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Complex Symbiotic Phenotypes Result from Gluconeogenic Mutations in *Rhizobium meliloti*

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Mutants of *Rhizobium meliloti* defective in one of the following enzyme activities have previously been reported: phosphoenolpyruvate carboxykinase (Pck^-), enolase (Eno^-), glyceraldehyde-3-phosphate dehydrogenase (Gap^-), and 3-phosphoglycerate kinase (Pgk^-) (Finan, Oresnik, and Bottacin. J. Bacteriol. 170:3396-3403). Here we report the symbiotic N_2-fixing activities (acetylene reduction and plant dry weight) and structure of alfalfa nodules induced by these gluconeogenic mutants. In addition, we have determined the activities of the above enzymes in extracts from wild-type bacteroids isolated from alfalfa nodules. The Gap^-, Eno^-, and Pgk^- mutants formed small white Fix^- nodules with acetylene-reducing activities of less than 5% of wild type. Each of these mutants induced nodules with a similar structure; the nodules lacked a distinct symbiotic zone and had relatively few infected cells. Plants inoculated with the Pck^- mutants had acetylene-reducing activities of approximately 60% of plants inoculated with the wild-type bacteria. No significant abnormalities in the ultrastructure of nodules induced by the Pck^- mutants were found. Paradoxically, no phosphoenolpyruvate carboxykinase activity was detectable in wild-type bacteroid extracts, indicating that gluconeogenesis does not occur in bacteroids. A possible role for phosphoenolpyruvate carboxykinase in controlling bacteroid proliferation is suggested.

Additional keywords: dicarboxylates, microscopy, symbiotic nitrogen fixation.

Leguminous root nodules are the result of a complex series of interactions between bacteria of the genus *Rhizobium* and their host plants. The differentiation of the bacteria from free-living to N_2-fixing cells called bacteroids is one of the last steps in the formation of effective root nodules. As N_2 fixation is a process requiring high energy, a continuous supply of carbohydrate to the nodules is required to maintain it at high rates. Much evidence suggests that photosynthate translocated to the nodules is metabolized to C_4-dicarboxylates (succinate, fumarate, and malate) and that these compounds are taken up and metabolized by the bacteroids. Bacterial mutants defective in α-ketoglutarate dehydrogenase and succinate dehydrogenase, respectively, form ineffective nodules (Duncan and Fraenkel 1979; Gardiol *et al.* 1982). Moreover, mutants that fail to transport C_4-dicarboxylates (Dct^-) as bacteroids fail to fix nitrogen (Ronson *et al.* 1981; Arwas *et al.* 1985; Bolton *et al.* 1986; Engelke *et al.* 1987; Finan *et al.* 1983; Watson *et al.* 1988; Yarosh *et al.* 1989). Early in development, the nodules induced by these Dct^- mutants are structurally similar to wild-type nodules. Bacteria surrounded by a plant-derived membrane (peribacteroid membrane) are released from the infection thread into the host plant cell cytoplasm and are similar to N_2-fixing bacteroids. Subsequently, bacteroid-containing cells senesce rapidly such that the nodules contain a narrow symbiotic zone and a large senescent zone. Because bacteroids initially survive in the infection threads and proliferate within the host cells, C_4-dicarboxylates do not appear to be required as an energy source by the bacteria during the early stages of infection. In contrast to C_4-dicarboxylate transport mutants, defined bacterial mutants that are defective in various steps in hexose or pentose metabolism generally form normal N_2-fixing nodules (Cervenansky and Arias 1984; Ronson and Primrose 1979; Glenn *et al.* 1984a; Glenn *et al.* 1984b; but see Arias *et al.* 1979, and Duncan 1981).

In addition to mutant analysis, there have been a few reports in which the metabolic status of N_2-fixing bacteroids has been examined by assaying bacteroid extracts for the activities of enzymes involved in carbon metabolism (Saroso *et al.* 1986; McKay *et al.* 1989). In general, these studies have shown that bacteroids have low activity levels for glycolytic enzymes and high levels of tricarboxylic acid cycle enzymes.

We have previously described mutants of *Rhizobium meliloti* Dangeard, each of which lack one of the following enzyme activities: phosphoenolpyruvate carboxykinase (Pck^-), enolase (Eno^-), glyceraldehyde-3-phosphate dehydrogenase (Gap^-), and 3-phosphoglycerate kinase (Pgk^-) (Finan *et al.* 1988). The phenotypes of these mutants coupled with enzyme analysis showed that gluconeogenesis in free-living cells of *R. meliloti* occurs via phosphoenolpyruvate carboxykinase and the enzymes of the lower half of the Embden-Meyerhoff-Parnas pathway. Here we have examined the role of these enzymes in root nodule development by determining the nodule structure and nitrogen-fixing activities of plants inoculated with these mutants. In addition, we have determined the activities of the enzymes in extracts of wild-type bacteroids. The Eno^-, Gap^-,

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and Pgk$^-$ mutants formed Fix$^-$ nodules, whereas the Pck$^-$ mutants formed partially effective nodules since plants inoculated with these mutants had acetylene-reducing activities of approximately 60% of wild type. The latter result is interesting as no Pck enzyme activity was detectable in wild-type bacteroid extracts.

MATERIALS AND METHODS

Bacterial strains, culture techniques, media, and plant growth. Bacterial strains used in this study were isolated previously (Finan *et al.* 1988) and are listed in Table 1. Bacterial growth media (Luria-Bertani [LB] and M9) and antibiotic concentrations were as described by Yarosh *et al.* 1989. The sensitivity of *R. meliloti* strains to the bacteriophage ΦM1, 5, 7, 9, 10, 11, 12, and 14 was determined by spot tests as previously described (Johansen *et al.* 1984). Exopolysaccharide synthesis on LB agar containing 0.02% Calcofluor (Cellufluor, Polyscience, Warrington, PA) was visualized under UV light as previously described (Leigh *et al.* 1985).

Revertants of strains Rm5065, Rm5418, and Rm5438 were isolated by spreading 10^8 cells on M9-succinate (15 mM) medium. After three single-colony purifications, the revertants were screened for the parental antibiotic resistance markers.

Plant nodulation experiments in Leonard jars, containing a 1:1 mixture of quartz sand and vermiculite, and acetylene-reduction determinations were done as previously described (Yarosh *et al.* 1989). Plant dry weights were determined on plant tops cut at the stem-root interface. Plants were dried for at least 2 wk at 70° C before weight determination. To isolate bacteria from nodules, the nodules attached to a piece of root were first surface sterilized by immersing them in 1% sodium hypochlorite for 2 min. The hypochlorite was then removed by washing the nodules in LB medium and water. The bacteria and bacteroids were released from the nodule by squashing in a drop an M9 salts solution containing 0.3 M sucrose and plated on LB agar.

Biochemical techniques. Bacteroids were isolated from alfalfa root nodules (var. Iroquois) 3–4 wk after inoculation with *R. meliloti* strain Rm1021. Nodules were removed from the roots and transferred to an ice-chilled petri plate containing MMS (40 mM 3-(4-morpholino)-propane sulfonic acid, 20 mM KOH, 2 mM $MgSO_4$, 0.3 M sucrose, pH 7.0). Nodules in 1 ml of MMS were then crushed gently with a chilled pestle and mortar until a fine slurry was formed; all subsequent steps were carried out at 4° C. After adding a further 7 ml of MMS to the slurry the mixture was passed through eight layers of cheesecloth. The filtrate was then centrifuged at 70 × *g* for 5 min and the bacteroids were removed from the resulting supernatant fraction by centrifugation at 2,200 × *g* for 5 min. The pellet fraction was resuspended in MMS and again centrifuged at 2,200 × *g* for 5 min. The resulting pellet fraction was resuspended in buffer containing 20 mM Tris-HCl (pH 8), 1 mM $MgCl_2$ (4 ml of buffer per gram wet weight of bacteroid) and stored at −70° C until needed. Cell-free bacteroid extracts were prepared by sonication after adding dithiothreitol (0.1 mM) to thawed cells as previously described (Finan *et al.* 1988). After centrifugation at 500 × *g* for 15 min at 4° C, the supernatant fraction was assayed for glucose-6-phosphate dehydrogenase (Lessie and Vander Wyk 1972), 6-phosphogluconate dehydrogenase (Lessie and Vander Wyk 1972), isocitrate dehydrogenase (Reeves *et al.* 1971), and malate dehydrogenase (Englard and Seigal 1969). Fructose bisphosphatase (Lynch *et al.* 1975), phosphoenolpyruvate carboxykinase (Hansen *et al.* 1976), and enolase, glyceraldehyde-3-phosphate dehydrogenase, and 3-phosphoglycerate kinase activities (Maitra and Lobo 1971) were determined following the referenced procedures, using the supernatant fraction obtained after centrifugation of the bacteroid extracts for 15 min at 14,000 × *g* at 4° C. The combined activities of 6-phosphogluconate dehydratase and 2-keto-3-deoxy-6-phosphogluconate aldolase (Entner-Douderoff pathway) were assayed by measuring the amount of pyruvate formed from 6-phosphogluconate as previously described (Finan *et al.* 1988). Protein concentrations were determined with a Coomassie blue reagent (Bio-Rad Lab-

Table 1. Bacterial strains used in this study

Rhizobium meliloti strains	Phenotype[a]	Source
RCR2011	Wild type, also called SU47	Laboratory collection
Rm1021	SU47 *str21*	Laboratory collection
Rm5012	Rm1021 Eno-1::Tn*5*	Finan *et al.* 1988
Rm5065	Rm1021 Pck-1::Tn*5*-132	Finan *et al.* 1988
Rm5234	Rm1021 Pck-2::Tn*5*-VB32	Finan *et al.* 1988
Rm5418	Rm1021 Gap-1::Tn*5*	Finan *et al.* 1988
Rm5438	Rm1021 Eno-2::Tn*V*	Finan *et al.* 1988
Rm5439	Rm1021 Pck-1::Tn*V*	Finan *et al.* 1988
RmF331	Rm1021 Pgk-11::Tn*V*	Finan *et al.* 1988
RmF307	Rm1021 Nm^s Eno$^+$	Revertant from Rm5438
RmF308	Rm1021 Nm^s Eno$^+$	Revertant from Rm5438
RmF311	Rm1021 Nm^s Gap$^+$	Revertant from Rm5418
RmF312	Rm1021 Nm^s Gap$^+$	Revertant from Rm5418
RmF362	Rm1021 Ot^s Pck$^+$	Revertant from Rm5065
RmF363	Rm1021 Ot^s Pck$^+$	Revertant from Rm5065

[a] Phenotypic designations: Eno, enolase; Gap, glyceraldehyde-3-phosphate dehydrogenase; Pck, phosphoenolpyruvate carboxykinase; Pgk, 3-phosphoglycerate kinase; Nm^s, sensitivity to neomycin; Ot^s, sensitivity to oxytetracycline. Tn*5*-132, Tn*5*-VB32, Tn*V*, and Tn*5*-233 are Tn*5* derivatives (Berg and Berg 1987; Bellofatto *et al.* 1984; Furuichi *et al.* 1985; DeVos *et al.* 1986).

Table 2. Symbiotic phenotype of *Rhizobium meliloti* mutants

Strain	Phenotype	Acetylene reduced[a] (nmoles/hr/plant)	Shoot dry weight[b] (mg/plant)	(% wt)
Rm1021	Wild type	1,148 ± 237	16.3	100
Rm5012	Eno$^-$	14 ± 1	ND[c]	
Rm5065	Pck$^-$	852 ± 200	ND	
Rm5418	Gap$^-$	25 ± 5	6.1	38
Rm5438	Eno$^-$	85 ± 65	6.6	40
Rm5439	Pck$^-$	719 ± 176	9.3	57
RmF331	Pgk$^-$	38 ± 26	6.0	36
Rm5234	Pck$^-$	ND	9.6	60
Uninoculated		30 ± 10	6.1	38

[a] Plants were assayed for acetylene reduction 50 days after inoculation. Data are expressed as the means of triplicate assays ± standard deviations.

[b] The mean dry weight values averaged from at least eight plants harvested 24 days after inoculation.

[c] Not determined.

oratories, Richmond, CA) (Bradford 1976). Enzyme-specific activities were expressed as nanomoles of product formed per minute per milligram of protein.

Microscopy. Plants were grown in tubes on agar slopes containing nitrogen-free growth media prepared as described previously (Selvaraj *et al.* 1987). At weekly intervals, nodules were excised, sliced, and fixed for 3 hr in 3% (w/v) glutaraldehyde in 0.05 M cacodylate buffer (pH 7.2). They were then washed three times in cacodylate buffer and postfixed in 1% OsO_4 for 1.5 hr. The nodules were stained overnight in 2% uranyl acetate, then dehydrated through an increasing ethanol series. They were embedded in Epon 812 resin polymerized at 70° C for 24 hr. Light microscopy sections were stained with 0.01% toluidine blue and photographed through a yellow filter. Thin sections were examined with a Philips EM300 transmission electron microscope after being stained with saturated uranyl acetate and lead citrate.

RESULTS

The Pck$^-$, Eno$^-$, Pgk$^-$, and Gap$^-$ mutants of *R. meliloti* used in this study (Table 1) were all transposon insertion mutants derived from the symbiotically effective parent strain Rm1021. The mutants, which are defective in gluconeogenesis, cannot grow on tricarboxylic acid cycle intermediates when these are supplied as the sole carbon sources (Finan *et al.* 1988). Transduction of the transposon-encoded antibiotic resistance marker from each of the mutants into Rm1021 resulted in 100% cotransfer of the inability to grow on succinate minimal medium (Finan *et al.* 1988); this strongly suggests that the gluconeogenic mutations resulted from single transposon insertions.

The symbiotic phenotypes of the mutants were investigated after inoculation of alfalfa seedlings growing under nitrogen-deficient conditions. All of the mutants formed root nodules on alfalfa. The symbiotic N_2-fixing activity as measured by acetylene-reduction and plant dry weight determinations, of representative mutants is shown in Table 2. Both measurements indicated that the symbiotic N_2-fixing activity of the Eno$^-$ (Rm5438), Pgk$^-$ (RmF331), and Gap$^-$ (Rm5418) mutants were less than 10% of wild type. Plants inoculated with these mutants were generally similar in appearance to the chlorotic and stunted plants of the uninoculated control. Some of the plants inoculated with strains Rm5418 and RmF331 were pale green, indicating a very low level of N_2-fixation may have occurred. The acetylene-reducing activity of plants inoculated with strains Rm5065 and Rm5439 carrying the Pck-1 allele were 74 and 64%, respectively, of wild type. In independent experiments, plants inoculated with Rm5065 or another Pck$^-$ mutant, strain Rm5234, always showed reduced plant growth compared with plants inoculated with Rm1021 (see shoot dry weights, Table 2).

To further establish the link between the transposon-induced mutations and the observed symbiotic defects, two

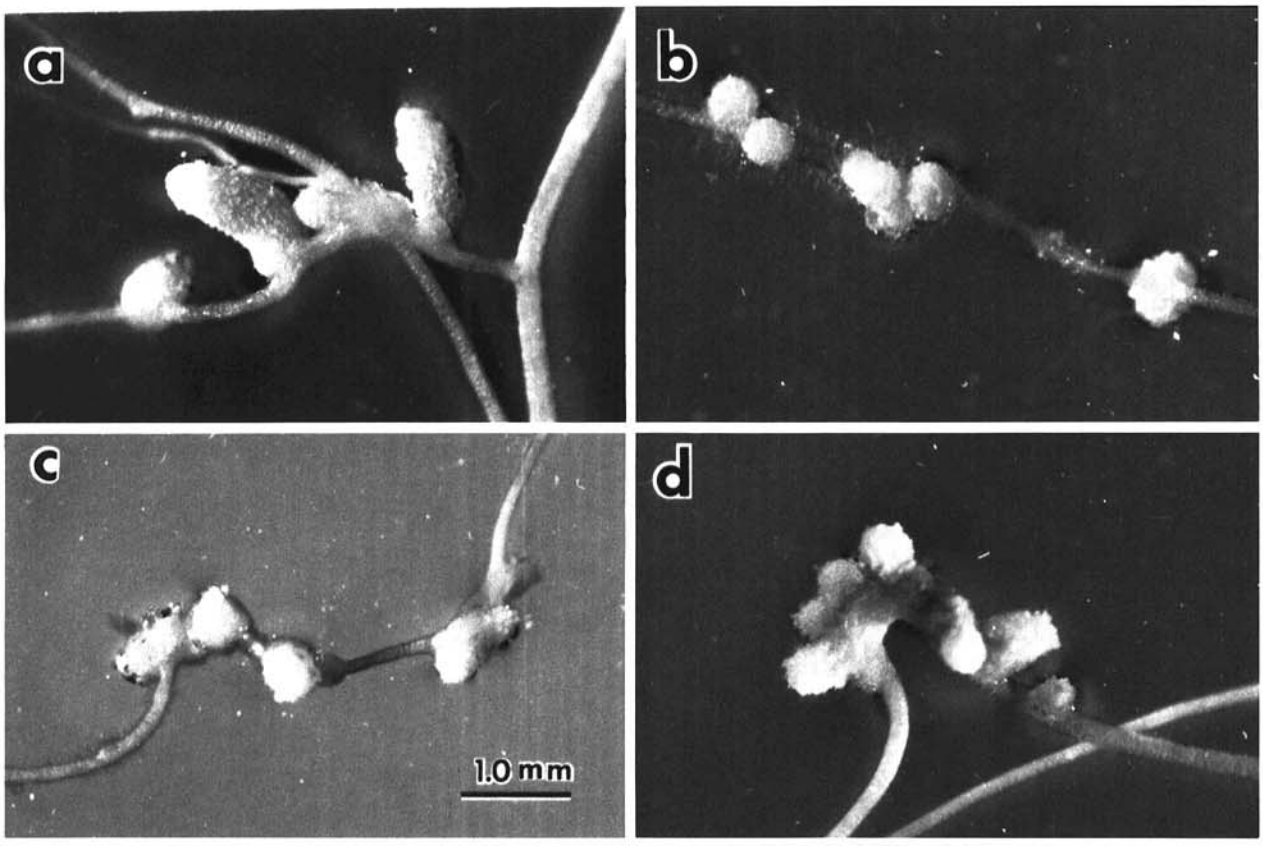

Fig. 1. Morphology of nodules formed on alfalfa roots by wild-type *Rhizobium meliloti* 1021 and Eno$^-$, Gap$^-$, and Pck$^-$ mutants. Nodules shown were photographed 28 days after infection by: **A,** *R. meliloti* 1021; **B,** Eno$^-$ mutant Rm5012; **C,** Gap$^-$ mutant Rm5418; **D,** Pck$^-$ mutant Rm5065.

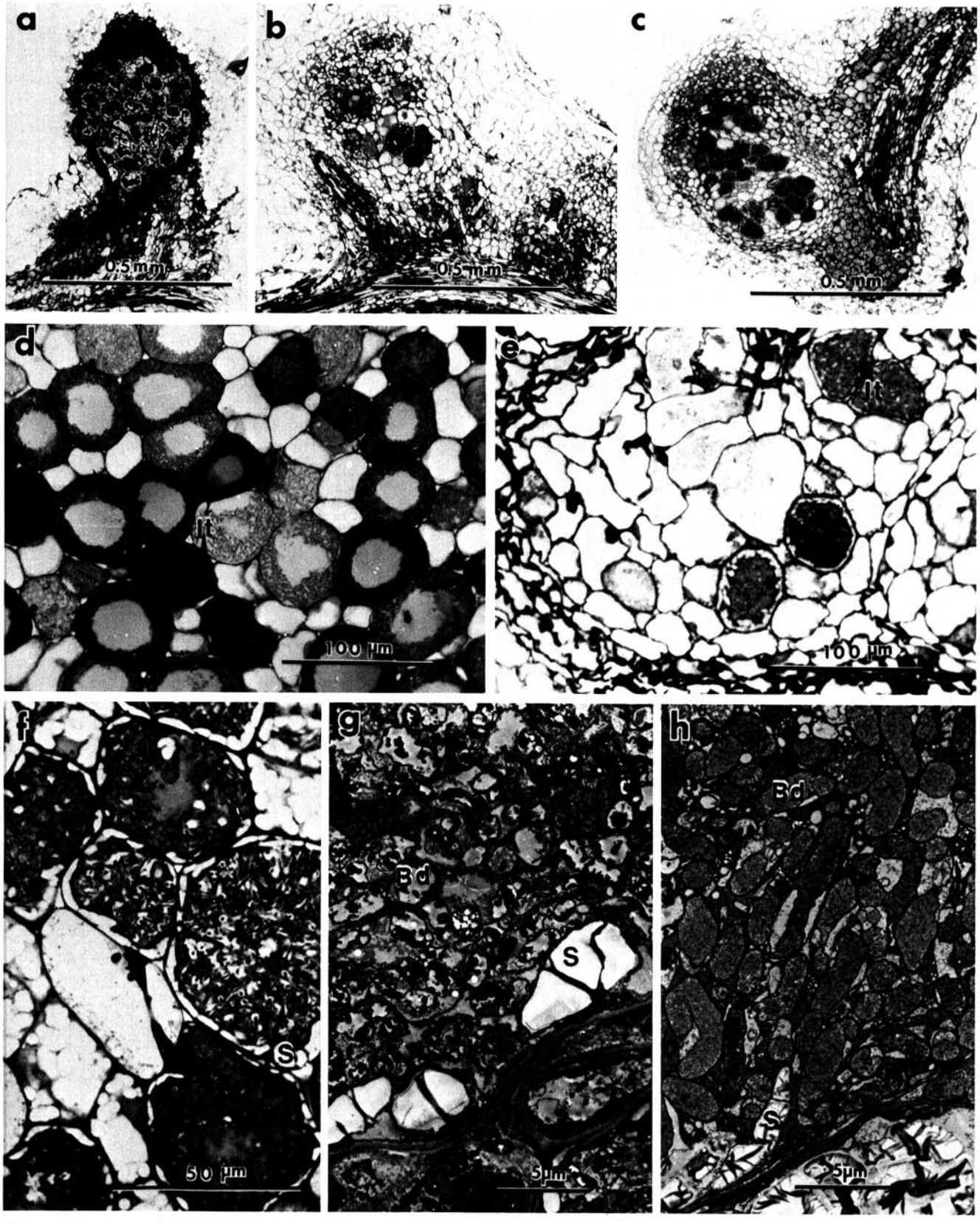

Fig. 2. Internal structure of nodules formed by Eno$^-$, Gap$^-$, and Pgk$^-$ mutants derived from *Rhizobium meliloti* 1021. **A–C,** Longitudinal sections of mutant nodules: **A,** Eno$^-$ mutant Rm5438, 3 wk; **B,** Gap$^-$ mutant Rm5418, 2 wk; **C,** Pgk$^-$ mutant F331, 2 wk. **D–F,** Light microscopy of bacteroid-containing cells within the symbiotic zone, showing infection threads (It) and starch granules (S): **D,** Rm1021 (wild type) 4 wk; **E,** Eno$^-$ mutant Rm5438, 3 wk; **F,** Pgk$^-$ mutant F331, 3 wk. *G and H,* Electron microscopy of cells containing degenerating bacteroids (Bd) and starch granules: **G,** Eno$^-$ mutant Rm5438, 3 wk; **H,** Gap$^-$ mutant Rm5418, 3 wk.

independent revertants of strains Rm5065, Rm5418, and Rm5438 were examined. These revertants, designated RmF307, RmF308, PmF311, RmF312, RmF362, and RmF363, were found to have lost their parental transposon antibiotic resistance marker, and reacquired wild-type activities of the enzymes absent in the parental strains. In plant nodulation experiments all of the revertants appeared to be fully symbiotically effective, as evidenced by the physical appearance of the plants and by plant dry weight determinations (data not shown). These data confirm that the symbiotic phenotypes observed in the mutants resulted from the various gluconeogenic mutations rather than possible secondary mutations. In summary, we conclude that Eno^-, Pgk^-, and Gap^- mutants of *R. meliloti* form nodules that are essentially Fix^- and that plants inoculated with Pck^- mutants have a reduced N_2-fixation phenotype.

Because the gluconeogenic mutations could result in cell surface alterations as previously reported in *Pseudomonas aeruginosa* (Banerjee *et al.* 1983), we screened all of the mutants for the presence of the Calcofluor-binding exopolysaccharide (Leigh *et al.* 1985) and for other cell surface alterations as revealed by altered sensitivity to eight bacteriophage (see Methods). No differences between the mutants and wild type were found.

Nodule structure. To determine at what stage the nodulation development was blocked in the various mutants we examined the structure of the nodules by light and electron microscopy. Nodules induced by Gap^- (Rm5418), Pgk^- (RmF331), and Eno^- (Rm5012 and Rm5438) mutants formed small, white, round nodules that were often misshapen and clustered (Fig. 1B and C). Older nodules frequently became yellowish brown and necrotic. In contrast, nodules induced by the wild-type strain were pink and cylindrical (Fig. 1A). Longitudinal sections through the nodules formed by the above mutants showed that development progressed through cortical cell division to produce small nodules with an endodermis, meristematic region, and vascular strands (Fig. 2A–C). Within 2 wk, infection threads were observed in the nodules formed by all three mutant classes (Fig. 2E and F). In most nodules, bacteria were released into a few of the host cells below the meristem, but further infections and development of a full symbiotic region did not occur. The infected cells and neighboring uninfected cells contained many starch granules near their peripheries (Fig. 2F–H). Some infected cells contained apparently mature bacteroids, whereas in others senescent bacteroids were observed (Fig. 2G and H). Unlike wild-type nodules, the distribution of cells containing bacteroids of a particular developmental stage was not related to their position within the nodule. That is, in the mutant nodules, cells containing senescent bacteroids were frequently observed to be mixed with cells containing bacteroids of earlier stages.

Nodules induced by the *pck* mutants Rm5065 and Rm5439 were examined to determine if their partial nitrogen-fixation ability was accompanied by any abnormality in nodule structure. Two to three weeks after infection, these mutants produced a mixture of small white and pink nodules, but pink nodules predominated after 4 wk. Most nodules examined had a morphology that was similar to wild-type nodules. Nodules formed by the Pck^- mutants appeared to contain more starch granules within the infected and uninfected cells of the symbiotic zone compared with wild-type nodules. Electron microscopy of Pck^- mutant bacteroids did not demonstrate any structural differences compared with wild-type bacteroids. In a few of the white nodules harvested 2 wk after inoculation, a large reduction in the number of infected cells was observed. In this respect these nodules were similar to those formed by the Eno^-, Gap^-, and Pgk^- mutants.

To investigate the possibility that the partial Fix^+ phenotype of the Pck^- mutants might be due to the presence of Pck^+ revertants, bacteria were isolated from 26 nodules induced by Rm5065, Rm5439, or Rm5234 by plating the nodule contents on LB. All of the resulting isolates grew on glucose but not on succinate minimal medium, demonstrating that no Pck^+ revertants were present.

Bacteroid enzyme activities. To relate the results of the above mutant analyses to carbon metabolism in wild-type bacteroids, we assayed the levels of gluconeogenic and other enzymes in bacteroids isolated from alfalfa nodules. For comparison, we also assayed the levels of these enzymes in the bacteria grown in minimal medium with either succinate or glucose as a carbon source (Table 3). To reduce variability due to assay conditions, enzyme activities in both bacteroid and free-living cell extracts were determined on the same days, using the same assay mixtures. The activities of enzymes in free-living cells have previously been reported (Finan *et al.* 1988), and, with the exception of lower glyceraldehyde-3-phosphate dehydrogenase and 3-P-glycerate kinaise activities reported in this paper, the activities obtained are similar to those previously reported. Except for phosphoenolpyruvate carboxykinase, the enzyme activities in bacteroids were similar to those of succinate-grown cells. In particular, the lack of detectable Entner-Douderoff pathway activity and the low glucose-6-phosphate dehydrogenase activities suggest that glucose or hexose sugars are not metabolized by bacteroids. Low activities for the latter enzyme activities have previously

Table 3. Enzyme activities in free-living cells and in bacteroids from alfalfa root nodules

	Specific activity (nmoles/min/mg protein)		
Enzyme	**Bacteroids[a]**	**Glucose grown[b]**	**Succinate grown[b]**
ED-Pathway[c]	1	25	3
Glucose-6-P DH (NADP-linked)	12	128	10
6-P-gluconate DH (NADP)	22	57	20
F-1,6-Bisphosphatase	4	6	8
Glyceraldehyde-3-P DH	16	31	21
3-P-Glycerate kinase	45	29	54
P-Glycerate mutase	5	13	15
Enolase	15	18	38
Isocitrate DH	432	300	241
Malate DH	2,968	467	651
PEP Carboxykinase	ND[d]	ND	91

[a] Data are expressed as the means of duplicate samples obtained from independently isolated bacteroids.
[b] Data were obtained from a single experiment.
[c] Entner-Douderoff (ED) pathway represents the combined activities of 6-phosphogluconate dehydratase and 2-keto-3-deoxy-6-phosphogluconate aldolase.
[d] No activity detected.

been reported in bacteroids of snakebean nodules (Saroso *et al.* 1986). Bacteroid malate dehydrogenase activity was more than fourfold higher than activities determined in succinate-grown free-living cells. A similar observation was recently reported for bacteroids from pea nodules (McKay *et al.* 1989). With reference to the enzyme activities absent in *R. meliloti* mutants, glyceraldehyde-3-phosphate dehydrogenase, 3-phosphoglycerate kinase, and enolase activities were low but readily detected in bacteroid extracts.

No phosphoenolpyruvate carboxykinase activity (PCK) was detected in alfalfa bacteroid extracts, and attempts to detect activity by increasing the concentration of extract assayed were unsuccessful. To determine whether PCK activity was inhibited or inactivated in the bacteroid preparations, we mixed extract from succinate grown cells (which had a PCK specific activity of 106) with bacteroid extract. After 30 min of incubation at 28° C, no loss of the PCK activity was observed. Thus, no evidence of inhibition or inactivation of PCK was obtained. Low phosphoenolpyruvate carboxykinase activity has previously been reported in bacteroids from pea and snakebean nodules (McKay *et al.* 1989; Saroso *et al.* 1986).

DISCUSSION

R. meliloti Gap$^-$, Pgk$^-$, and Eno$^-$ mutants formed ineffective nodules that were very similar in morphology. Each mutant lacks an enzyme activity of the lower half of the Embden-Meyerhoff-Parnas pathway, which results in a pleiotropic phenotype in free-living cells. Compared with wild type, the mutants grew poorly on glucose and glycerol and failed to grow on tricarboxylic acid intermediates as sole source of carbon (Finan *et al.* 1988). Nodules induced by these mutants contained few infected cells and lacked a distinct "symbiotic" zone. Nodule development appears to be altered at or near the stage when bacteria are released into the plant cells. In those cells that were infected, the bacteroids appeared normal; however, the plant cells contained many starch grains and appeared to senesce prematurely. The accumulation of starch granules within the infected and neighboring cells is probably an indication that energy reserves are not being consumed. Bacteroids do contain GAP, PGK, and ENO activities (Table 3) and thus these enzymes may play a role in bacteroid metabolism. It is not clear whether the symbiotic phenotypes of the mutants result from their defect in gluconeogenesis or their more general defect in intermediary carbohydrate metabolism, as evidenced by their slow growth on glucose and glycerol. Moreover, the gluconeogenic mutations could cause cell surface alterations, which in turn could result in a symbiotic defect. Whereas we failed to detect differences between the wild type and mutants in the synthesis of the Calcofluor-binding exopolysaccharide and in sensitivity to eight bacteriophage, it is possible that cell surface alterations that may have been present were not detected. It is also possible that the metabolic status of the bacteria during infection results in cell surface alterations in the mutant but not wild-type cells.

Phosphoenolpyruvate carboxykinase is a central enzyme controlling gluconeogenesis in *R. meliloti*. This enzyme does not play a role in general intermediary carbohydrate metabolism. Thus, growth of Pck$^-$ mutants on nongluconeogenic substrates such as glucose and glycerol as sole carbon source is indistinguishable from the wild type (Finan *et al.* 1988). Because Pck$^-$ mutants can fix N_2 symbiotically (albeit reduced), it is unlikely that the gluconeogenesis-deficient phenotype of the Gap$^-$, Pgk$^-$, and Eno$^-$ mutants is alone responsible for their failure to fix N_2 symbiotically.

Microscopy of nodules formed by C_4-dicarboxylic acid transport (*dctA*) mutants has revealed a nodule morphology with the major characteristics noted here for Eno$^-$, Gap$^-$, and Pgk$^-$ mutants: Few infected host cells are present within a poorly developed symbiotic zone, many starch granules are present in the nodule cells, and senescence of bacteroids occurs prematurely (Ronson *et al.* 1981; Watson *et al.* 1988; Engelke *et al.* 1989). The major differences are that *dctA* mutant nodules usually contain more infected cells, and in older cells a large senescent zone within an elongated nodule is frequently present. *R. meliloti* mutants that are symbiotically ineffective due to *nif* and *fix* mutations have also been shown to form nodules with similar characteristics to those noted here for Eno$^-$, Gap$^-$, and Pgk$^-$ mutants (Hirsch and Smith 1987).

As bacteroids lack PCK activity, we assume that the symbiotic defect resulting from the Pck$^-$ mutation occurs before bacteroid formation. Perhaps gluconeogenesis is required for efficient growth of *R. meliloti* in the infection thread. The ultrastructure of nodules induced by the Pck$^-$ mutants did not reveal any obvious reason for the partial symbiotic defect. The partially effective phenotype of the *R. meliloti* Pck$^-$ mutants differs from the fully effective phenotype of previously described Pck$^-$ mutants of *R. leguminosarum* bv. *viciae* (McKay *et al.* 1985; T. M. Finan, unpublished). The latter result may reflect slight differences among plants in the availability and/or composition of metabolites made available to the bacteria in nodules.

We are currently unable to rule out the possibility that the symbiotic defect in Pck$^-$ mutants results from a polar effect of the transposon insertions. Current evidence suggests that the *pck-1* and *pck-2* insertions examined in this study lie within the phosphoenolpyruvate carboxykinase structural gene *pckA* (T. M. Finan and M. Osteras, unpublished). The DNA region flanking the *pck-1*::Tn*5* insertion was recently used as a probe to clone the homologous region from the broad host range *Rhizobium* sp. strain NGR234. The DNA sequence of the NGR234 gene region (Osteras *et al.*, in press) revealed a strong transcriptional terminator immediately following the *pckA* gene. It is likely that the NGR234 and *R. meliloti* gene regions are similar in organization, and thus it is unlikely that Tn*5* insertions in the *pckA* gene of *R. meliloti* will have a polar effect on transcription of other genes. In view of the symbiotic phenotype of the *R. meliloti* and *R. leguminosarum* Pck$^-$ mutants described above, it is interesting that an NGR234 Pck$^-$ mutant was recently found to exhibit a host plant dependent symbiotic phenotype (Osteras *et al.*, in press).

Much evidence suggests that bacteroids use C_4-dicarboxylates as their primary carbon and energy source (see introduction). Thus, the lack of detectable PCK activity in bacteroids is surprising as this enzyme is highly induced in free-living cells growing on succinate as carbon source (Table 3, Finan *et al.* 1988; McKay *et al.* 1985). In phos-

phate-limited continuous cultures of *R. leguminosarum* grown with 10 mM fumarate as carbon source, the synthesis of PCK was shown to be completely repressed by 0.4 mM sucrose (McKay *et al.* 1985). That result together with the Fix^+ phenotype of a *R. leguminosarum* Pck^- mutant led the latter authors to suggest that pea bacteroids receive a low concentration of sucrose for the provision of sugar precursors for biosynthesis. Similar experiments on the regulation of PCK in *R. meliloti* have not yet been carried out. We note that in the absence of an alternative route for phosphoenolpyruvate synthesis, the lack of PCK activity in *R. meliloti* bacteroids should effectively prevent their growth on C_4-dicarboxylic acids in the nodule. Thus, the regulation of PCK synthesis may be very important in controlling bacteroid proliferation in alfalfa root nodules.

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