

# Increased pyruvate orthophosphate dikinase activity results in an alternative gluconeogenic pathway in *Rhizobium (Sinorhizobium) meliloti*

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**The formation of phosphoenolpyruvate (PEP) is a major step in the gluconeogenic pathway in which tricarboxylic acid (TCA) cycle intermediates are converted to hexose sugars. In *Rhizobium (now Sinorhizobium) meliloti* this step is catalysed by the enzyme PEP carboxykinase (PCK) which converts oxaloacetate to PEP. *R. meliloti* Pck<sup>-</sup> mutants grow very poorly with TCA cycle intermediates as the sole source of carbon. Here, the isolation and mapping of suppressor mutations which allow Pck<sup>-</sup> mutants to grow on succinate and other TCA cycle intermediates is reported. Tn5 insertions which abolished the suppressor phenotype and mapped to the suppressor locus were located within the *pod* gene encoding pyruvate orthophosphate dikinase (PPDK). Strains carrying suppressor mutations had increased PPDK activity compared to the wild-type. The suppressor phenotype was dependent on the combined activities of malic enzyme and PPDK, which thus represent an alternative route for the formation of PEP in *R. meliloti*. PPDK activity was not required for symbiotic N<sub>2</sub> fixation.**

**Keywords:** *Rhizobium (Sinorhizobium) meliloti*, pyruvate orthophosphate dikinase, gluconeogenesis

## INTRODUCTION

Pyruvate orthophosphate dikinase (PPDK, EC 2.7.9.1) catalyses the reversible reaction: pyruvate + ATP + P<sub>i</sub> ⇌ phosphoenolpyruvate (PEP) + AMP + PP<sub>i</sub> (Evans & Wood, 1968; Hatch & Slack, 1968; Reeves *et al.*, 1968). This enzyme has been found in plants, protozoa and several bacteria (Benziman & Palgi, 1970; Buchanan, 1974; Ernst *et al.*, 1986; Evans & Wood, 1968, 1971; Hatch & Slack, 1968; Matsuoka, 1995; Petzel *et al.*,

1989; Reeves, 1968, 1971; Reeves *et al.*, 1968; Schwitzguébel & Ettliger, 1979).

In C<sub>4</sub> plants, PPDK catalyses the regeneration of PEP, the primary acceptor of CO<sub>2</sub> in the C<sub>4</sub> photosynthetic pathway (Hatch & Slack, 1968; Edwards *et al.*, 1985). In *Entamoeba*, *Bacteroides* and *Asteroleplasma anaerobium*, PPDK functions in a glycolytic capacity, replacing pyruvate kinase activity in the conversion of PEP to pyruvate (Reeves, 1968; Reeves *et al.*, 1968; Petzel *et al.*, 1989). The primary function of PPDK in *Propionibacterium*, *Acetobacter* and the photosynthetic bacteria, however, appears to be in gluconeogenesis, as PPDK activity in these bacteria increases following growth on carbon sources which require gluconeogenesis (Benziman & Eizen, 1971; Evans & Wood, 1971).

Our interest in PPDK arose from metabolic studies of the soil bacterium *Rhizobium (now Sinorhizobium) meliloti*, which forms N<sub>2</sub>-fixing root nodules on alfalfa. There is much evidence that within nodules, the plant supplies these bacteria with C<sub>4</sub>-dicarboxylic acids, such as malate, as their principal source of energy for the N<sub>2</sub>-fixation process. In *Rhizobium leguminosarum*, *R.*

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**Abbreviations:** PEP, phosphoenolpyruvate; PPS, phosphoenolpyruvate synthase; PTS, phosphoenolpyruvate:carbohydrate transferase system; TCA, tricarboxylic acid; for other enzyme abbreviations see legend to Fig. 1.

The GenBank/EMBL accession numbers for *pod-5*, *pod-6* and the *Bam*HI *pod* gene fragment reported in this paper are U61377, U61376 and U61378, respectively.

**Table 1.** Bacterial strains and plasmids used in this study

Strain, plasmid or phage	Relevant characteristics	Reference
<b><i>Rhizobium meliloti</i></b>		
Rm1021	<i>R. meliloti</i> SU47, <i>str-21</i>	Meade <i>et al.</i> (1982)
Rm5000	<i>R. meliloti</i> SU47, <i>rif-5</i>	Finan <i>et al.</i> (1984)
Rm5065	Rm1021, <i>pckA1</i> ::Tn5-132	Finan <i>et al.</i> (1988)
Rm5234	Rm1021, <i>pckA2</i> ::Tn5-VB32	Finan <i>et al.</i> (1988)
Rm5439	Rm1021, <i>pckA1</i> ::TnV	Finan <i>et al.</i> (1988)
Rm6661	SU47, <i>his-39 trp-33 leu-53</i> <sup>+</sup> Ω601::Tn5- <i>mob</i> (-)	Klein <i>et al.</i> (1992)
Rm6662	SU47, <i>his-39 trp-33 leu-53</i> <sup>+</sup> Ω602::Tn5- <i>mob</i> (+)	Klein <i>et al.</i> (1992)
Rm6692	SU47, <i>his-39 leu-53 trp-33</i> <sup>+</sup> Ω611::Tn5- <i>mob</i> (+)	Klein <i>et al.</i> (1992)
Rm6693	SU47, <i>his-39 leu-53 trp-33</i> <sup>+</sup> Ω612::Tn5- <i>mob</i> (-)	Klein <i>et al.</i> (1992)
Rm6695	SU47, <i>his-39 trp-33 pyr-49</i> <sup>+</sup> Ω614::Tn5- <i>mob</i> (+)	Klein <i>et al.</i> (1992)
Rm6696	SU47, <i>his-39 trp-33 pyr-49</i> <sup>+</sup> Ω615::Tn5- <i>mob</i> (-)	Klein <i>et al.</i> (1992)
Rm6865	SU47, <i>his-39 trp-33 pyr-49 cys-11</i> <sup>+</sup> Ω637::Tn5- <i>mob</i> (+)	Klein <i>et al.</i> (1992)
RmF361*	Rm5065, <i>pod-1</i>	Driscoll & Finan (1993)
RmF871	Rm5065, Ω5208::Tn5-233	This work
RmF914	Rm5065, <i>pod-1</i> Ω5208::Tn5-233	This work
RmG115	Rm5065, <i>pod-2</i>	This work
RmG116	Rm5439, <i>pod-3</i>	This work
RmG117	Rm5439, <i>pod-4</i>	This work
RmG139	Rm1021, <i>pod-1</i>	Driscoll & Finan (1993)
RmG242	Rm1021, <i>pckA3</i> ::Tn3HoHoSp	This work
RmG243	RmG242, <i>pod-1</i>	Driscoll & Finan (1993)
RmG273	RmG243, <i>pod-5</i> ::Tn5	This work
RmG274	Rm1021, <i>pod-5</i> ::Tn5	This work
RmG316	RmG243, <i>pod-6</i> ::Tn5	This work
RmG416	Rm5065, <i>pod-6</i> ::Tn5	This work
RmG417	Rm1021, <i>pod-6</i> ::Tn5	This work
RmG443	RmG243, <i>dme-2</i> ::Tn5	Driscoll & Finan (1993)
RmG420	RmG243, <i>pod-7</i> ::Tn5	This work
RmG457	Rm1021, <i>pod-7</i> ::Tn5	This work
RmG458	RmF914, <i>pod-7</i> ::Tn5	This work
RmG566	Rm1021, <i>pod-5</i> ::Tn5-233	This work
RmH187	RmF361, <i>tme-4</i> ::ΩSp	Driscoll & Finan (1996)
RmH188	RmF361, <i>dme-1</i> ::Tn5	Driscoll & Finan (1996)
RmH194	RmF187, <i>dme-1</i> ::Tn5	Driscoll & Finan (1996)
RmH243	Rm1021, <i>pod-5</i> ::TnV	This work
<b><i>Rhizobium</i> sp. NGR234</b>		
NGRpckA1	NGR234R, <i>pckA</i> ::Ω	Østerås <i>et al.</i> (1991)
<b><i>Escherichia coli</i></b>		
EJ1321	<i>pck dme tme</i>	Hansen & Juni (1975)
DH5α	F <sup>-</sup> , <i>endA1 hsdR17 supE44 thi-1 recA1 gyrA96 relA1Δ(arg-lacZYA)</i>	BRL
MT607	<i>pro-82 thi-1 hsdR17 supE44 recA56</i>	Finan <i>et al.</i> (1986)
MT616	MT607, pRK600	Finan <i>et al.</i> (1986)
MT620	MT607, Rf <sup>+</sup>	T. M. Finan

Table 1 (cont.)

Strain, plasmid or phage	Relevant characteristics	Reference
<b>Plasmids</b>		
pRK7813	IncP cosmid cloning vector, Tc <sup>r</sup>	Jones & Gutterson (1987)
pRmT103	pLAFR1, <i>R. meliloti</i> cosmid clone with <i>pckA</i>	Finan <i>et al.</i> (1988)
pTH141	pJB3JI R-prime, <i>pod-1</i> , $\Omega$ 5208::Tn5-233	This work
pTH142	pJB3JI R-prime, <i>pod-1</i> , $\Omega$ 5208::Tn5-233	This work
pTH143	pJB3JI R-prime (pTH141), <i>pod-6</i> ::Tn5	This work
pTH243	pRK7813, 10 kb <i>EcoRI</i> fragment from pTH141 with <i>pod-1</i>	This work
pTH244	pRK7813, 10 kb <i>EcoRI</i> fragment from pTH141 with <i>pod-1</i>	This work
pTH245	pUC118, 10 kb <i>EcoRI</i> fragment from pTH141 with <i>pod-1</i>	This work
pTH246	pUC118, 10 kb <i>EcoRI</i> fragment from pTH141 with <i>pod-1</i>	This work
pTH247	TnV with flanking 2 kb <i>BamHI</i> fragment from RmH243	This work
<b>Phage</b>		
$\phi$ M12	<i>R. meliloti</i> transducing phage	Finan <i>et al.</i> (1984)

\* The second-site mutation in RmF361 was previously designated *spk-1*; here we change the designation to *pod-1*.

*meliloti*, *Rhizobium* sp. NGR234 and in many other bacteria, the first step in gluconeogenesis is the conversion of oxaloacetate to PEP by the enzyme phosphoenolpyruvate carboxykinase (PCK) (Finan *et al.*, 1988; McKay *et al.*, 1985; Østerås *et al.*, 1991). Mutants of *R. meliloti* which lack PCK activity grow poorly on minimal media containing succinate, or other tricarboxylic acid (TCA) cycle intermediates, as sole carbon source (Finan *et al.*, 1988). In previous work, we used an *R. meliloti pckA* mutant which grows normally on succinate because of a second-site mutation to identify mutants lacking NAD<sup>+</sup>-dependent malic enzyme (Driscoll & Finan, 1993). Here, we characterize *pckA* second-site suppressor mutations and show that these mutations result in increased PPDK activity. Transposon insertions which eliminate the suppressor phenotype and map to the suppressor locus are shown to be located within the PPDK gene (designated *pod*). Thus, in the *pckA* suppressor strain, gluconeogenesis from malate appears to proceed via the combined activities of malic enzyme and PPDK.

The gene encoding PPDK has been isolated from several plant species (Matsuoka, 1990; Rosche & Westhoff, 1990; Rosche *et al.*, 1994; Matsuoka, 1995; Usami *et al.*, 1995), two protozoa (Bruchhaus & Tannich, 1993; Nevalainen *et al.*, 1996; Saavedra-Lira & Pérez-Montfort, 1994) and one bacterium, *Bacteroides symbiosus* (Pocalyko *et al.*, 1990). The primary structure of the protein is well conserved, and shows homology with Enzyme I of the PEP:carbohydrate phospho-

transferase system (PTS) (Pocalyko *et al.*, 1990; Matsuoka, 1995).

The reaction catalysed by PEP synthase (PPS, EC 2.7.9.2), which converts pyruvate and ATP to PEP, AMP and P<sub>i</sub>, is analogous to that catalysed by PPDK, except that PPS does not require P<sub>i</sub> to synthesize PEP (Cooper & Kornberg, 1967). The genes encoding PPS from *Escherichia coli* and the archaeobacterium *Pyrococcus furiosus* have recently been characterized and both of the deduced PPS proteins contain regions homologous to PPDK and Enzyme I of the PTS system (Jones *et al.*, 1995; Niersbach *et al.*, 1992; Reizer *et al.*, 1993; Robinson & Schreier, 1994).

## METHODS

**Bacterial strains, plasmids and media.** Bacterial strains and plasmids are listed in Table 1. Luria-Bertani (LB) medium was used for *E. coli*, LBmc (LB supplemented with 2.5 mM MgSO<sub>4</sub> and 2.5 mM CaCl<sub>2</sub>) for *R. meliloti*, and TY (Beringer *et al.*, 1978) for *Rhizobium* sp. NGR234. M9 (Miller, 1972) was used as defined medium for all strains. When required, antibiotics were added at concentrations previously described (Finan *et al.*, 1986).

**Genetic techniques.** Bacterial matings,  $\phi$ M12 transductions, transposon mutagenesis and transposon replacements were performed as previously described (Finan *et al.*, 1984, 1986). Revertants were isolated by spreading approximately 10<sup>8</sup> cells of the Pck<sup>-</sup> mutant on succinate minimal medium. Isolation of Tn5-233 transposon insertions linked to the suppressor allele (*pod-1*) was performed as previously described (Oresnik *et al.*,

1994), with screening for lack of growth on M9-succinate after  $\phi$ M12 transduction of the insertion bank into RmF361. Genetic mapping using Tn5-*mob* followed procedures previously described (Finan *et al.*, 1988; Klein *et al.*, 1992). The mobilizing plasmid pGMI102 was transferred by conjugation into strains RmF914 ( $\Omega$ S208::Tn5-233) and RmG566 (*pod*-5::Tn5-233), and then the seven Tn5-*mob* insertions were transduced into each of the resulting strains.

Construction of R-prime plasmids was based on the ability of R68.45 to mobilize the DNA of the host strain at high frequency (Riess *et al.*, 1980). The R68.45 derivative pJB3JI was transferred into RmF914, and the resulting strain was used as donor in conjugational matings with *E. coli* MT620 as recipient. Transconjugants were selected on LB with rifampicin (20  $\mu$ g ml<sup>-1</sup>) and spectinomycin (50  $\mu$ g ml<sup>-1</sup>) for isolation of plasmids containing the transposon Tn5-233 (Gm<sup>r</sup> Sp<sup>r</sup>). Identification of R-prime plasmids carrying large fragments of *R. meliloti* DNA was done by size comparison on agarose gels.

Recombinants in which the *pod*-6::Tn5 allele was transferred to the R-prime via homologous recombination were identified as Km<sup>r</sup> Rf<sup>r</sup> transconjugants, following a mating between RmG416(pTH141) and *E. coli* MT620. The recombination event was confirmed by screening transconjugants for loss of Gm<sup>r</sup> Sp<sup>r</sup> encoded by the  $\Omega$ S208::Tn5-233 insertion. The R-prime plasmid isolated was designated as pTH143.

**DNA manipulations and sequencing.** Standard methods were used for plasmid DNA isolation, restriction analysis, agarose and polyacrylamide gel electrophoresis, Southern blot, DNA ligation and transformation (Sambrook *et al.*, 1989). Bacterial genomic DNA was isolated by the method described previously for *R. meliloti* (Østerås *et al.*, 1995). Hybridizations were performed with digoxigenin-labelled probe (Boehringer Mannheim). Unbound probe was removed by washing the filters twice at room temperature for 15 min with 5 $\times$ SSC, 0.1% SDS, followed by two washes for 15 min at 65 °C with 0.1 $\times$ SSC, 0.1% SDS. DNA sequencing techniques were as previously described (Østerås *et al.*, 1995). Nucleotide sequences were analysed using BLAST (Altschul *et al.*, 1990) and CLUSTALV (Higgins *et al.*, 1992).

The region flanking the *pod*-6::Tn5 insertion was subcloned from the R-prime pTH143 as a Km<sup>r</sup> *Bam*HI fragment into pUC118. The nucleotide sequence from the IS50 in the resulting plasmid was determined using a primer (5'-TCACATGGAAGTCAGATCCT-3') specific to the IS50 of Tn5 (see arrow labelled a in Fig. 2).

The *pod*-5::TnV insertion together with flanking DNA was cloned as the plasmid pTH247. To isolate this plasmid, *Bam*HI-digested genomic DNA from RmH247 was diluted, self-ligated and transformed into *E. coli* with selection for Km<sup>r</sup> [TnV lacks a *Bam*HI site and contains the pSC101 origin of replication (Furuichi *et al.*, 1985)]. A DNA fragment, from the *Hind*III site of the IS50 to a *Hind*III site in the genomic DNA, was subcloned from pTH247 into pUC119. The IS50-specific primer was used to obtain the nucleotide sequence indicated by arrow c in Fig. 2 from the resulting plasmid. An additional *Bam*HI-*Xho*I fragment from pTH247 was subcloned into pUC119. The nucleotide sequence from the *Bam*HI site of the resulting plasmid was determined using the universal -20 primer. This sequence is indicated by arrow b in Fig. 2.

**Biochemical techniques.** Cell growth and the preparation of cell-free sonicated extracts was performed as described previously (Finan *et al.*, 1988). Malate dehydrogenase (MDH), PCK and PPS activities were measured as described by Cooper

& Kornberg, (1967), Englard & Seigal (1969) and Hansen *et al.* (1976). As PPK from some sources has been reported to be cold-labile (Evans & Wood, 1971; Edwards *et al.*, 1985), cell extracts were prepared at 15 °C and kept at room temperature prior to assay. PPK activity, in the direction of PEP formation from pyruvate, was assayed by measuring the rate of NADH oxidation at 340 nm (Uvikon 930 spectrophotometer) in a coupled assay containing excess PEP carboxylase and MDH. The assay mixture contained 100  $\mu$ mol NaHCO<sub>3</sub>, 200  $\mu$ mol imidazole pH 6.6, 4  $\mu$ mol glutathione, 6 units MDH (0.5  $\mu$ g, Boehringer Mannheim), 0.5 units PEP carboxylase (Boehringer Mannheim), 0.2  $\mu$ mol NADH, 2  $\mu$ mol EDTA, 20  $\mu$ mol MgCl<sub>2</sub>, 20  $\mu$ mol NH<sub>4</sub>Cl, 10  $\mu$ mol sodium pyruvate, 20  $\mu$ mol ATP in a final volume of 2 ml. After addition of the extract, the background NADH oxidase and pyruvate carboxylase (PYC) activities were measured. The reaction was initiated by addition of 10  $\mu$ mol potassium phosphate pH 7. PPK activities were corrected for the background PYC activities in the extracts. PPK activity in the direction of pyruvate formation from PEP was determined by measuring the rate of NADH oxidation in a coupled assay containing excess lactate dehydrogenase (LDH). The assay mixture contained 200  $\mu$ mol imidazole pH 6.6, 4  $\mu$ mol glutathione, 0.2  $\mu$ mol NADH, 2  $\mu$ mol EDTA, 2.5  $\mu$ mol AMP, 20  $\mu$ mol MgCl<sub>2</sub>, 20  $\mu$ mol NH<sub>4</sub>Cl, 5 units LDH (Boehringer Mannheim), 5  $\mu$ mol PEP in a final volume of 2 ml. The background NADH oxidase was measured after adding the crude extract and the reaction was initiated by the addition of 10  $\mu$ mol pyrophosphate pH 7.

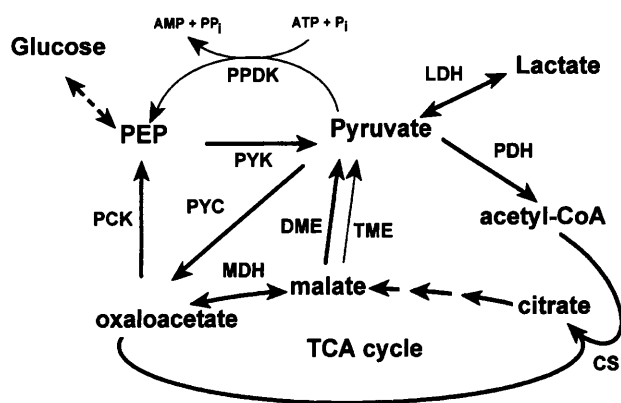
The protein concentration of the cell extracts was determined by the method of Bradford (1976) using the Bio-Rad protein assay (Coomassie Brilliant Blue G250) with BSA as a standard.

## RESULTS AND DISCUSSION

### Isolation and manipulation of *pckA* suppressor mutations

A schematic representation of the metabolic pathways and enzymic reactions referred to in this paper is shown in Fig. 1. *R. meliloti* Pck<sup>-</sup> mutants grow poorly on succinate and other TCA cycle intermediates as sole carbon sources (Finan *et al.*, 1988). While isolating revertants of *pckA* mutants, we identified four independent pseudorevertant strains, RmF361, RmG115, RmG116 and RmG117, which grew as well as the wild-type on succinate and other TCA cycle intermediates. The four pseudorevertant strains retained the antibiotic resistance marker of the *pckA* transposon insertion and extracts of these strains were found to lack PCK activity (data not shown). As the four second-site mutations appear to map to the *pod* locus (see below), we have designated the suppressor mutations in the four pseudorevertant strains RmF361, RmG115, RmG116 and RmG117, as *pod*-1, *pod*-2, *pod*-3 and *pod*-4, respectively (see Table 1). Strain RmF361 was previously employed during the isolation of NAD<sup>+</sup>-dependent malic enzyme mutants of *R. meliloti*; however the nature of the suppressor mutation was not examined (Driscoll & Finan, 1993).

Tn5-233 (Gm<sup>r</sup> Sp<sup>r</sup>) insertions linked in transduction to *pod*-1 were identified following phage  $\phi$ M12 transduction of a random *R. meliloti* Tn5-233 insertion bank into the suppressor strain RmF361. Gm<sup>r</sup> Sp<sup>r</sup> transductants



**Fig. 1.** Metabolic pathways in *R. meliloti*. CS, citrate synthase; DME, NAD<sup>+</sup>-dependent malic enzyme; LDH, lactate dehydrogenase; MDH, malate dehydrogenase; PCK, phosphoenolpyruvate carboxykinase; PDH, pyruvate dehydrogenase; PPK, pyruvate orthophosphate dikinase; PYC, pyruvate carboxylase; PYK, pyruvate kinase; TME, NAD<sup>+</sup>-dependent malic enzyme. Note that the TCA cycle reactions between citrate and malate are summarized by three arrows.

were screened for failure to grow on M9-succinate, and one such transductant, RmF871, carrying the Tn5-233 insertion designated  $\Omega 5208$  was used in further experiments. When Gm<sup>r</sup> Sp<sup>r</sup> was transduced from RmF871 into RmF361, with selection on LB (Gm Sp) medium, 55 of 95 of the resulting transductants failed to grow on M9-succinate. Thus,  $\Omega 5208::Tn5-233$  was 58% linked to *pod-1*. In similar crosses  $\Omega 5208::Tn5-233$  was found to be 54%, 69%, and 76% linked respectively to the suppressor mutations in strains RmG115, RmG116 and RmG117. Although more precise three-factor crosses were not done, the results suggested that the *pod-1*, *pod-2*, *pod-3* and *pod-4* suppressor alleles map to the same locus.

In the above crosses, when Gm<sup>r</sup> Sp<sup>r</sup> was transduced from RmF871 ( $\Omega 5208::Tn5-233$ ) into strains carrying *pckA::TnV* (Nm<sup>r</sup>), all of the transductants were Nm<sup>r</sup>, which showed that the suppressor mutations were extragenic with respect to *pckA* (see Fig. 2). Using transduction, we further constructed strains carrying *pod-1* and the *pckA* alleles *pckA2::Tn5-VB32* and *pckA3::Tn3HoHoSp*. These strains grew on succinate demonstrating that *pod-1* suppression was not *pckA*-allele-specific. We also transduced *pod-1* from RmF914 ( $\Omega 5208::Tn5-233$ , *pod-1*) into strains RmF331, Rm5418 and Rm5438 which lack genes encoding 3-phosphoglycerate kinase (*pgk*), enolase (*eno*), and glyceraldehyde-3-phosphate dehydrogenase (*gap*), respectively and fail to grow on M9-succinate (Finan *et al.*, 1988). None of the resulting transductants grew on succinate, which demonstrated that *pod-1* was not a general suppressor of gluconeogenic mutations but rather appeared to be *pckA*-specific.

#### Isolation of *pod::Tn5* insertion mutants

Four thousand Tn5 insertion mutants of the *pckA3::Tn3HoHoSp pod-1* double mutant, RmG243, were screened for reduced growth on M9-succinate. Among 30 such mutants, three carried insertions in the NAD<sup>+</sup>-dependent malic enzyme gene (*dme*), and others were identified as defective in C<sub>4</sub>-dicarboxylate transport, and glyceraldehyde-3-phosphate dehydrogenase or 3-phosphoglycerate kinase enzyme activities (see Driscoll & Finan, 1993). In addition to the mutants defective in

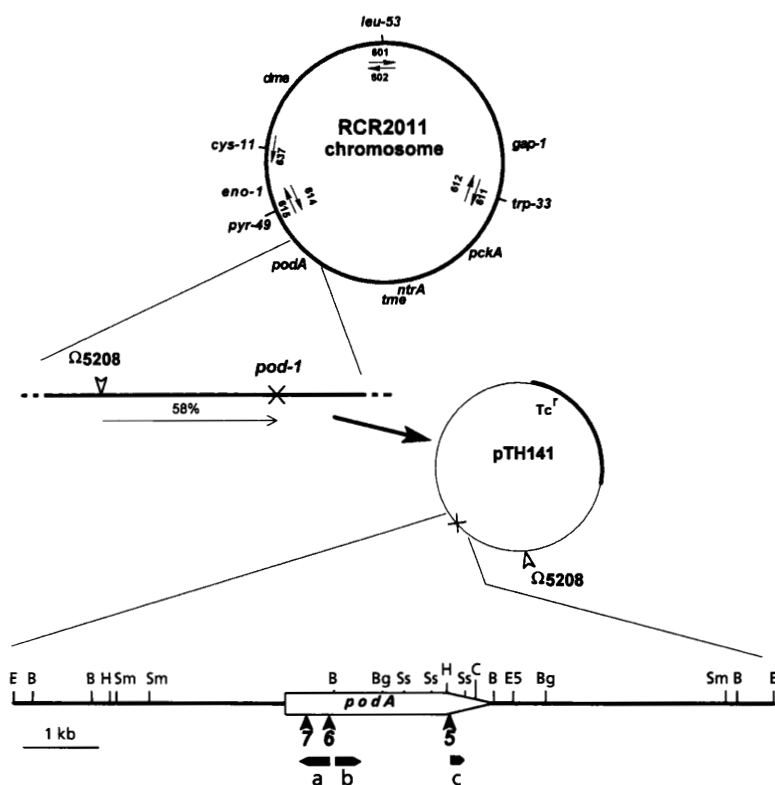
the known gluconeogenic enzymes, we also expected to isolate mutants in which Tn5 had disrupted the *pod-1* suppressor locus. In three of the mutants (RmG273, RmG316 and RmG420), the Nm<sup>r</sup> marker (encoded by Tn5) was on average 55% linked in transduction to the Gm<sup>r</sup> Sp<sup>r</sup> of  $\Omega 5208::Tn5-233$ . When Southern blots of total genomic DNA from these strains were hybridized to a Tn5-specific probe, the Tn5 insertions were all located within a 10 kb *EcoRI* fragment (Fig. 2). The positions of the transposon insertions in these strains were localized within the 10 kb fragment by Southern blot and restriction analysis of subclones (data not shown), and the insertions were designated *pod-5*, *pod-6* and *pod-7*.

#### Cloning of the *pod* suppressor allele

The *pod-1* suppressor allele was cloned by selecting for R-prime plasmids which carried genomic DNA contiguous with the Gm<sup>r</sup> Sp<sup>r</sup> insertion  $\Omega 5208::Tn5-233$ .  $\Omega 5208$  and *pod-1* are approximately 27 kb apart as deduced from the 58% linkage (Finan *et al.*, 1984). Two different R-prime plasmids, pTH141 and pTH142, which appeared to carry the *pod-1* locus were identified. These allowed the Pck<sup>-</sup> mutant Rm5439 to grow on succinate and restriction analysis revealed that, in addition to other fragments, both plasmids contained a 10 kb *EcoRI* fragment. This fragment was subcloned, in both orientations, into vector pRK7813 and the resulting plasmids (pTH245 and pTH246) both allowed the Pck<sup>-</sup> mutant Rm5065 to grow on succinate. Transfer of pTH141 into the *Rhizobium* sp. NGR234 *pckA1* mutant, and an *E. coli* Pck<sup>-</sup> Pps<sup>-</sup> double mutant HG4 (Goldie & Sanwal, 1980) allowed both these strains to grow on succinate. However transfer of pTH141 into the *E. coli* Pck<sup>-</sup> Dme<sup>-</sup> Tme<sup>-</sup> mutant EJ1321 (Hansen & Juni, 1975) did not allow this mutant to grow on succinate. This result confirmed our earlier finding that the *pod-1* gene product must work in concert with malic enzyme to suppress the *pckA* succinate-negative growth phenotype (Driscoll & Finan, 1993, 1996).

#### Molecular characterization of the *pod* locus

The nucleotide sequences from three regions of the *pod* locus were determined (see Methods and Fig. 2, *pod* regions a, b and c). GenBank searches with these



**Fig. 2.** Physical and genetic mapping of the *pod* locus. The location of *pod* is shown on the *R. meliloti* chromosome. Locations of *cys-11*, *pyr-49*, *trp-33*, *leu-53*, *gap-1*, *eno-1*, *ntrA* and *pckA* are as given by Finan *et al.* (1988) and Klein *et al.* (1992); that of *dme* as given by Driscoll & Finan (1993). Arrows with insertion numbers on the chromosome map indicate positions of Tn5-*mob* insertions. The arrowhead is the origin of transfer, and the arrow tail is the direction of transfer. The enlarged chromosome region shows the Tn5-233 insertion ( $\Omega$ 5208) linked to *pod* (with the frequency of transductional cotransfer on the arrow) which was used to isolate the R-prime (pTH141) containing *pod*. The bold region on the R-prime represents the pJB3JI vector DNA. The restriction map of the subcloned 10 kb *EcoRI* fragment is shown at the bottom, with the location and the orientation of the *pod* gene. The locations of the Tn5 insertions (5, 6 and 7) within *pod* are shown. The DNA fragments and the direction of sequencing are indicated by the arrows below the map labelled a, b and c. B, *Bam*HI; Bg, *Bgl*II; C, *Cl*I; E, *Eco*RI; E5, *Eco*RV; H, *Hind*III; Sm, *Sma*I; Ss, *Sst*I.

sequences revealed open reading frames which were very similar to PPDK proteins from other organisms. The three predicted amino acid sequences from *R. meliloti* aligned with the corresponding regions of the PPDK proteins from *B. symbiosus*, *Entamoeba histolytica*, *Flaveria trinervia*, *Mesembryanthemum crystallinum* and *Zea mays*, and these allowed the position and orientation of the *pod* gene within the 10 kb *EcoRI* fragment to be determined (Fig. 2). Alignment of the deduced amino acid sequences a, b and c with the corresponding sequences from the above PPDK proteins revealed that they were 37, 49, and 28% identical (multiple alignment not shown).

PPDK and two other enzymes which catalyse reactions between pyruvate and PEP, PPS and Enzyme I of the PTS, form a family of phosphohistidine proteins (Pocalyko *et al.*, 1990). These proteins have similar mechanisms of action, and several regions are conserved between their amino acid sequences (Pocalyko *et al.*, 1990; Niersbach *et al.*, 1992). *R. meliloti* sequences a and c (Fig. 2, alignment not shown), corresponding to residues 72–185 and 697–744 in *B. symbiosus*, were found to be outside the five regions common to PPDK, PPS and Enzyme I of the PTS (residues 207–224, 439–473, 552–566, 655–666 and 739–780). The last five residues of sequence c do overlap the 739–780 region (Niersbach *et al.*, 1992). *R. meliloti* sequence b includes the first region common to the phosphohistidine proteins (residues 207–224), but as in the case of the other PPDK proteins, the homology of sequence c to the *E. coli* PPS protein did not extend outside these 18 residues.

### Biochemical analysis of *pod* alleles

We determined the levels of PPDK activity in *R. meliloti* strains, using enzyme assays which measured PPDK activity in each physiological direction (see Methods). PPDK activity was detected in an assay of  $PP_i$ -dependent conversion of PEP to pyruvate, by coupling PPDK to LDH (Table 2). Extracts of the suppressor mutants RmG139 (*pod-1*), RmG115, RmG116 and RmG117 contained a higher level of PPDK activity than the wild-type (Rm1021) extract (Table 2). Conversely, no PPDK activity was detected in the extract of RmG274 (*pod-5::Tn5*) cells. The PPDK activity observed was shown to be strictly  $PP_i$ -dependent, as replacing  $PP_i$  with  $P_i$  in the assay eliminated nearly all detectable activity in RmG139 [ $P_i$   $0.9 \pm 0.5$  nmol  $\text{min}^{-1}$  (mg protein) $^{-1}$ ;  $PP_i$   $27.2 \pm 0.3$  nmol  $\text{min}^{-1}$  (mg protein) $^{-1}$ ].

Together, the data from the enzyme assays (Table 2) and the DNA sequence analysis suggest that the suppressor mutations result in increased activity of PPDK. Whether the increased enzyme activity is caused by mutations which activate the enzyme or increase *pod* gene transcription is not known. Three of the four suppressor strains showed a greater than fivefold increase in PPDK activity, but the level of PPDK activity in RmG115 was only twice the wild-type value (Table 2). This low activity could be due to an unstable mutant PPDK protein, or it may reflect the PPDK assay employed. When PPDK was assayed in the gluconeogenic direction, by following the  $P_i$ -dependent conversion of pyruvate to PEP, PPDK activity [ $3.5$  nmol  $\text{min}^{-1}$  (mg protein) $^{-1}$ ] was

**Table 2.** PPK and MDH activities detected in four independent *pod* alleles

Cells were grown in LBmc. Values are the means of triplicate measurements  $\pm$  standard error.

Strain	Relevant characteristics	Specific activity [nmol min <sup>-1</sup> (mg protein) <sup>-1</sup> ]	
		PPDK*	MDH
Rm1021	Wild-type <i>R. meliloti</i>	2.5 $\pm$ 0.2	596 $\pm$ 43
RmG139	<i>pod-1</i>	15.3 $\pm$ 0.9	ND
RmG115	<i>pckA1::Tn5-132 pod-2</i>	5.4 $\pm$ 0.8	498 $\pm$ 17
RmG116	<i>pckA1::TnV pod-3</i>	58.5 $\pm$ 1.6	514 $\pm$ 5
RmG117	<i>pckA1::TnV pod-4</i>	15.4 $\pm$ 1.1	657 $\pm$ 22
RmG274†	<i>pod5::Tn5</i>	0	ND

ND, Not determined.

\*PPDK activity was measured in the direction of pyruvate formation as described in Methods.

†PCK activity in this extract was 89 nmol min<sup>-1</sup> (mg protein)<sup>-1</sup>.

detected in the RmG139 (*pod-1*) mutant whereas no activity was detected in the wild-type.

We also assayed the *R. meliloti* mutant and suppressor extracts for PPS activity. None was detected in *R. meliloti* extracts under conditions where activity was readily detected in control *E. coli* extracts (data not shown).

### Symbiotic effects of *pod* alleles

When strain RmG274, bearing the *pod-5::Tn5* insertion in a wild-type genetic background, was inoculated onto alfalfa seedlings, no reduction in plant dry weight was observed after 28 d compared to plants inoculated with the wild-type strain (data not shown). The intact *pod* gene is therefore not required for N<sub>2</sub>-fixation by *R. meliloti*.

### Mapping of *pod* to the *R. meliloti* chromosome

Two Tn5-233 (Gm<sup>r</sup> Sp<sup>r</sup>) insertions in the *pod* gene region ( $\Omega$ 5208::Tn5-233 and *pod-5::Tn5-233*) were mapped by conjugation using a set of seven Hfr-like donor strains able to mobilize the *R. meliloti* chromosome (see Fig. 2; Finan *et al.*, 1988; Klein *et al.*, 1992). The 14 constructed donor strains were mated with Rm5000 (wild-type, Rf<sup>r</sup>), and Gm<sup>r</sup> Sp<sup>r</sup> Rf<sup>r</sup> transconjugants were selected. The results of the conjugations were expressed as number of transconjugants per 10<sup>8</sup> donor cells (Table 3). The *pod-5::Tn5-233* and  $\Omega$ 5208::Tn5-233 insertions both mapped in the region of the *R. meliloti* chromosome located between the markers *trp-33* and *pyr-49*, but closer to *pyr-49* (Fig. 2).

### Growth phenotype of strains carrying *pod* mutations

Since the *pod* suppressor mutations result in increased PPK activity, it was of interest to examine the growth phenotypes of various *pod*, *pckA*, *dme* and *tme* mutant derivatives with respect to the metabolic scheme outlined in Fig. 1. Thus, while the slow growth of the *pckA* mutant RmG242 on succinate, malate or pyruvate was restored to wild-type levels upon acquisition of the *pod-1* allele (RmG242 vs RmG243, Table 4), this slow growth was eliminated upon disruption of the *pod* gene (RmG273, *pckA pod-5::Tn5*). These results establish that the residual slow growth of Pck<sup>-</sup> mutants on succinate is due to the wild-type *pod* gene product, and that presumably the rate of conversion of pyruvate to PEP is increased in strains carrying the *pod-1* allele. The results did not reveal what role PPK normally plays in *R. meliloti* carbon metabolism as the disruption of *pod* in a wild-type background had no observed effect on growth (RmG274, Table 4).

The introduction of *dme* mutations, which eliminate NAD<sup>+</sup>-dependent malic enzyme activity, into the *pckA pod-1* suppressor strains severely reduced their ability to grow on succinate and malate (compare RmG243 with RmG443 and RmH188; Table 4). However, transfer of the *tme-4* mutation, which eliminates NADP<sup>+</sup>-dependent malic enzyme activity, into the *pckA pod-1* strains,

**Table 3.** Conjugal mapping of the *pod* gene

Conjugal matings were performed as described in Methods. The recipient strain was Rm5000. The donor strains were derivatives of RmF914 (Rm5065,  $\Omega$ 5208::Tn5-233, 62% linked to *pod-1*) and RmG566 (Rm1021, *pod-5::Tn5-233*, a Tn5-233 replacement of the *pod-5::Tn5* insertion), carrying the indicated Tn5-*mob* insertions and the mobilizing plasmid pGMI102. The plus and minus signs indicate transfer of DNA clockwise and counterclockwise, respectively, from the Tn5-*mob* insertion, relative to the map of the *R. meliloti* chromosome (Fig. 2). Recombinants were selected for Gm<sup>r</sup> Sp<sup>r</sup> Rf<sup>r</sup>. Donor cells were selected for Sm<sup>r</sup> Nm<sup>r</sup>.

Donor marker	Number of transconjugants per 10 <sup>8</sup> donor cells						
	$\Omega$ 601 (+)	$\Omega$ 602 (-)	$\Omega$ 611 (+)	$\Omega$ 612 (-)	$\Omega$ 614 (+)	$\Omega$ 615 (-)	$\Omega$ 637 (+)
<i>pod-5::Tn5-233</i>	15	0	325	21	3	6341	10
$\Omega$ 5208::Tn5-233	1	33	520	11	11	9341	10

**Table 4.** Growth of bacterial strains on minimal media with different carbon sources

Strains were streaked for single colonies on plates of minimal media containing the indicated carbon source (see Methods). Growth of the strains was scored after 4–6 d relative to that of the wild-type strain Rm1021 on succinate. ++, growth equal to the wild-type Rm1021; +, less than optimal growth, or less growth than the wild-type; +/-, leaky growth; -, no (or very poor) growth.

Strain	Relevant characteristics	Carbon source:*						
		Suc	Mal	Lac	Pyr	Ace	Hba	Glc
Rm1021	Wild-type	++	++	+	+	+	+	++
RmG242	<i>pckA3</i>	+/-	+/-	-**	+/-	-	+/-	++
RmG243	<i>pckA3 pod-1</i>	++	++	+	+	+	+	++
RmG273	<i>pckA3 pod-5::Tn5</i>	-	-	-	+/-	-	-	++
RmG274	<i>pod-5::Tn5</i>	++	++	+	+	+	+	++
RmG443	<i>pckA3 pod-1 dme-2</i>	+/-	+/-	+	+	+/-	+	++
RmH187	<i>pckA1 pod-1 tme-4</i>	+	ND	+	ND	+	ND	++
RmH188	<i>pckA1 pod-1 dme-1</i>	+/-	ND	+	ND	+	ND	++
RmH194	<i>pck1 pod-1 dme-1 tme-4</i>	-	ND	+	ND	-	ND	++

ND, Not determined

\* Suc, succinate; Mal, malate; Lac, lactate; Pyr, pyruvate; Ace, acetate; Hba,  $\beta$ -hydroxybutyrate; Glc, glucose.

\*\* RmG242 was slightly leaky on lactate, but not enough to be classified as +/- . RmG273 was not leaky.

had little observable effect on growth (see RmH187, Table 4), while *pckA pod-1* suppressor strains which lack both malic enzymes were completely unable to grow on M9-succinate (RmH194). These results indicate that in the *pckA pod-1* suppressor strains, the conversion of malate to pyruvate is primarily catalysed by the NAD<sup>+</sup>-dependent malic enzyme (Fig. 1). The growth of both the *pckA pod-1 dme* triple mutant and the *pckA pod-1 dme tme* quadruple mutant strains on lactate but not succinate is also consistent with the proposed role of the *pod-1* suppressor gene product in converting pyruvate to PEP (see Fig. 1).

The low PPDK activity detected in wild-type cells is evidently sufficient to allow *R. meliloti* mutants which lack PCK activity to grow slowly on succinate. In contrast, Pck<sup>-</sup> mutants of *R. leguminosarum* and *Rhizobium* sp. NGR234 do not grow at all on carbon sources which require gluconeogenesis (McKay *et al.*, 1985; Østerås *et al.*, 1991). In this respect, it is interesting that when a *Bam*H1 restriction fragment internal to the predicted *R. meliloti pod* gene was used as a probe, we detected strong hybridization to Southern blots of DNA from *Rhizobium* sp. NGR234 and other *R. meliloti* strains (data not shown). This result, combined with the succinate-negative growth phenotype of *Rhizobium* sp. NGR234 Pck<sup>-</sup> mutants, suggests the *pod* gene of NGR234 is not expressed in cells grown in minimal medium with succinate as sole carbon source.

In summary, our results indicate that the combined activities of malic enzyme and PPDK constitute a gluconeogenic pathway independent of PCK. Indeed, the ineffectiveness of this pathway in wild-type cells

(Pod<sup>+</sup>) may be a regulatory design for channelling the bulk of gluconeogenesis through PCK, which is known to be regulated at the transcriptional level (Finan *et al.*, 1988; Østerås *et al.*, 1995). It may be advantageous to have a secondary, low-flux, gluconeogenic (anapleurotic) pathway to maintain the balance of intermediary metabolites. As PPDK is known to replace pyruvate kinase in some organisms, a possible function for PPDK in *R. meliloti* growing under glycolytic conditions also cannot be ruled out. The existence of two routes for the synthesis of PEP from C<sub>4</sub>-dicarboxylates is not limited to *R. meliloti* as, for example, in *E. coli*, PEP synthesis can be catalysed by PCK or by the combined activities of the malic enzymes and PPS (Cooper & Kornberg, 1967; Goldi & Sanwal, 1980; Hansen & Juni, 1974).

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