lyse the cells. Cells and cell extracts were prepared in an identical manner to those for sonication.

Protein determination

The protein concentration of crude cell extracts was determined by the Bradford method (Bradford 1976) using a mini-protocol for the Coomassie blue R250 Bio-Rad Protein Assay Dye Reagent (Bio-Rad Laboratories). Bovine serum albumin (BSA) amounts ranging from 1 to 10 μg were used to establish a standard curve.

β-Galactosidase assays

Assays were performed in a protocol adapted from Miller (1972) as described by Driscoll (1995). The final assay volume was reduced to 1.5 ml. For assays of cell extract, the absorbance of the final reaction mix for each sample

was read at 420 nm in the Cary Varian spectrophotometer, and the specific activity was calculated as nmoles of o-nitrophenol produced/minute/mg protein using the following formula:

(A_{420} x reaction volume in ml) / reaction time in minutes / 0.0045 / mg protein.

For assays of permeabilized cells, the absorbance of the culture to be assayed was measured at 600 nm. The absorbance at 420 nm of the final reaction mix was read in the Cary Varian spectrophotometer, and Miller units were calculated using the following formula:

(1000 x A_{420}) / A_{600} / reaction time in minutes / volume of culture used in ml.

Malate dehydrogenase (MDH) assay

MDH catalyzes the reaction: malate + NAD^+ $\leftrightarrow$ OAA + NADH. Assays were performed as described by Driscoll (1995). The change in absorbance at 340 nm due to the reduction of NAD^+ to NADH was read in the Cary Varian spectrophotometer over a period of approximately 3 minutes. The specific activity was calculated as nmoles of NADH produced/minute/mg protein using the following formula:

slope (ΔA_{340}) / 0.00622 / mg protein.

Plant growth

Plants were grown in Leonard (1943) assembly pots as described by Cowie (1998) and Driscoll (1995). Alfalfa seeds (*Medicago sativa* cultivar Iroquois) were surface sterilized and germinated as described by Cowie (1998). Ten

seedlings were planted into each of the Leonard assembly pots. Plants were placed in a Conviron growth chamber covered for two days before inoculation. Overnight cultures of the strains to be used for inoculation were grown in LBmc with appropriate antibiotics. 0.1 ml of culture was diluted in 10 ml of sterile H_2O, and added to each pot. The seedlings were grown uncovered for 30 days with 16 hours of light at 23°C and 8 hours of dark at 18°C per day. The pots were watered as required with sterile distilled, deionized H_2O. 30 days after inoculation, the plants were harvested. The plants were separated from the roots, and the plants were dried at 70°C for approximately 1 week before the dry weight was measured.

Nodules were removed from the plant roots, and bacteroids isolated as described by Cowie (1998). Bacteroids were sonicated for 10 minutes (30 seconds pulse, 20 seconds off) in the Heat Systems model XL2020 sonicator. The extract was centrifuged for 15 minutes at 13,000 rpm at 4°C to remove cell debris.

Electrophoretic Mobility Shift Assay (EMSA)

Construction of probe

A probe used in EMSAs was an 89 bp fragment from the *pck*A promoter. A PCR product was generated using pG6 (Osteras *et al.* 1995) as a template by amplification between the promoter of *pck*A and a Tn3HoHo1 insert located

within the coding region of *pck*A. The PCR product was digested with *Eco*RI and isolated from a 4.5% NuSeive agarose gel. This 89 bp fragment was subcloned into pUC118, digested with *Eco*RI and *Sma*I, and the cloning confirmed by polyacrylamide gel electrophoresis. The fragment was subsequently excised for labeling by digestion with *Eco*RI and *Bam*HI.

Other probes used in EMSA were *Sst*I and *Eco*RI, or *Sst*I and *Hind*III fragments isolated from a large scale preparation of pTH137, which contains the promoter of *pck*A (Osteras *et al.* 1995).

Preparation of ^{32}P labeled DNA probe

Target DNA was excised from approximately 10 μg of plasmid DNA digested with a restriction enzyme, such as *Eco*RI or *Sst*I, which generates a 5' overhang with at least one residue in the overhang being dTTP. After digestion for at least 1 hour at 37°C, 1 unit of the Klenow fragment of DNA polymerase (Boehringer Mannheim), 50 μCi of 3000 Ci/mmol [α-^{32}P]-dATP (NEN™ Life Science Products), and 2 mM 3dNTP (dCTP, dGTP, dTTP) mix were added and incubated at 30°C for at least 1 hour. The digested DNA was precipitated to remove 99% of the unincorporated nucleotides and electrophoresed on a 4.5% NuSeive GTG agarose gel (FMC BioProducts). The probe was excised from the gel and purified using the QIAEX II Gel Extraction kit (QIAGEN). The rate of incorporation of radiolabel into the probe was determined by measuring the counts per minute (cpm) of 1 μl of probe in a scintillation counter (Beckman

LS1801). Very low incorporation efficiencies (less than 5%) were observed due to use of excess label.

11.1 μg of the 89 bp probe in pUC118 was initially digested during probe preparation. The 89 bp insert accounted for 2.7% of the total vector. Assuming 80% recovery, and knowing the final resuspension volume was 60 μl, the final concentration of the 89 bp fragment in the probe preparation can be estimated at approximately 4 ng/μl.

Gel preparation

The gel mix consisted of 8 ml 5X Tris-glycine stock (30.28 g/L Tris base, 142.7 g/L glycine, 3.92 g/L EDTA), 5.33 ml 30% acrylamide (filtered through Whatman #1), 1 ml 2% bisacrylamide, 2 ml 50% glycerol and 23.7 ml H_20 (Ausubel *et al.* 1989). 100 μl of 30% ammonium persulfate and 34 μl of N,N,N',N'-tetramethylethylenediamine (TEMED) was added immediately before pouring the gel.

The gel was cast between a flat 15.5 x 19 cm glass plate and a notched glass plate separated by 1.5 mm spacers. The gel was allowed to polymerize for 3 hours or overnight. Upon removing the comb, the wells were cleaned thoroughly with running buffer. The gel was pre-run for 1 hour at a constant 100 V.

Binding assay

1 μl containing approximately 10,000 cpm ^{32}P labeled DNA probe was used per binding reaction, with 2 μg poly(dI-dC)-poly(dI-dC) (Boehringer Mannheim), 4 mM Tris-HCl pH 8.4, 60 mM KCl, 1 mM EDTA, 1 mM DTT, 12% glycerol, and 4.5 μg BSA (modified from Ausubel *et al.* 1990). Crude cell extract was added and the volume was made up to 15 μl with buffer 1 for sonication. Binding assays were incubated at 30°C for 20 minutes. 3 μl of 50% glycerol with bromophenol blue was added, and the samples were loaded onto the gel. During all steps the samples were handled gently to prevent destabilization of DNA-protein complexes. The gel was run at room temperature in 1X Tris-glycine buffer prepared from a 5X stock. A constant current of 30 mA was applied for 2 to 3 hours depending on the size of the probe.

Upon disassembly, the gel was attached to Whatman 3MM paper and dried under vacuum for 1 hour at 70°C. Gels were exposed to autoradiograph film for 24 to 48 hours, and were quantified using the Molecular Dynamics PhosphorImager (Model 425B). Phosphoimager data was area integrated to determine the pixel values using Molecular Dynamics ImageQuant 3.3 software.

CHAPTER 3: RESULTS

The *rpk*-9 mutation results in the expression of the *pck*A gene in glucose-grown cells, a condition during which *pck*A is not normally expressed. Ten cosmid clones restored a wild type pattern of *pck*A regulation to the *rpk*-9 mutant strain on glucose minimal media. All of these cosmids, upon restriction analysis, were found to contain a common 2.5 kb *Eco*RI fragment. Two Tn*5* insertions in the complementing cosmid, pTH277, which eliminated the ability of this cosmid to complement, were isolated and found to be localized within the common *Eco*RI fragment. The 2.5 kb *Eco*RI fragment was subcloned into pUC118 (pTH296) for further sequencing and analysis. Sequence proximal to the Tn*5* insertions was determined (see Figure 2) and the gene disrupted by the Tn*5* insertions was found to be a member of the LacI-GalR family of DNA-binding transcriptional regulators. (Osteras *et al.* 1997)

3.1 *PCK*R IS A LACI-GALR-TYPE REGULATOR

Restriction mapping, subcloning and sequencing of *pck*R

A restriction map of pTH296 was constructed for purposes of identifying restriction enzyme sites, which could be used to construct smaller regions for sequencing. The location of the restriction sites for *Hind*III, *Xma*I, *Sph*I, and *Pst*I were determined by diagnostic digests and these sites were used for subsequent subcloning as indicated in Figure 2. The entire open reading frame encoding PckR was found within the 2.5 kb *Eco*RI fragment. The region upstream of the putative *pck*R start codon contains several short strings of A and T which may have an effect on the secondary structure (data not shown).

The complete sequence 5' to the deduced open reading frame of *pck*R was also determined (for some of this region only one strand was sequenced). The sequences immediately 5' to *pck*R (Figure 2) upon BLAST (Altschul *et al.* 1997) analysis were found to be homologous (approximately 50% similarity) to hypothetical proteins from *Synechococcus* PCC7942 (ORF 271) (271 amino acids), *Streptomyces* (SC5A7.04c) (273 amino acids), *Synechocystis* sp., and *Mycobacterium tuberculosis.* These predicted proteins show weak homology to creatinine amidohydrolase from *Pseudomonas* sp. This potential open reading frame (ORF) appears to be transcribed in an opposite direction to *pck*R as shown in Figure 2. All of this predicted ORF is not contained within the 2.5 kb *Eco*RI

fragment; approximately 80 amino acids of the C-terminus are predicted to extend beyond the *Eco*RI site.

Sequences were assembled as contiguous sequences using Genetic Data Environment (GDE) (S. Smith, unpublished results). The complete nucleotide sequence and conceptual translation of *pck*R has been submitted to Genbank/EMBL databank and assigned accession number AF004316. This sequence was published as part of Osteras *et al.* (1997).

PckR is a member of the LacI-GalR family of transcriptional regulators

The sequence of the predicted *pck*R open reading frame was analyzed by BLAST (Altschul *et al.* 1997) and found to be homologous to the LacI-GalR family of transcriptional regulators. The LacI-GalR family is a large group of transcriptional regulators that bind directly to operator sequences via a helix-turn-helix domain in the N-terminus (as reviewed by Weickert and Adhya 1992). This homology suggests that PckR is a transcriptional regulator that functions to regulate Pck expression by direct interaction with sequences in the *pck*A promoter. The amino acid sequence of PckR as determined by conceptual translation was aligned with other LacI-GalR-type proteins as shown in Figure 3.

Figure 2: Subcloning strategy used to sequence *pck*R

Dashed arrows represent the sequences determined in this study, and the plasmid from which the sequence was determined is written under the arrow. All subclones were sequenced using the M13 forward and reverse primers. Solid arrows represent those sequences obtained from the cosmids containing the Tn*5* insertions using a primer annealing to the IS*50* region (5'-TCACATGGAAGTCAGATCCT-3') (Osteras *et al.* 1997). Shaded triangles represent the two Tn*5* insertions. Small shaded rectangles represent the synthetic primers AB12070 (5'-CGTTCGAGGAAGCCCTGC-3') and AB12071 (5'-ACTTGCGGACGCCGAGTC-3') constructed to sequence the region 5' to *pck*R. The region with homology to hypothetical ORFs from *Synechocystis* sp., *Synechococcus* PCC7942 and *Mycobacterium tuberculosis* is shown. *Pst*I sites are indicated as P1, P2 and P3 moving away from the multiple cloning site. (E = *Eco*RI, P = *Pst*I, H = *Hind*III, Sp = *Sph*I, Sm = *Sma*I)

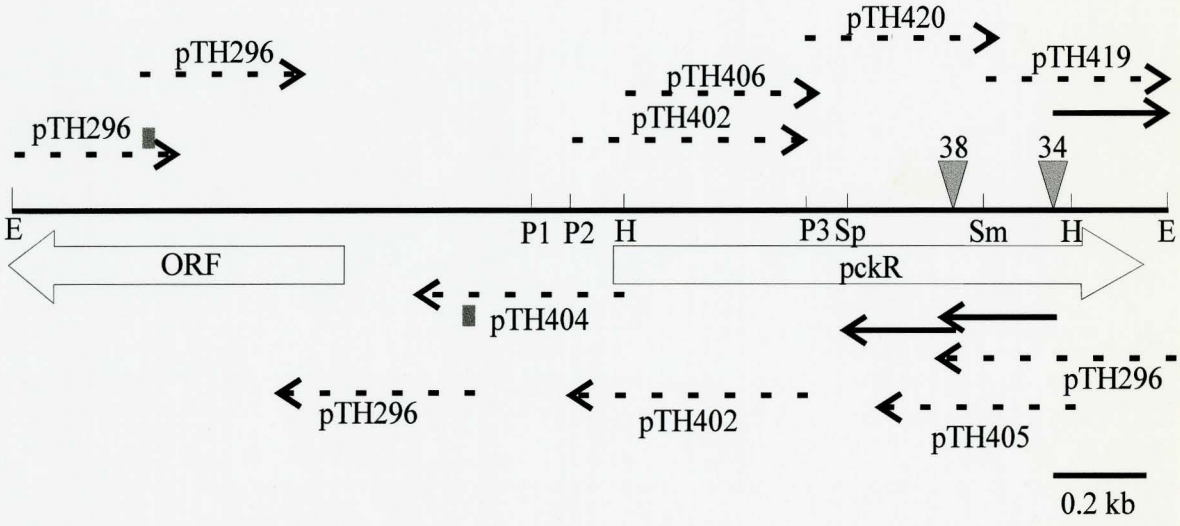
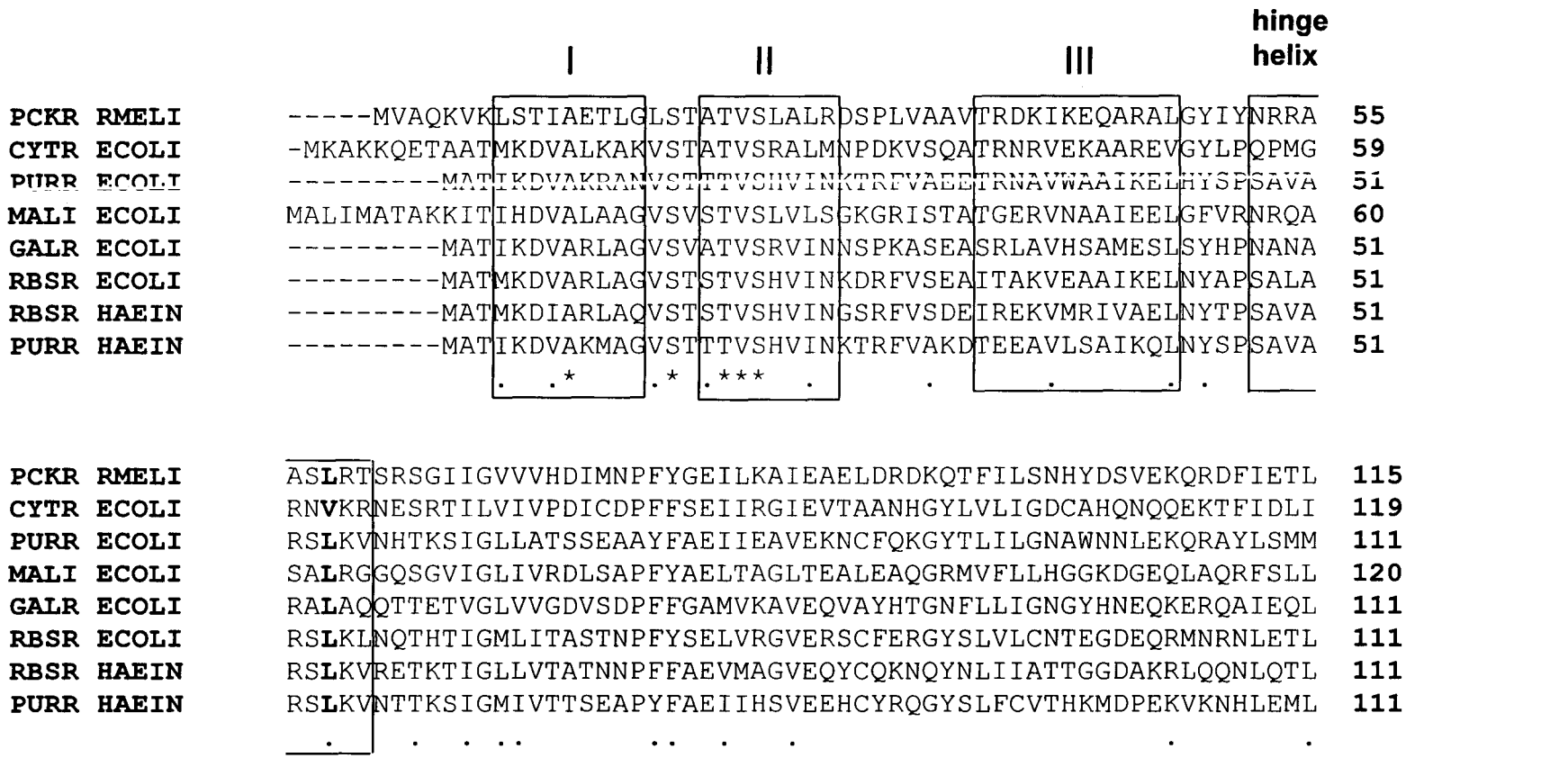
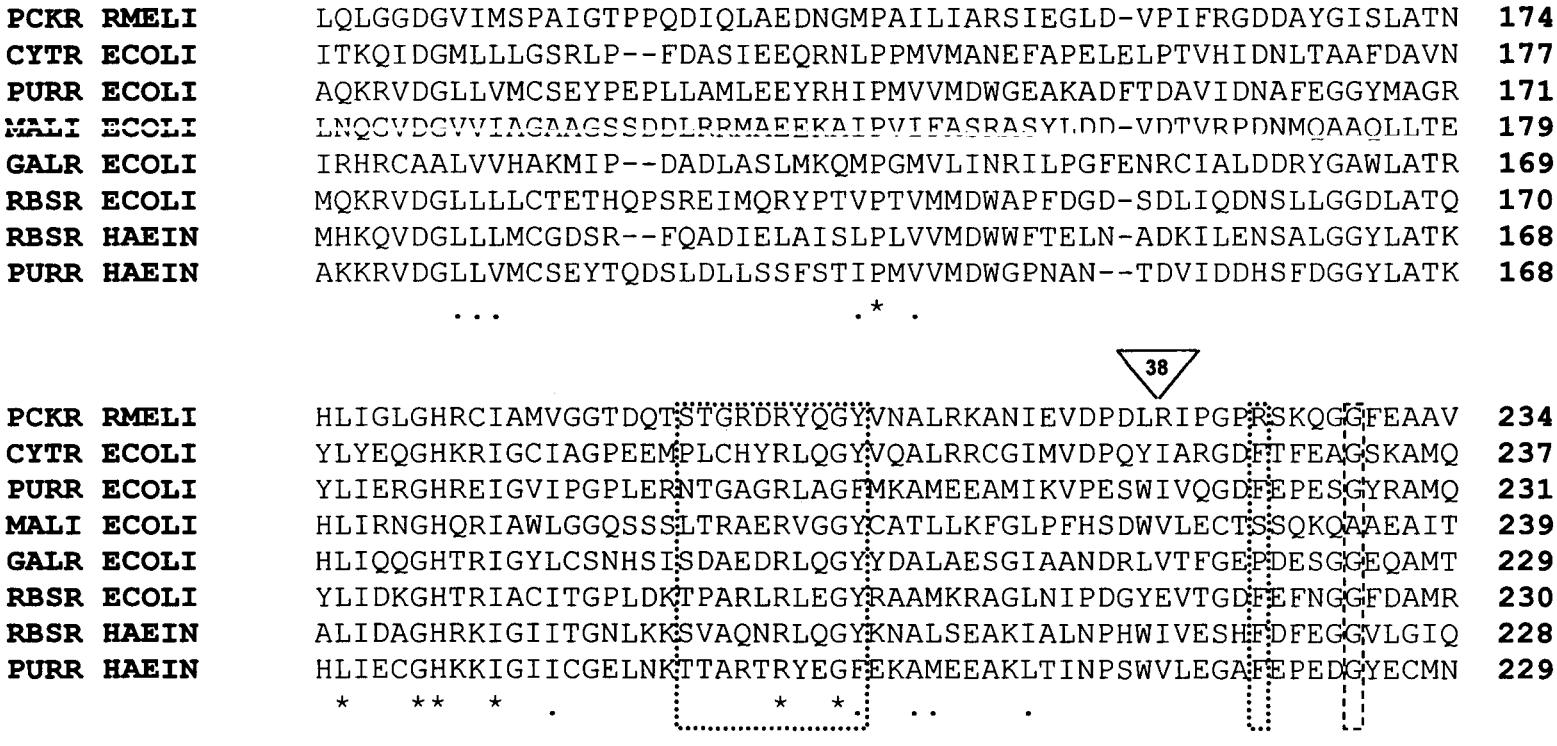

pTH420
pTH296
pTH419
pTH406
pTH296
pTH402
38
34
E
P1
P2
H
P3
Sp
Sm
H
E
ORF
pckR
pTH404
pTH296
pTH296
pTH402
pTH405
0.2 kb

Figure 3: ClustalW alignment of amino acid sequences from LacI-GalR-type proteins

Using ClustalW (Thompson *et al.* 1994), members of the LacI-GalR family of transcriptional regulators were aligned. PckR shows strong identity to *E. coli* CytR (30.3%), PurR (28.4%), MalI (28.1%), GalR (27.9%) and RbsR (27.5%), and *Haemophilus influenza* RbsR (27.9%) and PurR (23.5%). Stars indicate those residues that are identical for all sequences and conserved residues are indicated by dots. DNA-binding helix domains are boxed in solid lines. A solid box indicates the hinge helix. Leu54 (PurR) is indicated in bold (Schumacher *et al.* 1994). The locations of Tn*5* insertions 34 and 38 are indicated by triangles. Residues boxed with dots are believed to be involved in inducer binding, and residues boxed in dashed lines are believed to be involved in dimerization (Weickert and Adhya 1992). The numbers indicate the amino acid position.


hinge
helix
I
II
III
PCKR RMELI -----MVAQKVKLSTIAETLGLSTATVSLALRDSPLVAAVTRDKIKEQARALGYIYNRRA 55
CYTR ECOLI -MKAKKQETAATMKDVALKAKVSTATVSRALMNPDKVSQATRNRVEKAAREVGYLPQPMG 59
PURR ECOLI ---------MATIKDVAKRANVSTTTVSHVINKTRFVAEETRNAVWAAIKELHYSPSAVA 51
MALI ECOLI MALIMATAKKITIHDVALAAGVSVSTVSLVLSGKGRISTATGERVNAAIEELGFVRNRQA 60
GALR ECOLI ---------MATIKDVARLAGVSVATVSRVINNSPKASEASRLAVHSAMESLSYHPNANA 51
RBSR ECOLI ---------MATMKDVARLAGVSTSTVSHVINKDRFVSEAITAKVEAAIKELNYAPSALA 51
RBSR HAEIN ---------MATMKDIARLAQVSTSTVSHVINGSRFVSDEIREKVMRIVAELNYTPSAVA 51
PURR HAEIN ---------MATIKDVAKMAGVSTTTVSHVINKTRFVAKDTEEAVLSAIKQLNYSPSAVA 51
PCKR RMELI ASLRTSRSGIIGVVVHDIMNPFYGEILKAIEAELDRDKQTFILSNHYDSVEKQRDFIETL 115
CYTR ECOLI RNVKRNESRTILVIVPDICDPFFSEIIRGIEVTAANHGYLVLIGDCAHQNQQEKTFIDLI 119
PURR ECOLI RSLKVNHTKSIGLLATSSEAAYFAEIIEAVEKNCFQKGYTLILGNAWNNLEKQRAYLSMM 111
MALI ECOLI SALRGGQSGVIGLIVRDLSAPFYAELTAGLTEALEAQGRMVFLLHGGKDGEQLAQRFSLL 120
GALR ECOLI RALAQQTTETVGLVVGDVSDPFFGAMVKAVEQVAYHTGNFLLIGNGYHNEQKERQAIEQL 111
RBSR ECOLI RSLKLNQTHTIGMLITASTNPFYSELVRGVERSCFERGYSLVLCNTEGDEQRMNRNLETL 111
RBSR HAEIN RSLKVRETKTIGLLVTATNNPFFAEVMAGVEQYCQKNQYNLIIATTGGDAKRLQQNLQTL 111
PURR HAEIN RSLKVNTTKSIGMIVTTSEAPYFAEIIHSVEEHCYRQGYSLFCVTHKMDPEKVKNHLEML 111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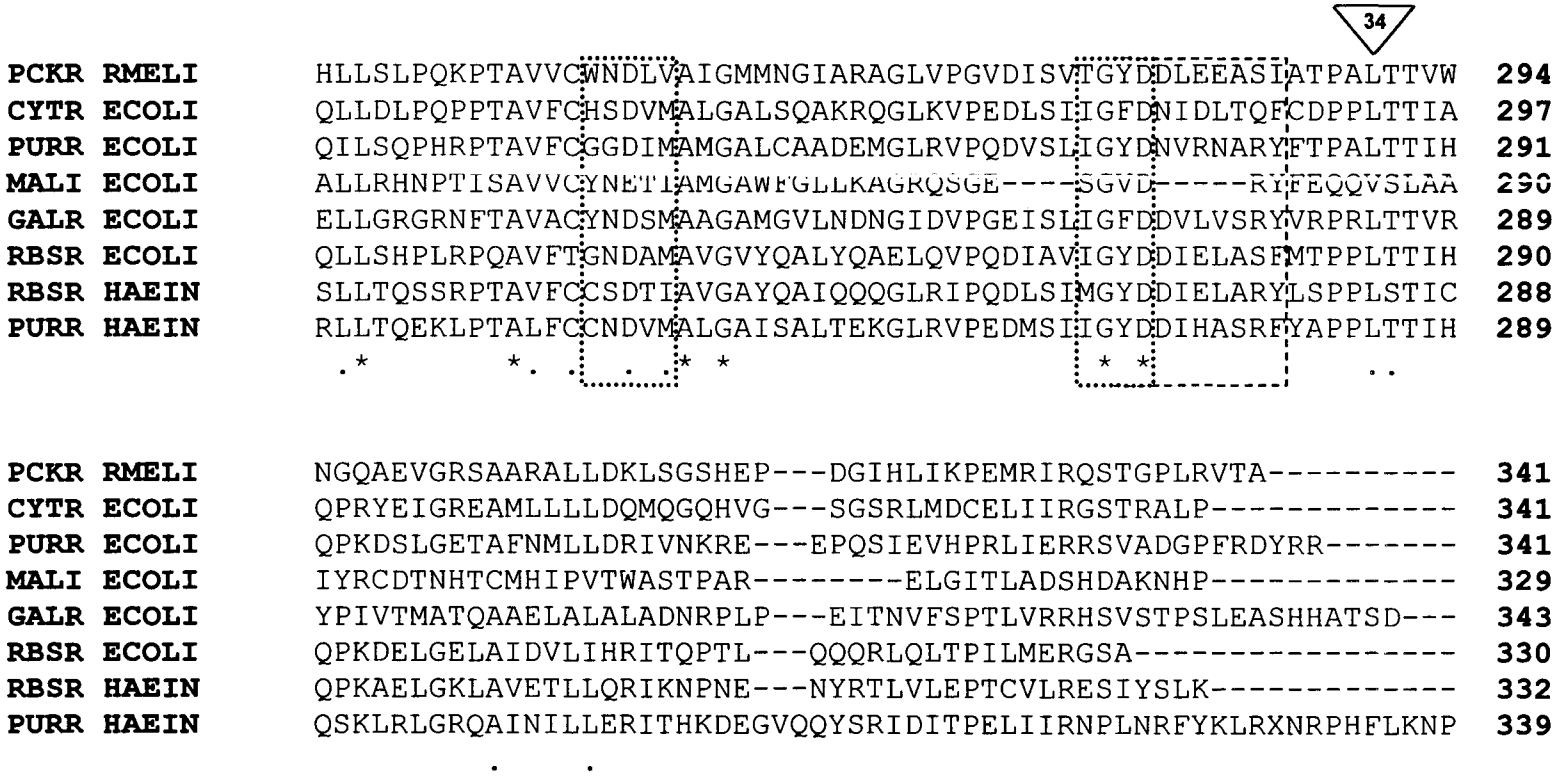
CYTR ECOLI ITKQIDGMLLLGSRLP--FDASIEEQRNLPPMVMANEFAPELELPTVHIDNLTAAFDAVN 177
PURR ECOLI AQKRVDGLLVMCSEYPEPLLAMLEEYRHIPMVVMDWGEAKADFTDAVIDNAFEGGYMAGR 171
MALI ECOLI LNQCVDGVVIAGAAGSSDDLRRMAEEKAIPVIFASRASYLDD-VDTVRPDNMQAAQLLTE 179
GALR ECOLI IRHRCAALVVHAKMIP--DADLASLMKQMPGMVLINRILPGFENRCIALDDRYGAWLATR 169
RBSR ECOLI MQKRVDGLLLLCTETHQPSREIMQRYPTVPTVMMDWAPFDGD-SDLIQDNSLLGGDLATQ 170
RBSR HAEIN MHKQVDGLLLMCGDSR--FQADIELAISLPLVVMDWWFTELN-ADKILENSALGGYLATK 168
PURR HAEIN AKKRVDGLLVMCSEYTQDSLDLLSSFSTIPMVVMDWGPNAN--TDVIDDHSFDGGYLATK 168
38
PCKR RMELI HLIGLGHRCIAMVGGTDQTSTGRDRYQGYVNALRKANIEVDPDLRIPGPRSKQGGFEAAV 234
CYTR ECOLI YLYEQGHKRIGCIAGPEEMPLCHYRLQGYVQALRRCGIMVDPQYIARGDFTFEAGSKAMQ 237
PURR ECOLI YLIERGHREIGVIPGPLERNTGAGRLAGFMKAMEEAMIKVPESWIVQGDFEPESGYRAMQ 231
MALI ECOLI HLIRNGHQRIAWLGGQSSSLTRAERVGGYCATLLKFGLPFHSDWVLECTSSQKQAAEAIT 239
GALR ECOLI HLIQQGHTRIGYLCSNHSISDAEDRLQGYYDALAESGIAANDRLVTFGEPDESGGEQAMT 229
RBSR ECOLI YLIDKGHTRIACITGPLDKTPARLRLEGYRAAMKRAGLNIPDGYEVTGDFEFNGGFDAMR 230
RBSR HAEIN ALIDAGHRKIGIITGNLKKSVAQNRLQGYKNALSEAKIALNPHWIVESHFDFEGGVLGIQ 228
PURR HAEIN HLIECGHKKIGIICGELNKTTARTRYEGFEKAMEEAKLTINPSWVLEGAFEPEDGYECMN 229


34
PCKR RMELI HLLSLPQKPTAVVCWNDLVAIGMMNGIARAGLVPGVDISVTGYDDLEEASIATPALTTVW 294
CYTR ECOLI QLLDLPQPPTAVFCHSDVMALGALSQAKRQGLKVPEDLSIIGFDNIDLTQFCDPPLTTIA 297
PURR ECOLI QILSQPHRPTAVFCGGDIMAMGALCAADEMGLRVPQDVSLIGYDNVRNARYFTPALTTIH 291
MALI ECOLI ALLRHNPTISAVVCYNETIAMGAWFGLLKAGRQSGE----SGVD-----RYFEQQVSLAA 290
GALR ECOLI ELLGRGRNFTAVACYNDSMAAGAMGVLNDNGIDVPGEISLIGFDDVLVSRYVRPRLTTVR 289
RBSR ECOLI QLLSHPLRPQAVFTGNDAMAVGVYQALYQAELQVPQDIAVIGYDDIELASFMTPPLTTIH 290
RBSR HAEIN SLLTQSSRPTAVFCCSDTIAVGAYQAIQQQGLRIPQDLSIMGYDDIELARYLSPPLSTIC 288
PURR HAEIN RLLTQEKLPTALFCCNDVMALGAISALTEKGLRVPEDMSIIGYDDIHASRFYAPPLTTIH 289
PCKR RMELI NGQAEVGRSAARALLDKLSGSHEP---DGIHLIKPEMRIRQSTGPLRVTA---------- 341
CYTR ECOLI QPRYEIGREAMLLLLDQMQGQHVG---SGSRLMDCELIIRGSTRALP---------- 341
PURR ECOLI QPKDSLGETAFNMLLDRIVNKRE---EPQSIEVHPRLIERRSVADGPFRDYRR------- 341
MALI ECOLI IYRCDTNHTCMHIPVTWASTPAR--------ELGITLADSHDAKNHP---------- 329
GALR ECOLI YPIVTMATQAAELALALADNRPLP---EITNVFSPTLVRRHSVSTPSLEASHHATSD--- 343
RBSR ECOLI QPKDELGELAIDVLIHRITQPTL---QQQRLQLTPILMERGSA---------- 330
RBSR HAEIN QPKAELGKLAVETLLQRIKNPNE---NYRTLVLEPTCVLRESIYSLK---------- 332
PURR HAEIN QSKLRLGRQAINILLERITHKDEGVQQYSRIDITPELIIRNPLNRFYKLRXNRPHFLKNP 339

The LacI-GalR-type proteins aligned in Figure 3 contain several conserved residues scattered throughout the proteins. Most members of the LacI-GalR family of transcriptional regulators bind DNA as dimers except for LacI and Cra/FruR. LacI contains a C-terminal extension that is believed to be the tetramerization domain (Weickert and Adhya 1992). PckR does not contain this C-terminal extension, and thus it likely acts as a dimer.

There is one conserved alanine residue in helix I which is widely conserved among this family (Weickert and Adhya 1992). PckR differs significantly in two residues of this helix that are conserved among the other seven proteins. This may suggest that PckR interacts differently with the operator DNA than do the other members of this family. Helix II is well conserved among all of the proteins examined, while helix III shows little conservation. Notably, in the hinge helix region, amino acid residue 54 of PurR (Leu58 of PckR) is leucine in all proteins examined in this study except CytR. This residue is highly conserved in this family (Nguyen and Saier 1995; Weickert and Adhya 1992) and is believed to act as a lever as it pries open and kinks the DNA (Schumacher *et al.* 1994).

Several residues are conserved for all eight proteins to which no known function has been attributed. In the domains believed to be involved in inducer binding, there are several residues conserved for all proteins examined, despite the fact that each protein has a different inducer.

3.2 PHENOTYPE OF *PCK*R MUTANTS

Construction of *pck*R insertion mutants

There is no direct evidence to suggest that the spontaneous mutant alleles, *rpk*-9, *rpk*-10 and *rpk*-15, are *pck*R null mutations. Further, it is not clear that the Tn*5* insertions in *pck*R (34 and 38) are *pck*R null mutations since both insertions fall within the C-terminal domain of the protein (see Figure 2). Therefore, in order to determine the phenotype of a *pck*R null mutant strain, I constructed a strain in which the ΩSp interposon was inserted into *pck*R such that the N-terminal DNA-binding domain was disrupted (Figure 4).

It was also necessary to construct *pck*R insertion mutants in a Lac$^-$ background for use in experiments where the expression of *pck*A would be measured from a *lac*Z fusion. Nm^R was transduced from RmH460 (*pck*R34::Tn*5*) and RmH461 (*pck*R38::Tn*5*), and Sp^R was transduced from RmK114 (*pck*R::ΩSp) into the Rm1021 Lac$^-$ derivative, RmG212, creating strains RmG853, RmG854 and RmK124 respectively.

The genomic *pck*R::Tn*5* and *pck*R::ΩSp insertions were confirmed to lie within the *pck*R gene (Figure 5, panel A). The structure of the *pck*R gene region, and the locations of the insertions are diagramed in Figure 5 (panel B).

Figure 4: Schematic of site-directed mutagenesis of *pck*R using ΩSp

The ΩSp fragment was gel isolated as a *Hind*III fragment from the plasmid pHP45Ω. pTH296, which carries the 2.5 kb *Eco*RI fragment shared by the ten cosmids complementing the *rpk*-9 phenotype, was partially digested with *Hind*III. The ΩSp fragment was ligated with this and the resulting construct, pTH475, was transformed into DH5α selecting for Sp^R. Arrows indicate the *Hind*III sites digested in pHP45Ω and pTH296. The 4.5 kb fragment containing the ΩSp fragment and the disrupted *pck*R gene region was cloned as an *Eco*RI fragment from pTH475 into pRK7813 selecting for Sp^R. The resulting plasmid, pTH476, was mated into Rm5000 and recombinants, in which the *pck*R::ΩSp allele was marker exchanged into the *S. meliloti* genome, were selected. (MCS = multiple cloning site; E = *Eco*RI; H = *Hind*III)

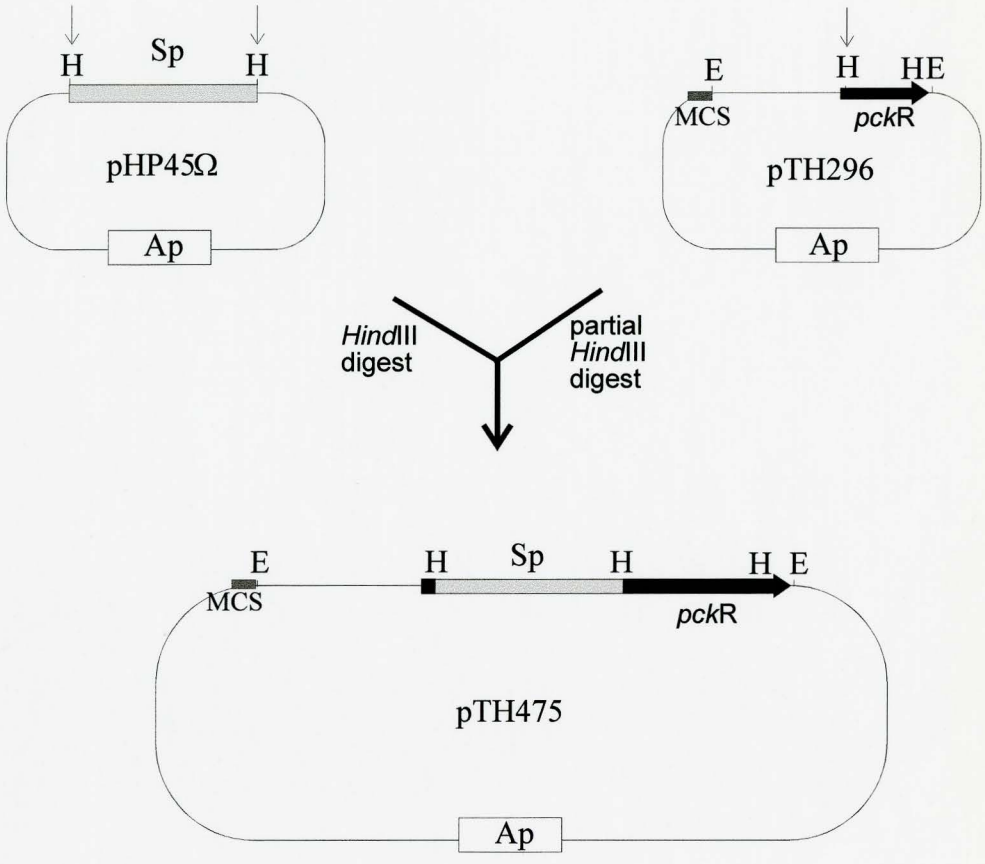
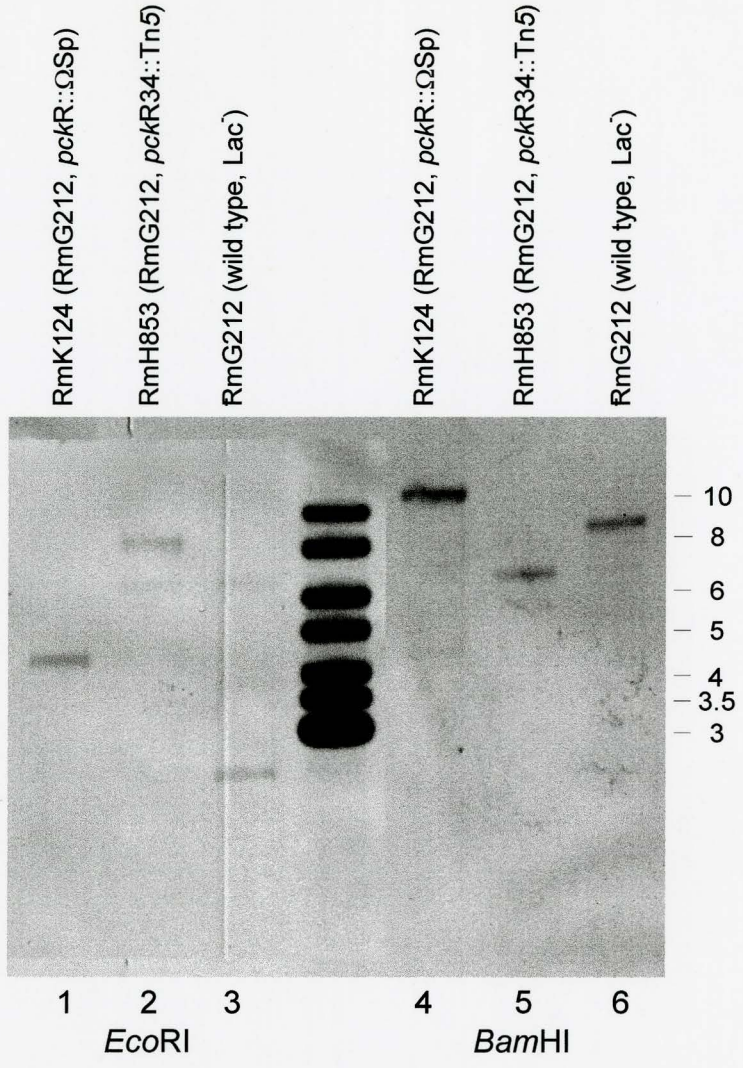

H
Sp
H
pHP45Ω
Ap
E
H
HE
MCS
pckR
pTH296
Ap
HindIII
digest
partial
HindIII
digest
E
H
Sp
H
H
E
MCS
pckR
pTH475
Ap

Figure 5: Confirmation of the genomic insertions in *pck*R

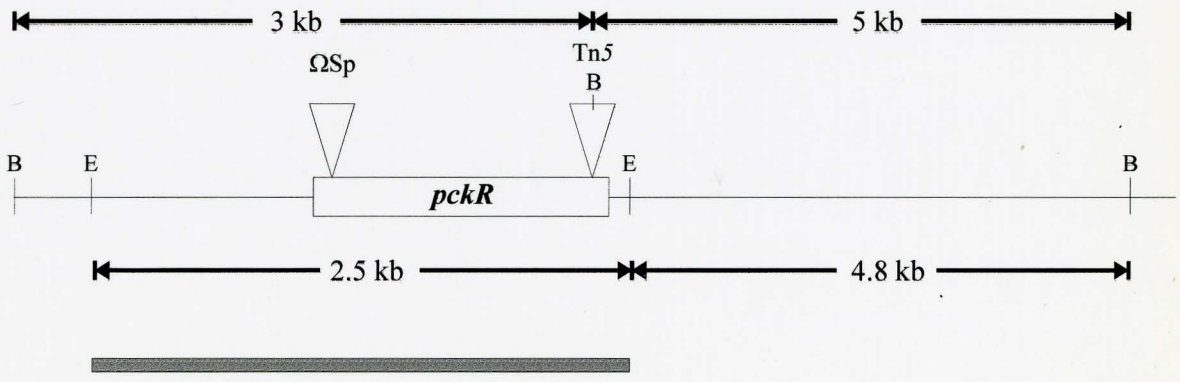
A. The insertions in *pck*R were confirmed by Southern blotting. 10 μg of genomic DNA from RmG212 (Rm1021, Lac$^-$), RmH853 (RmG212, *pck*R34::Tn*5*) and RmK124 (RmG212, *pck*R::ΩSp) was digested with *Eco*RI and *Bam*HI. The membrane was probed with the gel isolated 2.5 kb *Eco*RI fragment from pTH296 labeled with DIG. The band sizes of the ladder are indicated. The insertion in RmH854 (RmG212, *pck*R38::Tn*5*) was also confirmed to be within the *pck*R gene (data not shown). Lanes have been cropped from the original scanned membrane.

B. Schematic diagram of the *pck*R gene region indicating the location of the transposon and Ω insertions (triangles). The hybridizing probe is indicated by a shaded rectangle. *Eco*RI does not cut either Tn*5* or ΩSp. *Bam*HI cuts once in Tn*5*, but does not cut in ΩSp. Only the location of *pck*R34::Tn*5* is indicated for clarity. Figure 2 shows the relative location of *pck*R38::Tn*5*. (E = *Eco*RI; B = *Bam*HI)

A.


RmK124 (RmG212, pckR::ΩSp)
RmH853 (RmG212, pckR34::Tn5)
RmG212 (wild type, Lac⁻)
RmK124 (RmG212, pckR::ΩSp)
RmH853 (RmG212, pckR34::Tn5)
RmG212 (wild type, Lac⁻)
10
8
6
5
4
3.5
3
1
2
3
4
5
6
EcoRI
BamHI

B.


3 kb
5 kb
ΩSp
Tn5
B
B
E
E
B
pckR
2.5 kb
4.8 kb

*pck*R transposon insertions and group I spontaneous mutations are linked

Osteras *et al.* (1997) used chromosomal tranposon insertions to locate the spontaneous mutations on the *S. meliloti* chromosome and establish linkage groups. The group I spontaneous mutations, consisting of the *rpk*-9, *rpk*-10 and *rpk*-15 alleles, were found to be linked to Ω5345. In this study, linkage between the Tn*5* insertions in *pck*R and Ω5345 was examined by transduction. The results indicate that Ω5345 insertion and the Tn*5* insertions in *pck*R are tightly linked.

Table 2: Linkage of *rpk*-9 and *pck*R alleles as determined by transduction

	Linkage to Ω5345	
Allele	**Co-transduction frequency**	**Number of transductants screened**
rpk-9†	78%	40
rpk-10†	70%	40
rpk-15†	80%	40
*pck*R34::Tn*5*‡	95%	85
*pck*R38::Tn*5*‡	95%	148

† Experiment conducted by M. Osteras and published in Osteras *et al.* (1997). Lysate prepared from Ω5345 was used to transduce Nm^R into the recipient strains RmH147, RmH148 and RmH153 (*rpk*-9, *rpk*-10 and *rpk*-15 respectively). Transductants were screened for expression of the *pck*A::*lac*Z fusion on M9 glucose + X-Gal.

‡ Lysates prepared from RmH853 (*pck*R34::Tn*5*) and RmH854 (*pck*R38::Tn*5*) were used to transduce Nm^R into RmH443 (Ω5345::Tn*5*-233). Transductants were screened for loss of Gm^RSp^R.

Complementation of *rpk*-9, *rpk*-10 and *rpk*-15 by *pck*R

In order to determine if the 2.5 kb *Eco*RI fragment alone complemented the *rpk*-9, *rpk*-10 and *rpk*-15 mutations, β-galactosidase activity was measured in these mutant strains carrying pTH277, pTH277::Tn*5*, and pTH446, the 2.5 kb *Eco*RI fragment encoding *pck*R in pRK7813.

All three mutations showed approximately 5-fold increased *pck*A expression in glucose-grown cells compared to wild type, and 19 to 26-fold increased expression in LBmc-grown cells. As expected, the *rpk*-9 mutation was complemented by the cosmid pTH277 in both glucose and LBmc-grown cells, and this complementation was abolished by the transposon insertion in pTH277. The *rpk*-10 and *rpk*-15 mutations were complemented by pTH277 in glucose, however the *rpk*-15 mutation was not complemented by pTH277 in LBmc. Plasmid pTH446, which contains the 2.5 kb *Eco*RI fragment, complemented the *rpk*-10 mutation n glucose and LBmc-grown cells. The *rpk*-9 and *rpk*-15 mutations were similarly complemented for *pck*A expression by pTH446 in glucose-grown cells, but neither of these mutations were complemented in LBmc-grown cells.

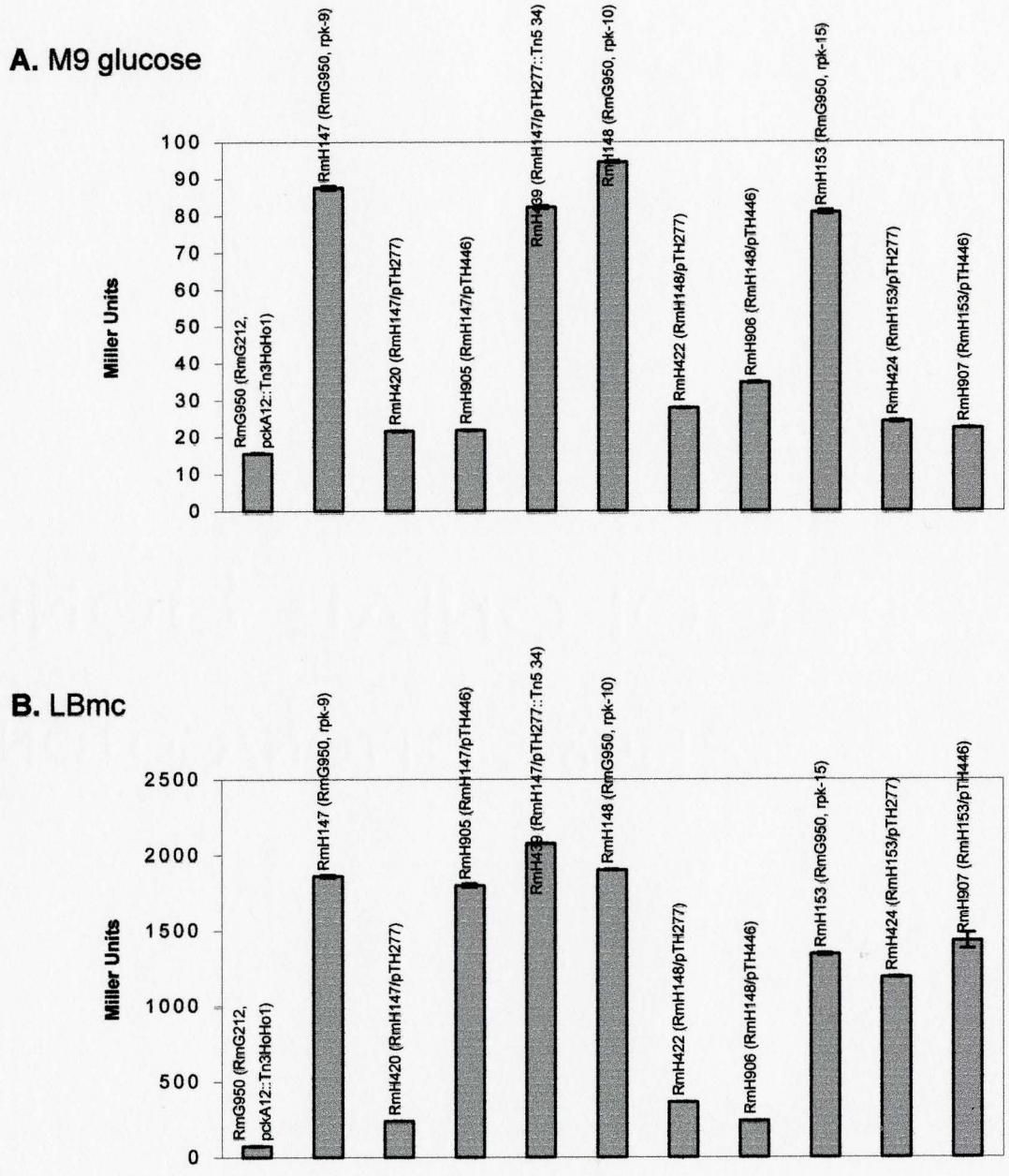
Table 3: Complementation of the *rpk*-9, *rpk*-10, *rpk*-15 mutations in glucose and LBmc-grown cells†

Strain	Genotype	Miller Units‡	
		M9 glucose	LBmc
RmG950	RmG212, *pck*A12::Tn3HoSp	15.6±0.2	72.3±1.5
RmH147	RmG950, *rpk*-9	87.6±0.5	1858.1±9.5
RmH420	RmH147/pTH277	21.6±0.1	238.6±1.7
RmH905	RmH147/pTH446	21.9±0.1	1797.4±12.2
RmH439	RmH147/pTH277,*pck*R34::Tn*5*	82.3±0.4	2075.1±1.0
RmH148	RmG950, *rpk*-10	94.5±0.3	1899.5±3.7
RmH422	RmH148/pTH277	27.9±0.1	363.1±1.5
RmH906	RmH148/pTH446	34.8±0.2	239.6±0.8
RmH153	RmG950, *rpk*-15	80.9±0.5	1340.7±8.7
RmH424	RmH153/pTH277	24.2±0.3	1188.9±3.7
RmH907	RmH153/pTH446	22.3±0.1	1429.2±53.2

† Miller Units of β-galactosidase activity was measured from permeabilized cells. Starter cultures of the strains to be tested were grown for 2 days in LBmc containing 2 μg/ml Tc for those strains with plasmids. These cultures were washed with 0.85% NaCl and used to inoculate 5 ml cultures of M9 glucose and LBmc (containing 2 μg/ml Tc for those strains with plasmids). The M9 glucose and LBmc cultures were grown overnight, and the β-galactosidase activity was measured from cells collected the following day.

‡ Each assay represents the mean for triplicate assays ± the standard error of the mean.

Figure 6: Histographical presentation of data in Table 3



Time course of *pck*A expression in mutant backgrounds

In order to examine the expression of *pck*A in the *pck*R null and *rpk*-9 mutant backgrounds during growth on different carbon sources and throughout the growth cycle, a time course experiment was conducted as described by Osteras *et al.* (1997). The pF94 plasmid (Tc^R) (Osteras *et al.* 1995) carrying *pck*A6::Tn3HoHo1 was mated into the *pck*R34::Tn5, *pck*R::ΩSp and *rpk*-9 containing strains in a Lac⁻ background, and expression of *pck*A was measured as β-galactosidase activity from this plasmid throughout the growth cycle on M9 glucose, M9 succinate and LBmc. The results in Figure 7 clearly indicate that the phenotype of the *pck*R null mutations is dramatically different from the *rpk*-9 spontaneous mutations.

The growth curves of all strains examined in Figure 7 were similar, indicating that none of the strains have a retarded growth phenotype on any media tested. Cells grown in minimal media did not reach as high of an optical density as did cells grown in complex media. As expected, growth in M9 glucose and succinate occurred slower than in LBmc. Stationary phase was reached in the LBmc grown cells by 30 hours, however it took about 40 hours for cell growth in M9 to plateau.

Wild type (RmG212) M9 glucose-grown cells had very low levels of *pck*A expression (panel A), whereas during growth in M9 succinate, this same strain expressed *pck*A to 4000 Miller Units (Figure 7, panel B). In contrast, the strain containing the *rpk*-9 spontaneous mutation (RmG914) had 1500 Miller Units of

*pck*A expression during growth in glucose (Figure 7, panel A). *pck*A expression levels in the *rpk*-9 mutant strain during growth in succinate was comparable to wild type (Figure 7, panel B). The strains carrying insertion mutations in *pck*R showed low levels (less than 100 Miller Units) of *pck*A expression during growth on both glucose and succinate (Figure 7, panels A and B).

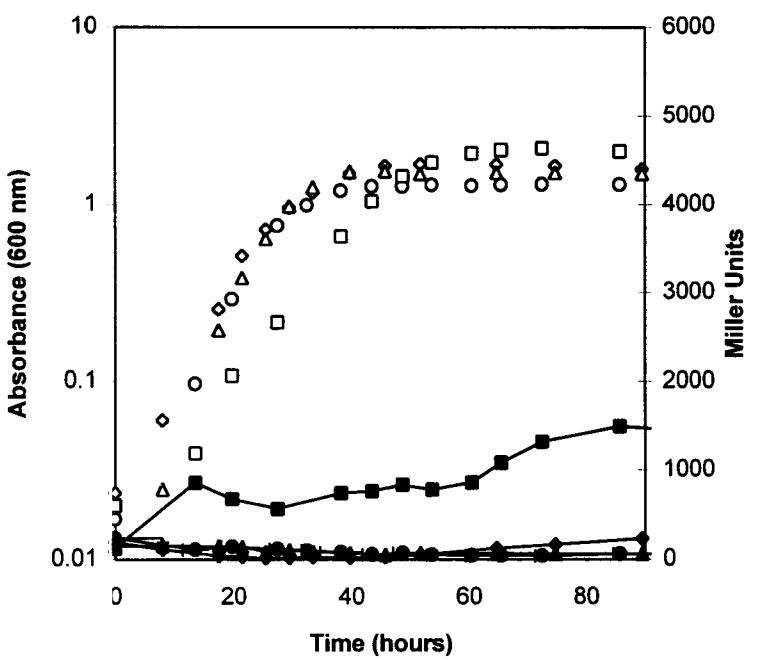
Levels of *pck*A expression in LBmc-grown wild type cells reached almost 8000 Miller Units (Figure 7, panel C)—twice the levels observed during growth in M9 succinate (Figure 7, panel B). The *rpk*-9 mutation had 6500 Miller Units of *pck*A expression in LBmc-grown cells. In contrast, levels of *pck*A expression in the strains containing the *pck*R insertions never rose above 1000 Miller Units. However, this level of expression was 10-fold higher than the level observed in minimal media.

The levels of *pck*A expression in the strain carrying the *rpk*-9 mutation are comparable to wild type (Figure 7). The shape of the *pck*A induction curve during growth in LBmc for the *rpk*-9 mutant strain was different from stationary phase induction observed in wild type cells. The cell density in wild type cells was at saturation for 10 hours before the *pck*A expression dramatically increased. In contrast, the expression of *pck*A in the *rpk*-9 mutant strain increased steadily with growth. Specifically, at 30 hours, the *pck*A expression in the *rpk*-9 mutant strain was 6-fold greater than the wild type.

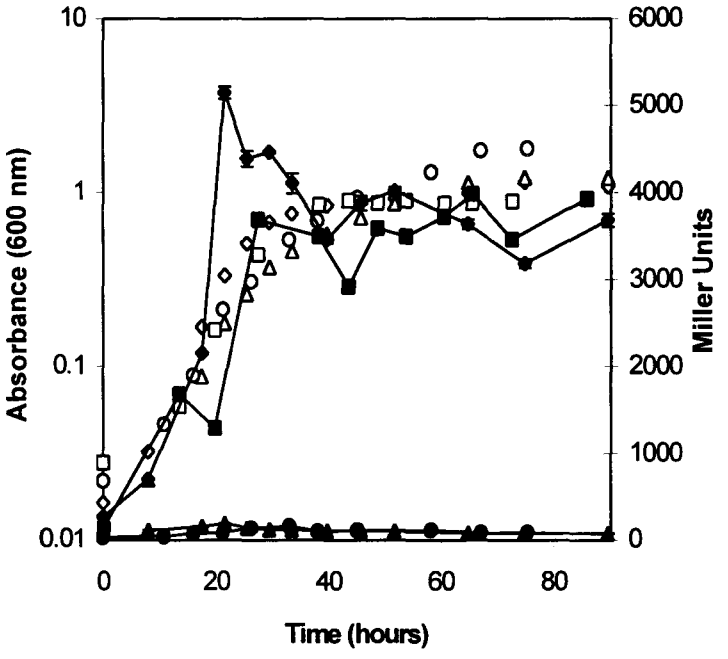
Figure 7: Relationship between growth and *pck*A in A. M9 glucose, B. M9 succinate, and C. LBmc

The relationship between growth and *pck*A::*lac*Z fusion expression for RmG319 (RmG212/pF94) (diamonds), RmG914 (RmG212, *rpk*-9, Ω5345::Tn*5*/pF94) (squares), RmK141 (RmG212, *pck*R::ΩSp/pF94) (circles), and RmG857 (RmG212, *pck*R34::Tn*5*/pF94) (triangles) is plotted. The method used for this experiment was as described by Osteras *et al.* (1995). The OD_{600} and β-galactosidase activity of each sample at each time point was determined. Miller Units indicated are the average of three assays conducted on an aliquot collected from the same flask. Standard error bars are shown for Miller Units. Unfilled symbols are OD_{600} values plotted on a logarithmic scale. Filled symbols are β-galactosidase activities in Miller Units plotted on a linear scale.

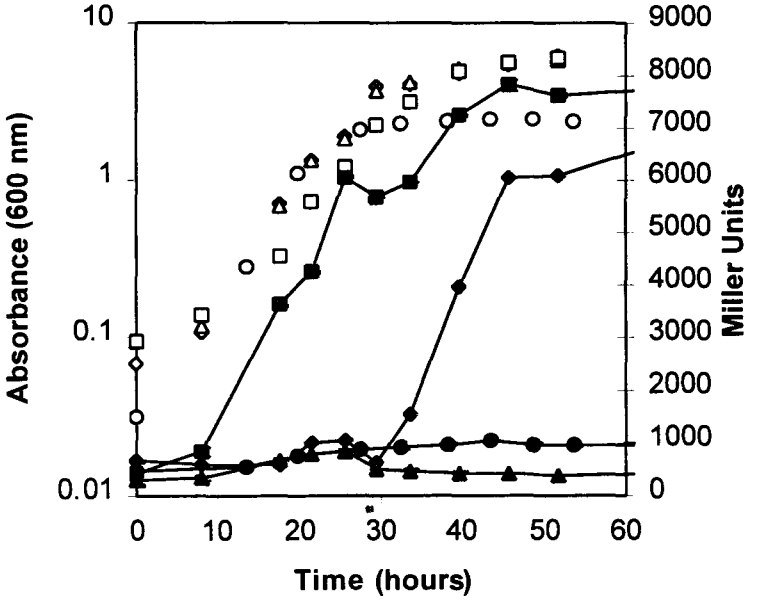
A. M9 glucose



B. M9 succinate



C. LBmc



Requirement of Pck for growth of *pck*R insertion mutants in succinate

The strains containing *pck*R insertions showed low-level *pck*A expression in succinate despite the fact that their growth on this carbon source was not retarded. The growth of these mutant strains in the absence of a functional *pck*A gene was investigated.

The results shown in Table 4 indicate that in the absence of *pck*A expression, strains were unable to grow in succinate, while growth in glucose was identical to wild type. When plasmid pRmT103 was added to Pck$^-$ strains growth was restored to wild type levels. However, strains with *pck*R::Tn*5*-233 mutations (RmH928 and RmH929) had 25% of wild type growth in succinate without Pck. In contrast, the *rpk*-9 mutation did not allow any growth without a functional *pck*A gene.

Table 4: Growth of Pck$^+$ and Pck$^-$ strains in glucose and succinate‡

Strain	Description	A_{600}	
		M9 glucose	M9 succinate
RmG212	wild type, Lac$^-$	1.80	1.15
RmG263	*pck*A6::Tn3HoHo1	1.75	0.14
RmG950	*pck*A12::Tn3HoHo1	1.75	0.14
RmH147	*rpk*-9, *pck*A12::Tn3HoHo1	1.80	0.15
RmH166	*rpk*-9, *pck*A Ω5315::Tn*5*†	1.75	1.05
RmH464	*rpk*-9, *pck*A6::Tn3HoHo1	1.80	0.10
RmH466	RmH464/pRmT103	1.75	1.05
RmH928	RmG263, *pck*R34::Tn*5*-233**	1.75	0.30
RmH964	RmH928/pRmT103	1.50	1.15
RmH929	RmG263, *pck*R38::Tn*5*-233**	1.75	0.28
RmH965	RmH929/pRmT103	1.50	1.15

‡ Starter cultures of strains to be tested were grown overnight in LBmc (2 μg/ml Tc for those strains carrying a plasmid). These cultures were spun down, washed with 0.85% NaCl, and resuspended in 5 ml 0.85% NaCl. 100 μl was used to inoculate 5 ml cultures of M9 containing glucose or succinate to give a starting OD_{600} of approximately 0.1. The M9 cultures were grown for 72 hours after which the absorbance at 600 nm was measured as an indicator of growth.
† The wild type *pck*A gene was transduced into this strain linked to the Tn*5* insertion Ω5315, which is located 500 bp downstream of *pck*A (Osteras *et al.* 1997).
** The transposons insertions in *pck*R (34 and 38) were replaced with Tn*5*-233, and the Gm^RSp^R insertions were transduced into a *pck*A6::Tn3HoHo1 (Nm^R) background for use in this experiment and subsequently described plant studies.

*pck*A expression in the bacteroid

The lack of Pck activity in *S. meliloti* bacteroids has been previously documented (Finan *et al.* 1991). Although Pck activity in *S. meliloti* is believed to be controlled at the level of *pck*A transcription (Osteras *et al.* 1995; Osteras *et al.* 1997), the level of *pck*A expression in the bacteroid has not been examined.

To measure *pck*A expression in bacteroids, I employed a chromosomal *pck*A::*lac*Z transcriptional fusion. In addition, the wild type *pck*A gene was transferred into the strains on the pLAFR1 cosmid clone (pRmT103). Strains examined include those containing the *pck*R34::Tn*5*-233 mutation and those with the *rpk*-9 spontaneous mutation. The dry weights of the plants are given below in Table 5.

Table 5: Dry weight of plants inoculated with strains containing chromosomal *pck*A::*lac*Z fusions‡

Strain	Genotype	Dry wt. (mg/plant)	% wild type
RmG212	wild type, Lac$^-$	47.5	100
RmG295	*pck*A6::Tn3HoHo1/pRmT103	25.2	53
RmH466	*pck*A6::Tn3HoHo1, *rpk*-9/pRmT103	25.0	53
RmH964	*pck*A6::Tn3HoHo1, *pck*R34::Tn*5*-233/pRmT103	26.2	55

‡ Alfalfa seedlings were inoculated with the indicated strains and plants were harvested 30 days after inoculation. The shoot dry weights are as determined from approximately 30 plants. Uninoculated plants had a dry weight of 5.7 mg per plant (12% of wild type).

The results in Table 5 indicate that the strains containing the plasmid pRmT103 are not fully complemented for the reduced symbiotic phenotype that results from the *pck*A mutation (60% of wild type) (Finan *et al.* 1991). This could be due to some loss of the plasmid, or perhaps a copy number effect.

Bacteroid extracts were prepared from the nodules harvested from the roots of the plants in Table 5. Before sonication, dilutions of the washed bacteroids

were prepared and plated on LB plates. The concentration of cells in the bacteroid extract before sonication was approximately 5 x 10^7 colony forming units per ml. Colonies were patched onto LB containing Nm and LB containing Nm and Tc to examine retention of the plasmid carrying the wild type *pck*A gene (pRmT103). Of 49 colonies screened from the RmG295 bacteroids, 36 were Tc^R, indicating 73% retention of the plasmid.

Table 6: *pck*A expression in the bacteroid‡

Strain	Genotype	MDH activity (nmol/min/mg)	β-gal activity (nmol/min/mg)
RmG212	wild type, Lac$^-$	718.8±7.2	12.1±0.6
RmG295	*pck*A::Tn*3*HoHo1/pRmT103	1078.2±37.8	77.1±6.4
RmH466	*pck*A::Tn*3*HoHo1, *rpk*-9/pRmT103	1091.8±50.5	944.9±15.6
RmH964	*pck*A::Tn*3*HoHo1, *pck*R34::Tn*5*-233/pRmT103	555.0±19.8	39.9±4.9

‡ Alfalfa plants were inoculated with the indicated strains and root nodules were harvested 30 days after inoculation. β-galactosidase and MDH activities of the bacteroid extracts were measured.

The levels of expression from the *pck*A::*lac*Z fusion in RmG295 and RmH964 are low as expected, however RmH466, containing the *rpk*-9 mutation, has significantly higher levels of *pck*A expression confirming the previous reports regarding the Pck activity levels in *rpk*-9 mutant bacteroids (Osteras *et al.* 1997). The expression of *pck*A in the bacteroid therefore parallels the activity of Pck in the bacteroid (Finan *et al.* 1991).

3.3 INTERACTION OF PROTEINS WITH THE *PCK*A PROMOTER

In this study, physical evidence was sought through electrophoretic mobility shift assays (EMSAs) to support the hypothesis that interaction occurs between PckR, which has DNA-binding helices like those of the LacI-GalR family of transcriptional regulators (Figure 3), and the *pck*A promoter, which appears to require PckR for activation. In addition, a site at –76 relative to the transcriptional start site in the *pck*A promoter matches with the consensus binding site established for LacI-GalR-like proteins (5' - NNNAANCGNTTNNN - 3') (Weickert and Adhya 1992) (see Figure 8). It is also worth noting that at approximately -90 relative to the transcriptional start site there is a region containing longs strings of A and T which may have effects on secondary structure.

Electrophoretic mobility shift assay (EMSA) analysis of the *pck*A promoter

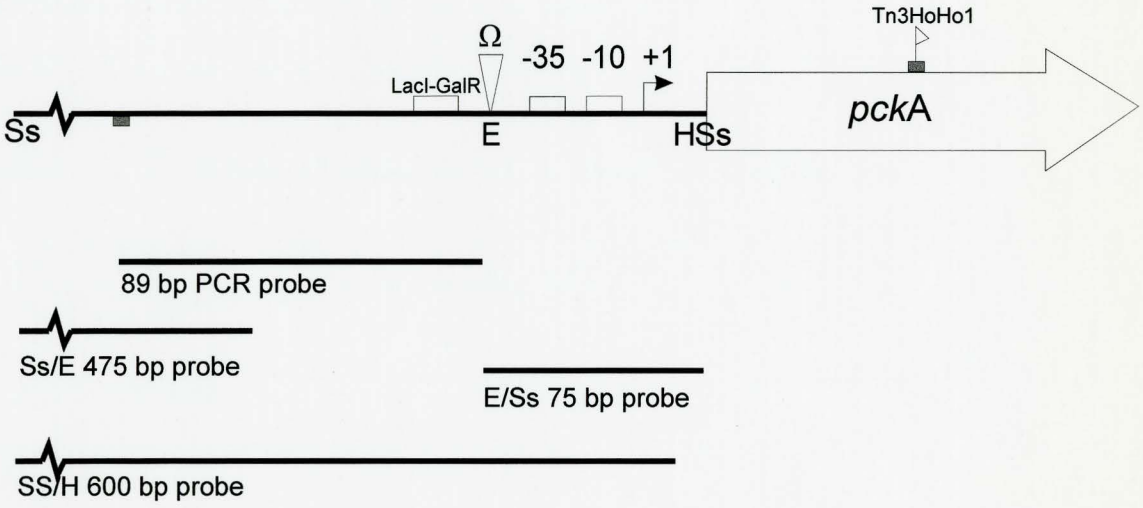
Electrophoretic mobility shift assays (EMSAs) (also called band shift assays or gel retardation assays) were used to identify proteins in crude cell extract that bound to fragments from the *pck*A promoter. A non-denaturing polyacrylamide gel was used to separate DNA-protein complexes from free probe. The gel matrix is believed to provide a "caging" effect whereby DNA-protein complexes are stabilized (Carthew *et al.* 1985; Hendrickson 1985).

Figure 8: The *pck*A promoter

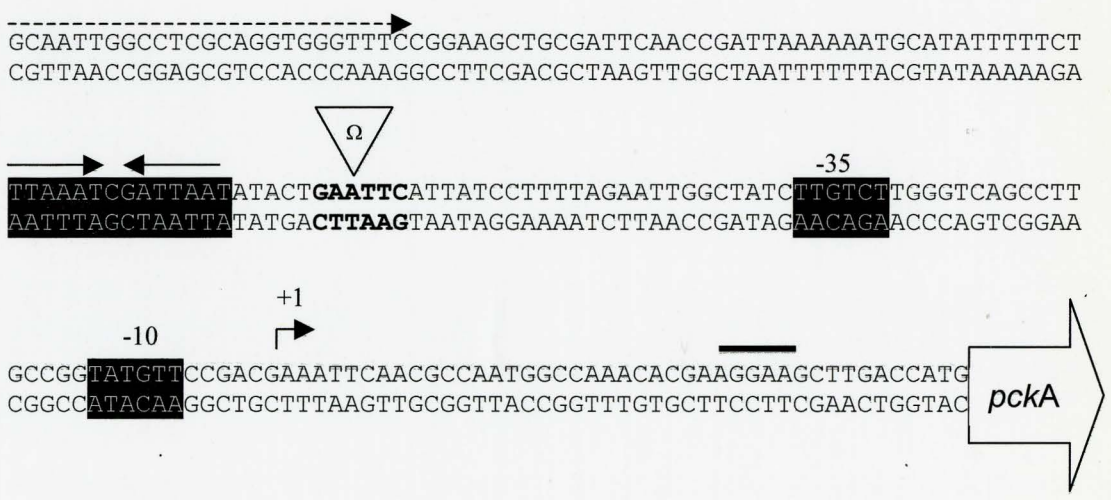
A. A schematic of the *pck*A promoter region is shown. The position of the transcriptional start site (+1) and the -10/-35 regions are diagrammed. The location of the insertion of the ΩSp interposon disrupting the promoter (Osteras *et al.* 1997) is indicated. The Tn3HoHo1 insertion (pG6) used for PCR amplification in probe construction is shown, and the shaded boxes represent the primers used in PCR. The putative binding site for a LacI-GalR-like transcriptional regulator is indicated. Restriction sites used in probe construction are shown. Symbols used for restriction enzymes are as follows: Ss = *Sst*I, E = *Eco*RI, H = *Hind*III. Diagram is not drawn to scale.

B. The nucleotide sequence of the *pck*A promoter is shown. A dashed arrow indicates the primer used in PCR. The putative LacI-GalR operator site and its palindromic structure is indicated. Solid line indicates the ribosome binding site (Osteras *et al.* 1997). The *Eco*RI restriction site is indicated in bold.

A.


Tn3HoHo1
Ω
-35 -10 +1
LacI-GalR
Ss
E
HSs
pckA
89 bp PCR probe
Ss/E 475 bp probe
E/Ss 75 bp probe
SS/H 600 bp probe

B.


GCAATTGGCCTCGCAGGTGGGTTTCCGGAAGCTGCGATTCAACCGATTAAAAAATGCATATTTTTCT
CGTTAACCGGAGCGTCCACCCAAAGGCCTTCGACGCTAAGTTGGCTAATTTTTTACGTATAAAAAGA
Ω
-35
TTAAATCGATTAATATACTGAATTCATTATCCTTTTAGAATTGGCTATCTTGTCTTGGGTCAGCCTT
AATTTAGCTAATTATATGACTTAAGTAATAGGAAAATCTTAACCGATAGAACAGAACCCAGTCGGAA
+1
-10
GCCGGTATGTTCCGACGAAATTCAACGCCAATGGCCAAACACGAAGGAAGCTTGACCATG
CGGCCATACAAGGCTGCTTTAAGTTGCGGTTACCGGTTTGTGCTTCCTTCGAACTGGTAC
pckA

Interaction of proteins from crude cell extracts with a *pck*A promoter fragment containing the putative LacI-GalR operator site

Crude cell extracts were prepared by French Press from M9 cultures with either glucose or succinate as the carbon source. EMSAs were performed by incubating crude cell extract with an 89 bp ^{32}P-labeled fragment (probe) from the *pck*A promoter as indicated in Figure 8. Subsequently, the binding reaction was electrophoresed to separate bound and unbound probe. The intensity of each band of shifted probe was quantified by area integration of phosphoimager data (Table 6).

The observed EMSA pattern from the wild type French-pressed crude cell extracts consists of four shifted bands, independent of the carbon source available for growth. The metabolic state of the cell—growth on glucose or growth on succinate—does not affect which proteins were observed to bind to the *pck*A promoter. However, shifted bands **a** and **b** were less intense in assays using extract prepared from glucose-grown wild type cells compared to all other strains.

Crude cell extract prepared from the *pck*R insertion mutant strains (RmK124 and RmH853) contain all of the shifted bands present with wild type extract, indicating that PckR is not one of the proteins observed to bind to the 89 bp fragment from the *pck*A promoter. Shifted band **c** was 2-fold less intense in succinate-grown cells compared to glucose-grown *pck*R insertion mutants, and shifted band **d** is 5 to 11-fold less intense in succinate-grown cells.

Figure 9: Interaction of proteins from crude cell extract of A. glucose and B. succinate-grown cells with the 89 bp *pck*A promoter fragment

Numbers above each lane indicate the amount of protein added to the binding reaction from French-pressed crude cell extract prepared from glucose or succinate-grown cells. Each lane contains 10,000 cpm (approximately 4 ng) of an 89 bp probe isolated from the *pck*A promoter region. The shifted probe bands are labeled **a**, **b**, **c** and **d**, corresponding to the bands whose intensity was quantitated by area integration (Molecular Dynamics ImageQuant 3.3), and whose values are given in Table 7. The lane labeled "probe" contained all components of the binding reaction except crude cell extract, which was replaced with an equal amount of buffer 1 (extract buffer). The lane labeled "probe + BSA" contained 15 μg bovine serum albumin. Two gels were run simultaneously and assembled from the original scans to create this figure.

A. glucose
probe
probe + BSA
0.1μg
1 μg
10 μg
RmH853
(RmG212,pckR34::Tn5)
RmK124
(RmG212, pckR::ΩSp)
RmG212
(wild type, Lac⁻)
RmH172
(rpk-15)
RmH167
(rpk-10)
RmH166
(rpk-9)
a
b
c
d
B. succinate
probe
probe + BSA
0.1 μg
1 μg
10 μg
a
b
c
d

Table 7: Area integration† values for the EMSA in Figure 9

		a‡		b		c		d	
		A. M9 G	**B. M9 S**	**A. M9 G**	**B. M9 S**	**A. M9 G**	**B. M9 S**	**A. M9 G**	**B. M9 S**
probe		212	709	672	733	614	1612	245	1931
probe + BSA		212	211	672	308	622	1849	1568	1399
RmH853	0.1 μg	3540	3917	1129	966.5	1221	2483	8502	2315
	1 μg	33975	22983	6269	4850	3785	4446	40561	7350
	10 μg	152736	89631	22777	14693	14271	5197	79329	7013
RmK124	0.1 μg	5347	1832	1691	1172	1740	2383	9839	1623
	1 μg	42144	32202	7872	7751	4658	4470	35405	7687
	10 μg	163357	93790	15834	14973	16075	7994	83540	18011
RmG212	0.1 μg	1138	3353	1116	1917	1506	2792	2518	3001
	1 μg	12301	53613	3360	10086	3202	5185	9976	10538
	10 μg	17538	146956	7228	18382	12177	14720	51755	57684
RmH166	1 μg	34673	62914	3430	10254	2792	2173	18967	3679
	10 μg	78948	211868	8687	21252	8894	9649	51794	30215
RmH167	1 μg	35197	25834	4410	5581	3420	1617	22196	10015
	10 μg	67632	164366	10409	19197	16792	10892	60565	86215
RmH172	1 μg	34840	61042	3689	10228	3587	2046	22107	5515
	10 μg	36174	196976	6881	22398	10351	7203	49886	19339

† The pixels along an imaginary x-axis are graphed, and the area under each peak is determined to give the pixel value for each shifted band.

‡ Shifted probe bands labeled in Figure 9.

Extracts prepared from *rpk* mutant strains also caused the same four shifted probe bands as did the wild type cell extract, suggesting that these spontaneous mutations do not eliminate any of the proteins observed to bind directly to 89 bp *pck*A promoter fragment. In the assays performed using crude cell extract prepared from the *rpk* mutant strains (RmH166, RmH167 and RmH172) grown in succinate, shifted bands **a** and **b** were 3 to 5-fold more intense than in assays performed using extract prepared from glucose-grown cells. For assays performed using the crude cell extract prepared from the strain containing the *rpk*-15 mutation, shifted band **d** is 3-fold less intense in succinate compared to glucose.

These results suggest that all extracts contain the proteins causing the observed shifted bands, however the quantities of these proteins may differ between the various mutant strains and preparations. It is probable that the EMSA conditions used in this study do not allow visualization of all of the DNA-protein complexes that exist between the *pck*A promoter and proteins found in the crude cell extract.

Interaction of proteins from crude cell extract with other fragments of the *pck*A promoter

In order to investigate the possibility that PckR binds to a region of the *pck*A promoter other than the 89 bp region upstream of the *Eco*RI site, EMSAs were

performed using other fragments (Figure 8, panel A) from the *pck*A promoter (Figure 10).

The 600 bp *Sst*I/*Hind*III probe was used to examine the interaction of proteins with the promoter of *pck*A because it overlaps the *Eco*RI site. If there is a protein that interacts with the *pck*A promoter at, or bisecting the *Eco*RI site, fragments that end at this site may be incapable of any interaction. The large size of this probe makes resolution difficult due to reduced probe mobility and the gel must be run for a longer period of time (3 hours) leading to warming of the gel and breakdown of the running buffer.

There are two shifted bands visible when crude cell extract was assayed with the 600 bp *Sst*I/*Hind*III probe, however these bands are not well resolved. There does not appear to be any difference between the EMSA pattern observed with wild type (RmG212) extract or any of the mutant extracts. Again, this suggests that none of the bands observed in EMSA analysis with this *Sst*I/*Hind*III fragment were caused by an interaction between PckR and the *pck*A promoter.

There was one distinct band visible in the EMSA pattern of the 475 bp *Sst*I/*Eco*RI probe. The shifted band in the assay performed using crude cell extract prepared from *pck*R::ΩSp strains was at least 2.5-fold the intensity of this band in the assays performed using any of the other strains. This band was present in all strains, indicating that PckR was not the protein causing this probe shift.

Figure 10: Interaction of proteins in crude cell extract with A. the *Sst*I/*Hind*III and B. the *Sst*I/*Eco*RI fragments from the *pck*A promoter

The approximately 75 bp *Sst*I/*Eco*RI, 475 bp *Sst*I/*Eco*RI and 600 bp *Sst*I/*Hind*III fragments were isolated from plasmid pTH137 as indicated in Figure 7 (panel A). The plasmid was digested with the two enzymes (*Sst*I/*Eco*RI or *Sst*I/*Hind*III), labeled with [α-^{32}P]-dATP. Each lane contains approximately 10,000 cpm of the indicated probe isolated from the *pck*A promoter region. The *Sst*I/*Hind*III probe contains the complete sequence of the 89 bp probe, and overlaps the *Eco*RI site, whereas the *Sst*I/*Eco*RI probes are the two pieces that make up the *Sst*I/*Hind*III probe. 10 μg of protein in French-pressed crude cell extract prepared from glucose-grown cells was added to each of the assays. The lane labeled "probe" contained all components of the binding reaction except crude cell extract, which was replaced with an equal volume of buffer 1. The lane labeled "probe + BSA" contained 15 μg bovine serum albumin. The shifted bands in **A.** are labeled **a** and **b**, corresponding to the bands whose intensity were quantitated by area integration (Molecular Dynamics ImageQuant 3.3) and whose values are given in Table 8.

A. SstI/HindIII
probe
probe + BSA
RmG212 (Lac-, wild type)
RmK124 (RmG212, pckR::ΩSp)
RmH853 (RmG212, pckR34::Tn5)
RmH166 (rpk-9, pckA+)
RmH167 (rpk-10, pckA+)
RmH172 (rpk-15, pckA+)
b a
B. SstI/EcoRI
probe
probe + BSA
RmG212
RmK124
RmH853
75 bp
probe
probe + BSA
RmG212
RmK124
RmH853
475 bp

Table 8: Area integration† values for the EMSA in Figure 10

	SstI/*Hind*III		*SstI*/*Eco*RI	
	600 bp			
	a‡	b	475 bp	75 bp
probe	1806	2065	496	298
probe + BSA	2310	2310	651	509
RmG212	19454	24423	5870	1131
RmK124	23520	19330	14925	1791
RmH853	20140	14656	4409	1425
RmH166	25485	18456	nd	nd
RmH167	29213	21447	nd	nd
RmH172	19691	15639	nd	nd

nd = not determined

† The pixels along an imaginary x-axis are graphed, and the area under each peak is determined to give the pixel value for each shifted band.
‡ Shifted probe bands labeled in Figure 9.

There are several faint bands visible in the EMSA pattern of the 75 bp *SstI*/*Eco*RI probe. Once again however, these faint shifted bands are present in the *pck*R insertion mutant strains indicating that PckR binding was not the cause of the observed probe shift.

The multiple shifted bands, representing DNA-protein complexes, observed with the non-overlapping *pck*A promoter fragments (Figure 7, panel A) suggests that several proteins are capable of binding to the *pck*A promoter.

It is perhaps worth noting that the conditions employed above for the EMSA were determined following experimentation with different running buffers and binding buffers of different ionic strengths. For example, a low ionic strength buffer (6.7 mM Tris-Cl, 3.3 mM sodium acetate, 1 mM EDTA) (Ausubel *et al.*

1990) was tested, but was found to be unstable over the length of electrophoresis required for sufficient separation of bound and unbound probe. Binding buffers tested used Tris-Cl or Tris-acetate buffering systems, with or without EDTA, varying concentrations of salts (KCl, Mg-acetate, potassium glutamate, MgCl, NaCl), and glycerol or sucrose; however the same pattern of EMSA was observed for all binding buffering systems. French pressed extracts were found to produce cleaner, more consistent EMSA results than extracts prepared by sonication.

CHAPTER 4: DISCUSSION

PckR is an activator of *pck*A expression

In *S. meliloti*, addition of succinate to glucose-grown cells causes a 58-fold increase in *pck*A expression compared to cells growing on glucose alone (Osteras *et al.* 1995). Results presented in this thesis suggest that *pck*R encodes a positive-acting regulator of *pck*A transcription that may be activated during growth in succinate. *pck*R null mutations (insertion mutations) cause low-level *pck*A expression in glucose and succinate-grown cells, suggesting that induction during growth in succinate has been eliminated. PckR may be activated by an elevated or reduced concentration of some cellular metabolite during growth in succinate, leading to induction of *pck*A expression.

Despite the fact that *pck*R null mutant strains show only low *pck*A expression, they have a wild type growth phenotype in succinate. Interestingly, *pck*R *pck*A double mutant strains show more growth in succinate than *pck*A insertion mutant strains. This suggest that PckR may regulate expression of other metabolic genes such as pyruvate orthophosphate dikinase (POD),

a PckR null mutation would allow pyruvate regeneration for the TCA cycle via malic enzyme and POD (see Figure 1) (Osteras *et al.* 1997b). This hypothesis could be tested by assaying POD activity in strains with a PckR mutation.

Evidence suggests that the group I spontaneous mutations—*rpk*-9, *rpk*-10 and *rpk*-15—are alleles of *pck*R. First, the *pck*R gene was able to complement the high *pck*A expression observed in these mutant strains during growth in glucose minimal media. Second, it can be extrapolated from transduction data that the spontaneous *rpk* mutations and the *pck*R Tn*5*-insertions are located within 15 kb on the *S. meliloti* chromosome.

***rpk*-9 mutant strains constitutively express *pck*A**

While the *rpk*-9, 10 and 15 mutations appear to be alleles of *pck*R it is intriguing that the *pck*R null and *rpk* mutant strains have very different *pck*A expression phenotypes. In glucose-grown cells, the *pck*R null mutations resulted in low-level *pck*A expression, whereas *pck*A expression in the *rpk*-9 mutant strain was comparable to succinate-grown wild type cells. An explanation for this observation is that *rpk*-9 results from a mutation that causes the PckR protein to always be in the active conformation. If this were the case, the *rpk* mutations would be expected within the inducer binding domains of PckR (see Figure 3). Alternatively, the *rpk* mutations could eliminate auto-regulation of PckR causing constitutive induction. Complementation with wild type PckR would, in this case, cause a reduction in *pck*R expression. Mutation of a positive regulator in either

of these ways could increase *pckA* transcription under non-inducing conditions, such as growth in glucose. Furthermore, the method used to isolate strains carrying the *rpk* mutations specifically selected for those mutations that caused high levels of *pckA* expression on glycolytic carbon sources, therefore it would not have been possible to isolate *pckR* null mutations.

This model that the *rpk* mutations are different *pckR* alleles is complicated by the observation that *pckR* was only conditionally able to complement the *rpk* mutations. Each of the group I mutations was complemented by *pckR* in glucose-grown cells, however only the *rpk*-10 mutation was complemented in LBmc-grown cells. It is not clear whether the high level of *pckA* expression observed in LBmc-grown cells in stationary phase is due to the same process that causes the succinate induction effect, or whether another mechanism is operating. To some extent, however, *pckA* expression in LBmc-grown cells is independent of *pckR*, as evidenced by 10-fold higher *pckA* expression levels in LBmc-grown, rather than glucose or succinate-grown, *pckR* insertion mutants. In addition, the lag in *pckA* expression relative to growth phase observed in wild type LBmc-grown cells was not observed for the *rpk*-9 mutant strain. Further research could address whether a quorum sensing mechanism involving a diffusible cellular signal is responsible for the induction of *pckA* expression in stationary phase. If this were the case, spent culture supernatant from cells in stationary phase may be sufficient to induce the expression of *pckA* via a LuxR-type transcriptional activator.

Despite the belief that succinate is a major carbon source in *S. meliloti* bacteroids (Ronson *et al.* 1981; Finan *et al.* 1983; Watson *et al.* 1988; Yarosh *et al.* 1989: Stowers *et al.* 1985), little Pck activity is observed (Finan et al. 1988). In this study, low-level *pck*A expression was observed in bacteroids, suggesting that, in accord with free-living cells, regulation of Pck activity is at the level of *pck*A transcription. Therefore, the expression of *pck*A in *S. meliloti* bacteroids appears to be independent of the available carbon sources. High *pck*A expression levels were observed in bacteroids harboring the *rpk*-9 mutation, suggesting that this mutation affects *pck*A expression in both the bacteroid and free-living states. A positive regulator that is constitutively in the active conformation, like hypothesized for *rpk*-9, would perhaps be incapable of responding to factors in the bacteroid state which would otherwise inactivate it. In bacteroids with a *pck*R null mutation, *pck*A is expressed at wild type levels.

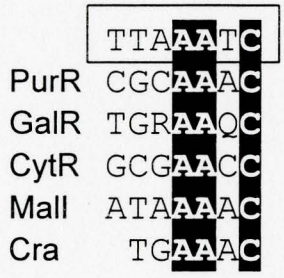
A possible role for PckR in global metabolism of *S. meliloti* is an avenue of future possible research. An in-depth examination of the activities of various metabolic indicator enzymes (such as those examined by Finan *et al.* 1988) could be conducted on strains containing *pck*R null mutations. A different approach could be use of a specialized technique called genomic SELEX (systematic evolution of ligands by exponential enrichment) to identify PckR binding sites from *S. meliloti* genomic DNA. Genomic SELEX involves cycles of PCR and binding assays to select and amplify those fragments that interact with a protein of interest (Gold *et al.* 1997; Singer *et al.* 1997).

A putative operator binding site is identified in the *pck*A promoter

A putative LacI-GalR-type consensus binding sequence (operator) has been identified in the *pck*A promoter at position –76 relative to the transcriptional start site (see Figure 8). Insertion of an ΩSp interposon into the *pck*A promoter, downstream of this putative operator, lead to high levels of *pck*A expression in glucose-grown cells (Osteras *et al.* 1997). This phenotype may result from displacement of a *cis*-acting operator site for a negative regulator as originally hypothesized (Osteras *et al.* 1997), or as the results presented here suggest, the phenotype may result from an increased affinity of a positive regulator for the promoter. The putative operator in the *pck*A promoter is homologous to operators identified for other members of the LacI-GalR family (Schumacher *et al.* 1994) (see Figure 11). It is possible that PckR, which we have identified as a LacI-GalR-type transcriptional regulator, interacts with this operator site in the *pck*A promoter region.

Figure 11: Alignment of the operator half sites of the LacI-GalR-type regulators

Contact is believed to occur between a LacI-GalR-type protein (see Section 3.1) and the aligned operator half sites. The boxed top sequence is the operator half site identified at –76 relative to the transcriptional start site in the *pck*A promoter. Conserved residues are highlighted. Symbols are as follows: Q = any base, R = guanine or thymidine. (Saier and Ramseier 1996; Schumacher *et al.* 1994)



Cra, a LacI-GalR-type protein, can act either as an activator or a repressor of transcription in *E. coli*. *pck*A gene expression and genes encoding other gluconeogenic enzymes are activated by Cra. The distance between the operator site for Cra binding and the transcriptional start site of the regulated gene determines whether Cra acts as an activator or a repressor (Ramseier *et al.* 1995). A model of promoter structure as the key determinant of activation or repression has been suggested for Cra (Saier and Ramseier 1996). According to this model, if the binding site is upstream of the –10/–35 regions of the σ^{70} promoter, Cra mediates activation by looping the DNA between the binding site and the transcriptional start site, allowing RNA polymerase to bind. However, if the binding site is between the –10/–35 regions of the σ^{70} promoter and the

transcriptional start, Cra mediates repression by preventing RNA polymerase binding. In accordance with this model, Cra binds to the *pck*A promoter of *E. coli* at position –130 relative to the transcriptional start site (Ramseier *et al.* 1995). Similarly in *S. meliloti*, the putative LacI-GalR-type operator site is located upstream of the –10/–35 regions in the σ^{70}-like promoter, hence supporting the hypothesis that *pck*A is subject to positive regulation by a LacI-GalR-type protein.

Based on sequence homology to the DNA-binding helices of other known DNA-binding proteins (see Figure 2), it is probable that PckR is a DNA-binding protein. However the electrophoretic mobility shift assays (EMSAs) performed in this report found no band shift differences between extracts from *pck*R$^+$ and *pck*R$^-$ strains. There are several possible explanations for these observations made in this study. First, the conditions employed might not have stabilized the interaction between the *pck*A promoter fragment and the PckR protein. Second, other proteins in the crude cell extract preparations may have interfered with the DNA-protein interaction. Third, the observed inducing effect of PckR upon *pck*A transcription may be indirect. In order to determine if there is an interaction between any region of the *pck*A promoter and PckR, DNaseI-footprinting analysis could be used with purified PckR protein.

Many questions remain unanswered regarding the regulation of *pck*A in *S. meliloti*. Particularly, the cause of the stationary phase induction effect observed in free-living cells growing in complex media has yet to be addressed. The

factors regulating *pck*A expression in the bacteroid, as well as the role of Pck in nitrogen fixation, remain to be determined. Furthermore, the functional role of the *pck*R gene product, identified as a positive regulator of *pck*A expression, in overall cellular metabolism remains to be determined. PckR is the first member of the LacI-GalR family to be identified in *S. meliloti*, and it is possible that its role in global metabolic control and carbon flow will prove wide reaching.

APPENDIX I

The following experiments were conducted for two purposes:

1. To overproduce PckR in a pET vector system (Novagen) with an inducible promoter. In this system, a His-Tag adds a translational fusion to the C-terminus of the protein for use in subsequent purification using commercially available Ni columns.

2. To sequence the *rpk*-9, *rpk*-10 and *rpk*-15 alleles to determine if the spontaneous mutations are within the *pck*R gene.

PCR primers were designed to amplify the *pck*R gene with introduced restriction sites and to then clone the product into the pET vector. The nucleotide sequence of the *pck*R gene, with applicable restriction sites, is provided in Appendix II.

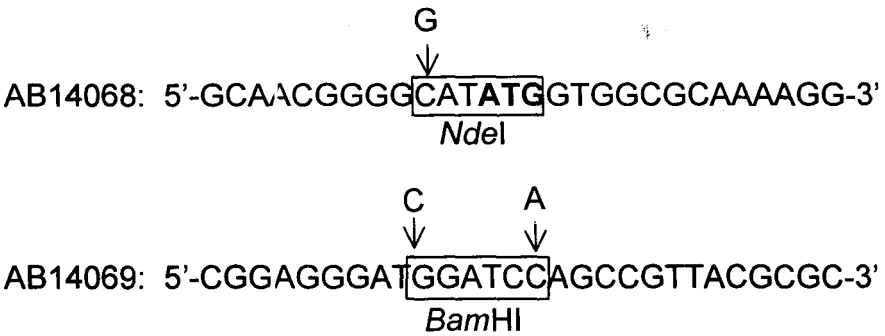


Figure 12: PCR primers used to amplify the *pck*R gene

The introduced restriction sites and the mismatches required to introduce these sites are indicated. The nucleotide shown above the primer is the base found in the *pck*R gene. The ATG start codon of the *pck*R gene is indicated in bold.

Overexpression of PckR

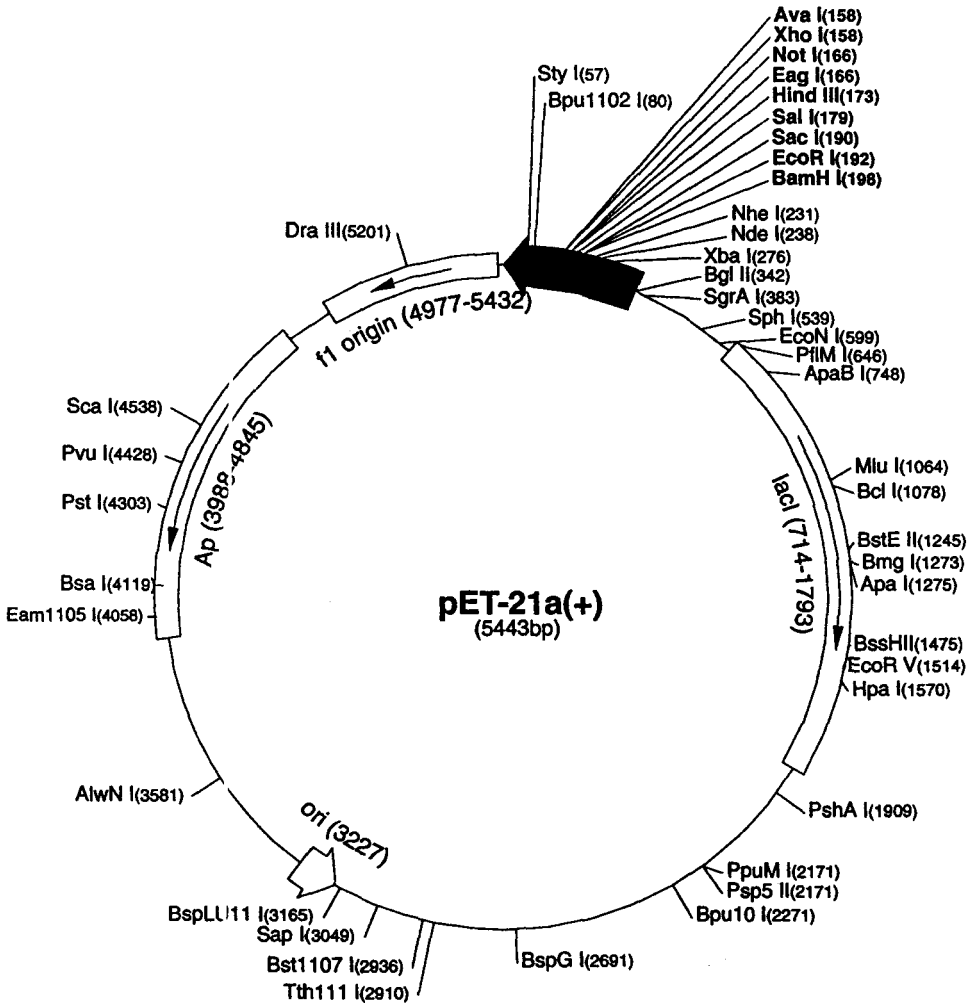


Figure 13: The pET vector system (Novagen)

The *pckR* PCR product was to be cloned into the pET21a vector as an *NdeI/BamHI* fragment in order to create a translation protein with a His-Tag for subsequent use in purification.

The PCR product and the pET21a vector were digested with *Bam*HI and *Nde*I, extracted with phenol, and ligated. Five transformants were pooled to inoculate 24 cultures (120 total transformants). Undigested plasmid preparations from the pooled transformants are shown in the above figure. One preparation contained a plasmid of the expected size (approximately 5 kb). This band was excised from the gel and isolated using a modified freeze and squeeze protocol. The DNA was re-transformed into DH5α cells, and the resultant colonies checked for the presence of the 5 kb plasmid. However, none of the resulting colonies contained this plasmid of interest. Instead all colonies appeared to contain DNA like the original pET vector.

A new plasmid preparation of the pET vector was prepared, and more of *pck*R was amplified by PCR. For PCR, the concentration of plasmid template was increased to 84 ng and 420 ng per reaction, and the number of cycles was increased to 43 in order to increase the yield. The PCR product and the pET vector were digested with *Bam*HI and *Nde*I, and electrophoresed. One half of the DNA was gel isolated using the modified freeze and squeeze protocol, and one half was isolated using QiaexII (pET vector, 4 kb; *pck*R, 1 kb). Ligations using both methods of preparation were conducted, but no colonies were retrieved. The isolated DNA was digested again with *Nde*I, ligated with the pET vector and transformed. No colonies above the background of religation were observed.

In order to determine if the *Bam*HI site in the PCR product was successfully digested, I attempted to clone both *Bam*HI/*Sac*I and *Sac*I/*Nde*I fragments of the PCR product (see Appendix II) into pET. Gel isolated DNA digested with *Bam*HI and *Nde*I, was further digested with *Sac*I, ligated into pET either digested with *Bam*HI/*Sac*I or *Sac*I/*Nde*I. For the *Bam*HI/*Sac*I cloning, many more transformants were observed compared with the religation of the pET vector, indicating that the cloning may have been successful. No colonies were observed for the *Sac*I/*Nde*I cloning. These results indicate that the *Nde*I site on the *pck*R PCR product was not digested. It is necessary to design new PCR primers with a greater number of nucleotides extending beyond the *Nde*I site in order to clone the *pck*R gene as an *Nde*I/*Bam*HI fragment into the pET vector system.

Sequencing of group I rpk alleles

The primers described for the cloning of *pck*R for overproduction (AB14068/AB14069) were used to amplify the group I *rpk* alleles (*rpk*-9, *rpk*-10 and *rpk*-15) from genomic DNA. The sequence obtained proved inconclusive in determining the site of the spontaneous mutations for *rpk*-15 and *rpk*-15 (see Figure 16). No sequence was obtained from *rpk*-9. Southern blotting confirmed that these spontaneous mutations do not contain any major rearrangements within the *pck*R gene (see Figure 17). In order to obtain sequence from the *pck*R gene region of these spontaneous mutations, new PCR primers should be designed to include the *pck*R promoter.

Figure 16: Alignments of the *pck*R sequence from strains containing the *rpk*-10 and *rpk*-15 alleles

Sequence obtained from primer AB14069 (3' end of gene) is given in A, while sequence obtained from primer AB14068 (5' end of gene) is given in B. Clustal W (Thompson *et al.* 1994) alignments are to the published *pck*R wild type gene (Appendix II). Possible nucleotide substitutions are indicated by boxes. No sequence was obtained from the PCR product obtained from RmH147 (*rpk*-9).

A

CLUSTAL W (1.74) multiple sequence alignment

```
WT              ATGGTGGCGCAAAAGGTCAAGCTTTCTACAATCGCGGAAACACTCGGCCTTTCGACGGCG
rpk10-3'        ------------------------------------------------------------
rpk15-3'        ------------------------------------------------------------

WT              ACGGTATCCCTGGCATTGCGCGACAGCCCGCTGGTGGCGGCGGTCACACGCGACAAGATC
rpk10-3'        ----------------------------------------------------ANNAAAATC
rpk15-3'        ----------------------------------------------------AGGANA
                                                                     *  *

WT              AACGAACAGGCGCGCGCACTCGGCTACATCTACAACCGCCGTGCCGCAAGTCTCAGGACG
rpk10-3'        AACAAAANGGGGTGC--ATNGGG--ANANGNACAACGG--NGNCAGCAAGTTTAAGAA-N
rpk15-3'        ANTTAATANNTGGGG--NATAGN--AGAT-NACAACAN---GGTNNAAAATATNAGAACT
                *   **     * *       *   * *   *****           ** * * ** *

WT              TCCCGCTCGGGCATCATCGGTGTCGTCGTGCACGACATCATGAACCCGTTCTACGGTGAG
rpk10-3'        TANNATT--GGCANCAT-GGTGT-NTNGGGCA-NACATAAGGA--CCNGTNTA-GGT-AA
rpk15-3'        TCCGANNA-GGANTNATGGGTTT-NTGTGGGN-CANATCANNAACCCGNNATNGGGT-AA
                *        **    ** *** *  *   *    * ** *  *  **    *  *** *

WT              ATCCTCAAGGCGATCGAGGCTGAGCTCGATCGCGACAAGCAGACCTTCATTCTGTCCAAC
rpk10-3'        AACCTAANGCGNNTTNAGGNTAAGCT-GANCGCGACAAGCAGNCNTTCATTNTGTCCAAC
rpk15-3'        NTCGNGAAG---GTTNANGTTNNAGTGNTTAGAGN-AANCAGACNTTNATTNTGTCCAAC
                   * *    *  * * *    *     * *  ** *** * ** *** ********

WT              CACTACGATTCCGTCGAGAAGCAGCGCGATTTCATCGAGACGCTGCTGCAGCTCGGTGGC
rpk10-3'        CANTAGGATTCCGTCAAAAA-CAGCG-GATTT-ATNGAAA-GATTGTNAAGTTGGGTGGA
rpk15-3'        ATNTAAAATCCANTNGANAAGNAGGGNTATTTAANAAGAAGGNTGTTGNAGGTAGGTGAA
                   **  ** *  *  * **  ** *  **** *     * * *  *  ** * ****

WT              GACGGCGTGATCATGTCGCCCGCTATCGGCA-CGCCGCCGCAGGACA-TTCAGCTTGCCG
rpk10-3'        AANGGGGGNATCATGTCGCC-GCTANNGGCAANGCNGCCGCAG-ACA-TTCAGCTTTCCG
rpk15-3'        GAGGGGTGATNATTGTCGCCNGCNATGGGCA-NGCAGCNGCAGAGCAGTTNAGCTTACGG
                 * **        ******* ** *  ****  ** ** ****  ** ** ***** * *

WT              AGGACAACGG-CATGCCGG-CGATCCTGATCG-CCCGCTCGATCGAGGGGCTCGACGTCC
rpk10-3'        AGGACAANGG-CATNCNGGGCGATCCTGATCGGCCCGCTCGATAGAGGGGCTNGACGTCC
rpk15-3'        GGANCAAAGGGCATNCGGG-CGAATCATANTGGCCCGCTCGATNAAAGGGCTAGAAGTCC
                 *  *** ** *** * ** ***  *  *  * **********  * ***** ** ****

WT              CCATCTT-CCGCGGCGACGACGCCTATGGCATTTCGCTGGCGACCA-ATCATCTCATCGG
rpk10-3'        CCATCTTTCCGGGGCGACGACGCCTATGGCATTTCGCTGGCGACCA-ATCATCTCATCGG
rpk15-3'        CCATATTTNGGGGNGANAAACGCCTATGGCATTTCGCTGNGGACCCGATCATCTCATCGG
                **** **   * *      ******************** ****  ************

WT              G-CTCGGCCATCGCTGCAT-CGCGATGGTCGGGGGAACGGACCA-GACCT-CGA-CCGGC
rpk10-3'        G-CTCGGCCATCGCTGCAT-CGCGATGGTCGGGGGAACGGACCA-GACCT-CGA-CCGGC
rpk15-3'        NGNTCGGCCATCGCTGCATTNGCGATGGTCGGGGGAACGAACCAAGACCNTCGANCCGGC
                   ***************  ********************** ****  ***  *****

WT              CGC--GACCG-CTACCAGGGCTACGTCAACGCGCTGCGCAAGGCGAATATCGAGGTCGAC
rpk10-3'        CGC--GACCG-CTACCAGGGCTACGTCAACGCGCTGCGCAAGGCGAATATCGAGGTCGAC
rpk15-3'        CNGCGGACCGGCTACCAGGGCTAGGTCAACGCGCTGCGCAAGGCGAATATCGAGGTCGAC
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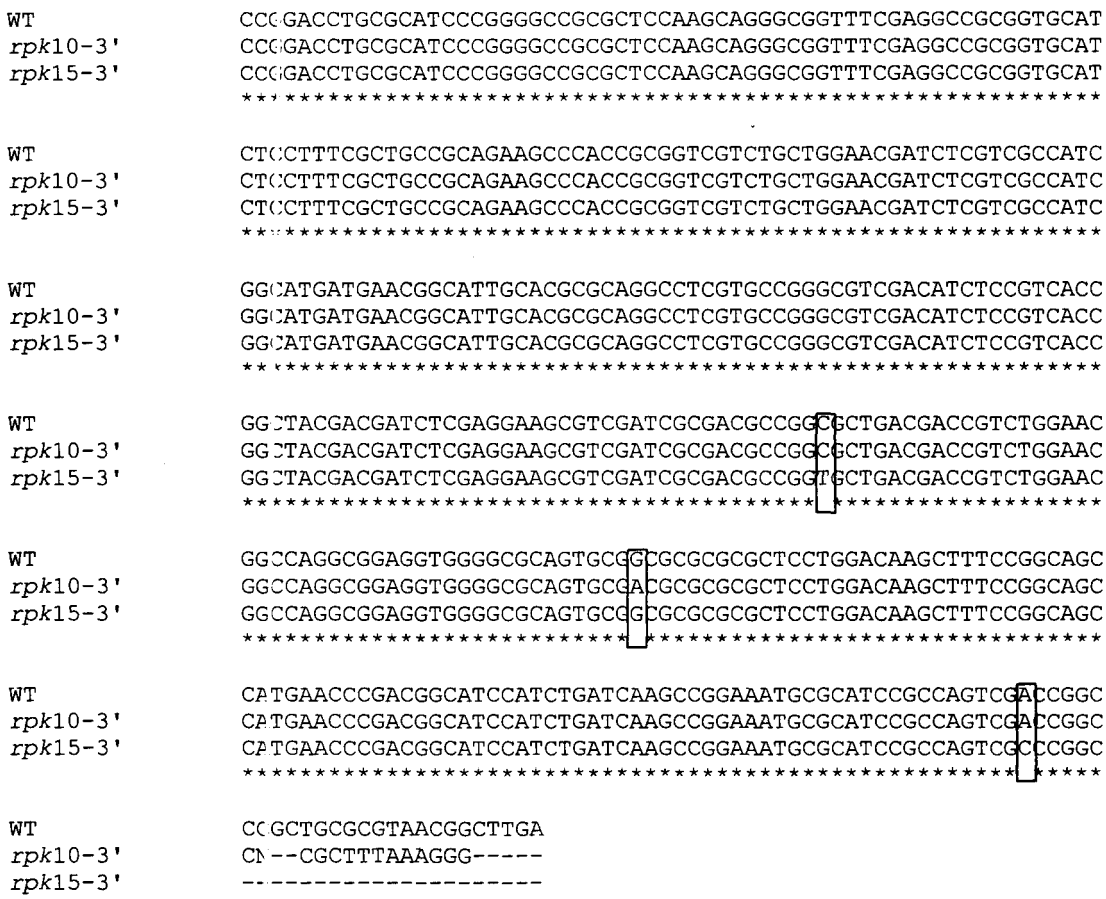
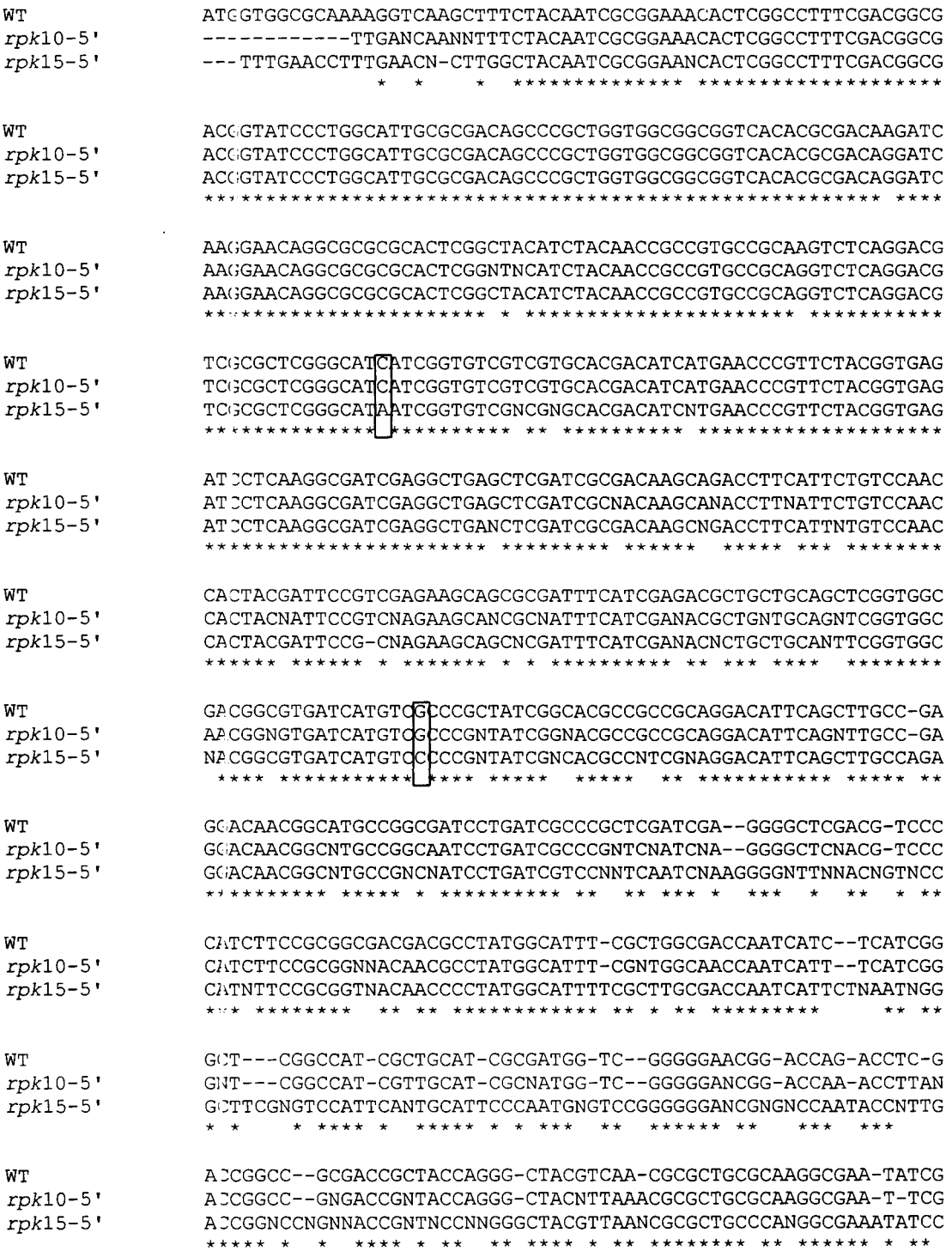

WT
rpk10-3'
rpk15-3'

B

CLUSTAL W (1.74) multiple sequence alignment



```
WT              AGG--TCGACCCGGA-CCTGCGCATCCCGGGGCCGCGCTCCAAGCAGGGCGGTTTCGAGG
rpk10-5'        AGG--TCAACCCGGA-CTTNC-CATNCCGGG-CCGNGCTTCAAN-AGGGCGGTTTNAAGG
rpk15-5'        ANNGCTCTACCCGGANCNTGCNCATTCCNGGGANNNNTCTNNAACANGGCGGTNTNNAAG
                *    ** ******* * * * *** ** **           *  * ****** *  * *

WT              CCCCGGTGCATCTCCTTTCGCTGCCGCAGAAGCCCACCGC-GGTCGTCTGCTGGAACGAT
rpk10-5'        CCCNGGGG--NTNNCTTTNTTGNCCAAAG----CCACCG--GGCCGTTTGCTNGAACAAT
rpk15-5'        CCCCGGNC-ATTCTCTTTCNNTGCCGCACAANCCNACNGCGGGNCGTTTGCTNGAACANN
                **  **         ****     **  *     * ** *  ** *** **** ****

WT              CTCGTCGCCATCGGCATGATGAACGGCATTGCACGCGCAGGCCTCGTGCCGGGCGTCGAC
rpk10-5'        TT-GTNNTCTTCNGCTTGATNA-CGGCATTNCACNC-CAGGCCTNNTGCCCGGCNNTNAA
rpk15-5'        TTCATCNNCNTGNGCATGATAAAACNCTTTCNNCCC--AAGNTTCTNNCC-NGCNTNAAT
                 *  *   * *  ** **** *    * **   * *  * *  *    **  **    *

WT              ATCTCCGTCACCGGCTACGACGATCTCGAGGAAGCGTCGATCGCGACGCCGGCGCTGACG
rpk10-5'        NATTTCCT-ACCGNTT-CAAC-ATTTNGTNGAAGC-TTTATNGCNAC-CCGGA-CTTNC-
rpk15-5'        TTTTCCAC--CTGGCTTNTAACAATTTGNNCANCN-TTNTCCNCNACCTCGTN-TTAACA
                   * *    * *  *   *  * * *  *    *      * **  **    *  *

WT              ACCGTCTGGAACGGC-CAGGCGGAGGTGGGGCGCAGTGCGGCGCGCGCGCTCCTGGACAA
rpk10-5'        ACCTTTTG-AACGNC-C--TCNNANGTGGNCCANNNTACCCCCCTTNNT-----------
rpk15-5'        ACC-TCTNTATGGACTCCANTNGTGGGNNNACTNNNNCTNCCCCTTNCT-----------
                ***  * *  *  * * *        *     *         * *

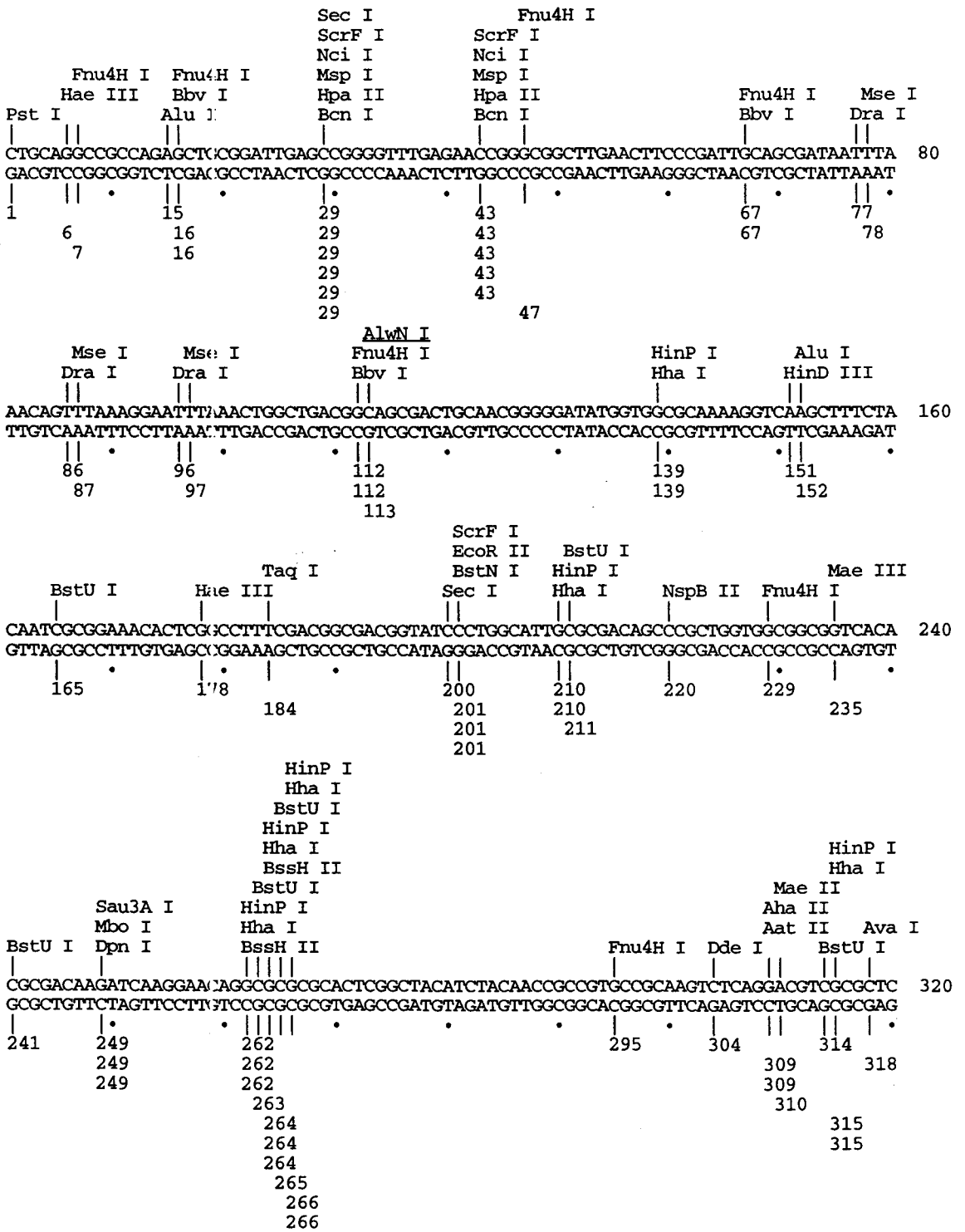
WT              GCTTTCCGGCAGCCATGAACCCGACGGCATCCATCTGATCAAGCCGGAAATGCGCATCCG
rpk10-5'        ------------------------------------------------------------
rpk15-5'        ------------------------------------------------------------


WT              CCAGTCGACCGGCCCGCTGCGCGTAACGGCTTGA
rpk10-5'        ---------------------------------
rpk15-5'        ---------------------------------
```

APPENDIX II

Figure 18: Restriction map of the *pckR* gene sequence as found in Genbank

Unique restriction sites are underlined. The length of the DNA sequence is 1250 kb. DNA Strider 1.1 was used to generate this map.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Hae III
Fnu4H I
Bbv I
Alu I
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Bbv I
Mse I
Dra I
Pst I
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7 16 29 43
29 43
29 43
29 47
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Mse I
Dra I
Mse I
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Fnu4H I
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HinP I
Hha I
Alu I
HinD III
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86 96 112 139 151
87 97 112 139 152
113
ScrF I
EcoR II
BstN I
Sec I
BstU I
HinP I
Hha I
Taq I
Hae III
BstU I
NspB II
Fnu4H I
Mae III
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201 210
201 211
201
HinP I
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HinP I
Hha I
BssH II
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BssH II
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Aha II
Aat II
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Mbo I
Dpn I
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Fnu4H I
Dde I
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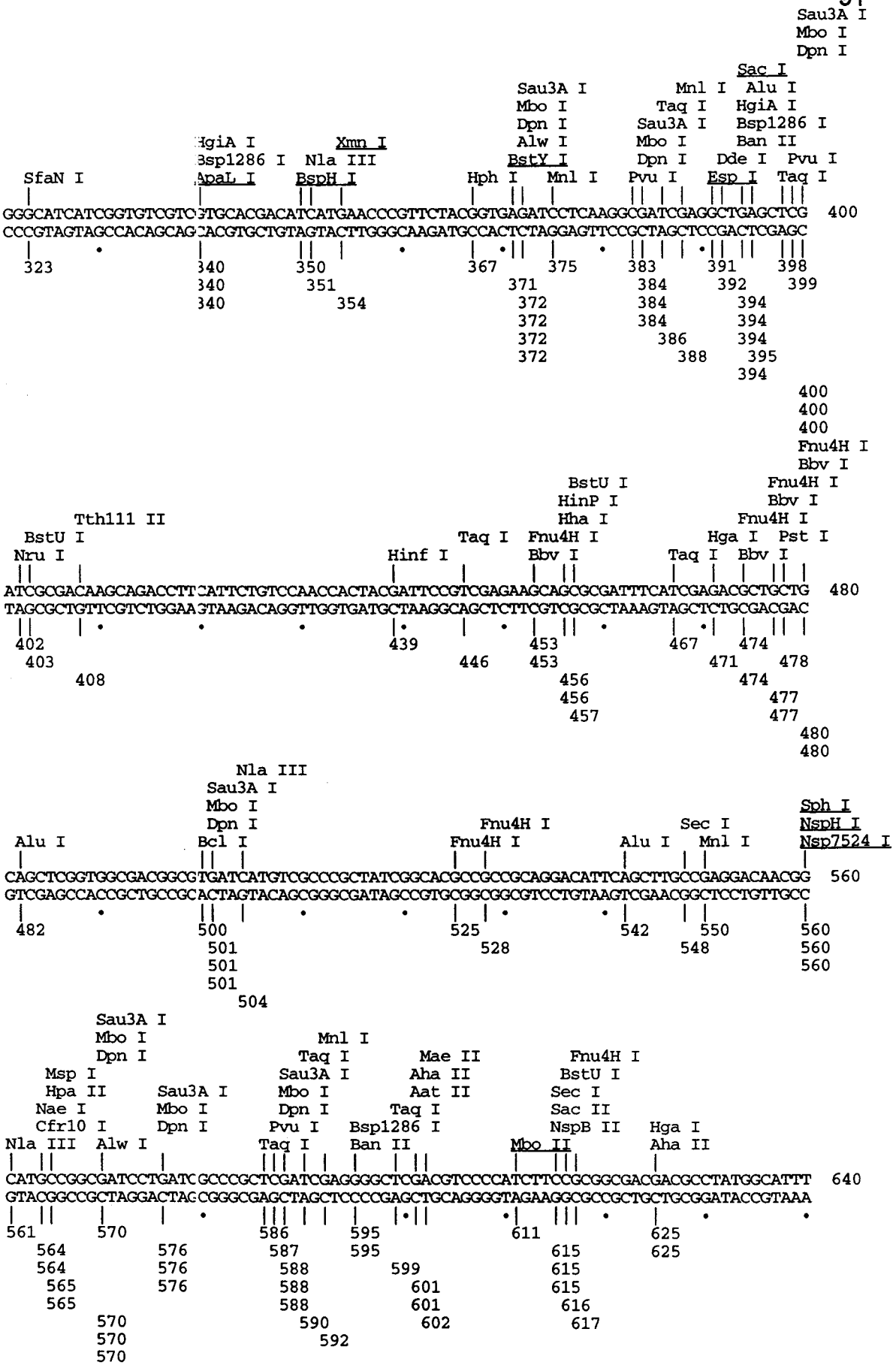
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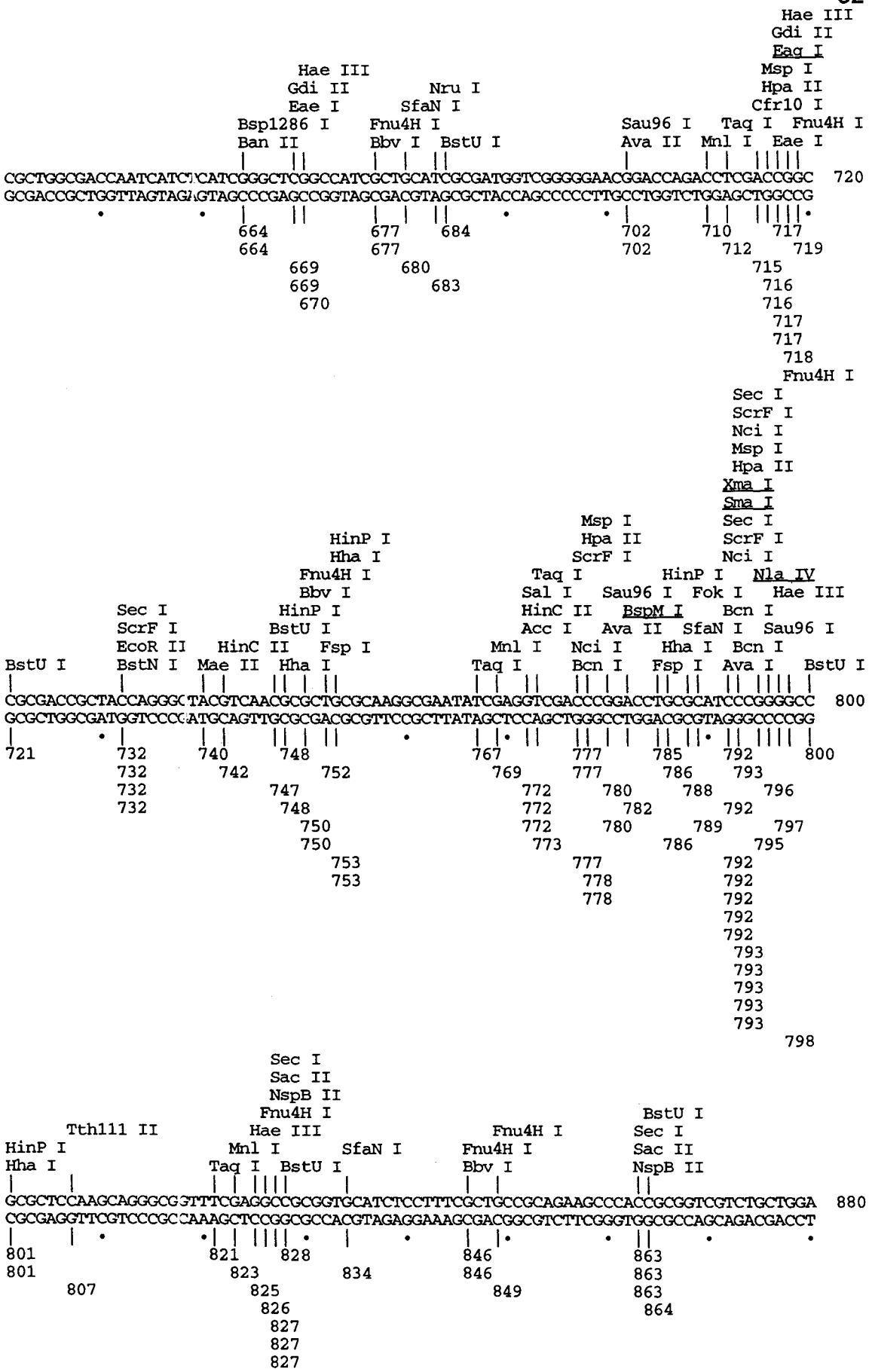
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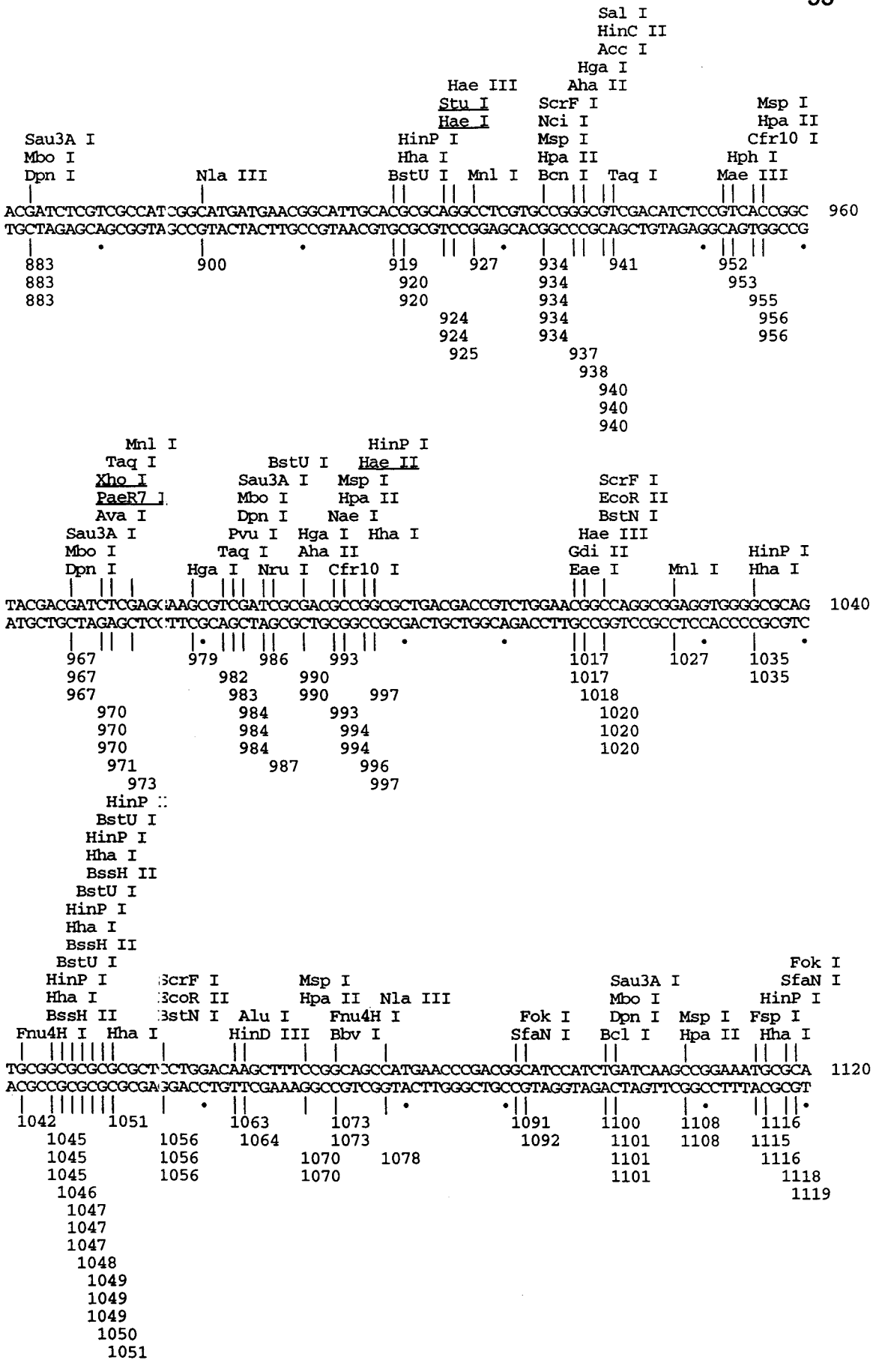
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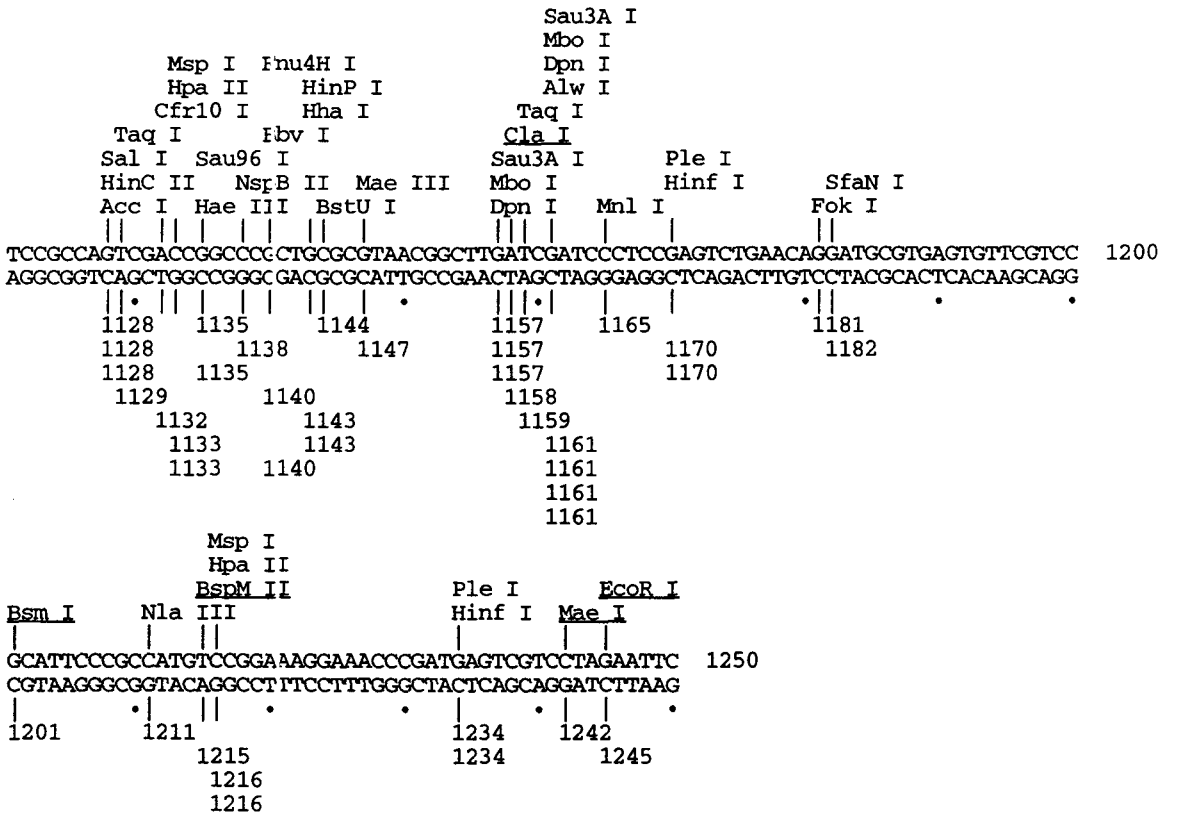
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Ple I
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Acc I
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