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

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INTRODUCTION

Pyruvate orthophosphate dikinase (PPDK, EC 2.7.9.1) catalyses the reversible reaction: pyruvate + ATP + P → phosphoenolpyruvate (PEP) + AMP + PP, (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 & Ettlinger, 1979).

In C₃ plants, PPDK catalyses the regeneration of PEP, the primary acceptor of CO₂ in the C₃ 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 N₂-fixing root nodules on alfalfa. There is much evidence that within nodules, the plant supplies these bacteria with C₃-dicarboxylic acids, such as malate, as their principal source of energy for the N₂-fixation process. In *Rhizobium leguminosarum*, R.
Table 1. Bacterial strains and plasmids used in this study

<table>
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<th>Strain, plasmid or phage</th>
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<td>T. M. Finan</td>
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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 P₇, is analogous to that catalysed by PPDK, except that PPS does not require P₇ 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 MgSO₄ and 2.5 mM CaCl₂) 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, *φM12* transductions, transposon mutagenesis and transposon replacements were performed as previously described (Finan et al., 1984, 1986). Revertants were isolated by spreading approximately 10⁶ cells of the *pckA* 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., 1984).

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* The second-site mutation in RmF361 was previously designated *spk-1*; here we change the designation to *pod-1*.
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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 pGM102 was transferred by conjugation into strains RmF914 (Ω5208::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 pJB3J1 was transferred into RmF914, and the resulting strain was used as donor in conjugal matings with E. coli MT620 as recipient. Transconjugants were selected on LB with rifampicin (20 \( \mu \)g ml\(^{-1} \)) and spectinomycin (50 \( \mu \)g ml\(^{-1} \)) for isolation of plasmids containing the transposon Tn5-233 (Gm\(^{+}\) Sp\(^{+}\)). 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\(^{+}\) R\(^{+}\) transconjugants, following a mating between RmG416(pTH141) and E. coli MT620. The recombination event was confirmed by screening transconjugants for loss of Gm\(^{+}\) Sp\(^{+}\) encoded by the \( \Omega 5208::Tn5-233 \) 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 1 x SSC, 0.1% SDS, followed by two washes for 15 min at 65 \(^{\circ}\)C with 0.1 x 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\(^{+}\) BamHI 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, BamHI-digested genomic DNA from RmH247 was diluted, self-ligated and transformed into E. coli with selection for Km\(^{+}\). TnV lacks a BamHI site and contains the pSC101 origin of replication (Furuchi et al., 1985). A DNA fragment, from the HindIII site of the IS50 to a HindIII 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 BamHI-Xhol fragment from pTH247 was subcloned into pUC119. The nucleotide sequence from the BamHI 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 & Seigel (1969) and Hansen et al. (1976). As PPDFK from some sources has been reported to be cold-labile (Evans & Wood, 1971; Edwards et al., 1985), cell extracts were prepared at 15 \(^{\circ}\)C and kept at room temperature prior to assay. PPDFK 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\(_3\), 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 Mg\(_2\)Cl\(_2\), 20 \( \mu \)mol NH\(_4\)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 oxidation and pyruvate carboxylase (PYC) activities were measured. The reaction was initiated by addition of 10 \( \mu \)mol potassium phosphate pH 7. PPDFK activities were corrected for the background PYC activities in the extracts. PPDFK 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 Mg\(_2\)Cl\(_2\), 20 \( \mu \)mol NH\(_4\)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\(^{-}\) 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\(^{+}\)-dependent malic enzyme mutants of R. meliloti; however the nature of the suppressor mutation was not examined (Driscoll & Finan, 1993).

Tn5-233 (Gm\(^{+}\) Sp\(^{+}\)) 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\(^{+}\) Sp\(^{+}\) transductants

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were screened for failure to grow on M9-succinate, and
one such transductant, RmF871, carrying the Tn5-233
insertion designated О5208 was used in further exper-
iments. When GmN SpY 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, О5208::Tn5-233 was 58% linked to
pod-1. In similar crosses О5208::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 GmN SpY was transduced from
RmF871 (О5208::Tn5-233) into strains carrying
pсkA::TnV (NmY), all of the transductants were NmY,
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
(О5208::Tn5-233, pod-1) into strains RmF331, RmS418
and RmS438 which lack genes encoding 3-phospho-
glycerate kinase (pgk), enolase (eno), and glyceralde-
hyde-3-phosphate dehydrogenase (gap), respect-
ively 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::
RmJHoHoSp pod-1 double mutant, RmG243, were
screened for reduced growth on M9-succinate. Among
30 such mutants, three carried insertions in the NAD+-
dependent malic enzyme gene (dme), and others were
identified as defective in C2-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 NmY marker (encoded by
Tn5) was on average 55% linked in transduction to the
GmN SpY of О5208::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 con-
tiguous with the GmN SpY insertion О5208::Tn5-233.
О5208 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 identi-
fied. These allowed the Pck- 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-
mutant Rm5065 to grow on succinate. Transfer of
pTH141 into the Rhizobium sp. NGR234 pckA1 mu-
tant, and an E. coli Pck- Pps- double mutant HG4
(Goldie & Sanwal, 1980) allowed both these strains to
grow on succinate. However transfer of pTH141 into the
E. coli Pck- Dme- Tme- 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

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
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 PPi-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 PPi-dependent, as replacing PPi with Pi in the assay eliminated nearly all detectable activity in RmG115 [Pi 0.9±0.5 nmol min⁻¹ (mg protein)⁻¹; PPi 0.3±0.1 nmol min⁻¹ (mg protein)⁻¹]. This low activity could be due to an unstable mutant PPDK protein, or it may reflect the PPDK assay employed.

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, following the Pi-dependent conversion of pyruvate to PEP, PPDK activity [3.5 nmol min⁻¹ (mg protein)⁻¹] was
mapping of the pod gene

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 N₂ fixation by R. meliloti.

**Symbiotic effects of pod alleles**

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).

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

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant characteristics</th>
<th>Specific activity [nmol min⁻¹ (mg protein)⁻¹]</th>
<th>PPDK*</th>
<th>MDH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rm1021</td>
<td>Wild-type R. meliloti</td>
<td>2.5 ± 0.2</td>
<td>596 ± 43</td>
<td></td>
</tr>
<tr>
<td>RmG139 pod-1</td>
<td></td>
<td>15.3 ± 0.9</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>RmG115 pckA::Tn5-132 pod-2</td>
<td></td>
<td>15.4 ± 0.8</td>
<td>498 ± 17</td>
<td></td>
</tr>
<tr>
<td>RmG116 pckA::TnV pod-3</td>
<td></td>
<td>58.5 ± 1.6</td>
<td>514 ± 5</td>
<td></td>
</tr>
<tr>
<td>RmG117 pckA::TnV pod-4</td>
<td></td>
<td>15.4 ± 1.1</td>
<td>657 ± 22</td>
<td></td>
</tr>
<tr>
<td>RmG274† pod-5::Tn5</td>
<td></td>
<td>0</td>
<td>ND</td>
<td></td>
</tr>
</tbody>
</table>

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

*PCK activity in this extract was 89 nmol min⁻¹ (mg protein⁻¹).

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

Two Tn5-233 (Gm’ Sp’) insertions in the pod gene region (Ω5208::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’), and Gm’ Sp’ RF’ transconjugants were selected. The results of the conjugations were expressed as number of transconjugants per 10⁸ donor cells (Table 3). The pod-5::Tn5-233 and Ω5208::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 PPDK 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⁻ 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 PPDK 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⁺-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⁺-dependent malic enzyme activity, into the pckA pod-1 strains,
Table 4. Growth of bacterial strains on minimal media with different carbon sources

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

ND, Not determined

* Suc, succinate; Mal, malate; Lac, lactate; Pyr, pyruvate; Ace, acetate; Hba, β-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+-dependent malic enzyme (Fig. 1). The growth of both the pckA pod-1 dme-1 triple mutant and the pckA pod-1 dme tame 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- mutants of R. leguminosarum and Rhizobium sp. NGR234 do not grow at all on carbon sources which require gluconeogenesis (McKay et al., 1985; Østérás et al., 1991). In this respect, it is interesting that when a BamH1 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- 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+) 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; Østérás 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 C4-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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