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The Formation of Nitrogen-Fixing Bacteroids Is Delayed but Not Abolished in Soybean Infected by an α-Ketoglutarate Dehydrogenase-Deficient Mutant of *Bradyrhizobium japonicum*[1]

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A mutant strain of *Bradyrhizobium japonicum* USDA 110 devoid of α-ketoglutarate dehydrogenase activity (LSG184) was used to test whether this tricarboxylic acid cycle enzyme is necessary to support nitrogen fixation during symbiosis with soybean (*Glycine max*). LSG184 formed nodules about 5 d later than the wild-type strain, and the nodules, although otherwise normal in structure, contained many fewer infected host cells than is typical. At 19 d after inoculation cells infected with the mutant strain were only partially filled with bacteroids and showed large accumulations of starch, but by 32 d after inoculation the host cells infected with the mutant appeared normal. The onset of nitrogen fixation was delayed about 15 d for plants inoculated with LSG184, and the rate, on a per nodule fresh weight basis, reached only about 20% of normal. However, because nodules formed by LSG184 contained only about 20% of the normal number of bacteroids, it could be inferred that the mutant, on an individual bacteroid basis, was fixing nitrogen at near wild-type rates. Therefore, the loss of α-ketoglutarate dehydrogenase in *B. japonicum* does not prevent the formation or the functioning of nitrogen-fixing bacteroids in soybean.

Nitrogen-fixing symbioses between legumes and rhizobia have become important model systems for the study of plant-microbe interactions. Great strides have been taken toward delineating the early signaling events leading to successful infection of the host plant by its bacterial partner. However, much less is known about the processes that support proliferation and differentiation of the bacteria during nodule development and nitrogen fixation by bacteroids in the mature nodule. Conditions within the legume nodule differ radically from those encountered by free-living rhizobia, including, for example, a very low free oxygen concentration and a limited array of carbon substrates. Therefore, an integral part of the development of effective nitrogen-fixing bacteroids is the metabolic adjustments they must make to acclimate to their new environment. Determining what carbon metabolic pathways are used by bacteroids and how they are regulated is essential to an understanding of how free-living rhizobia differentiate into effective symbiotic partners.

The main carbon compounds used by mature bacteroids are the TCA cycle intermediates malate and succinate, a view supported by ample physiological, biochemical, and genetic data (for review, see McDermott et al., 1989; Day and Copeland, 1991; Streeter, 1991). For example, dicarboxylic acids readily pass through the plant-derived symbiosome membrane that surrounds the bacteroids and controls their supply of nutrients from the plant (Udvardi et al., 1988). Malate and succinate are also among the best carbon substrates for supporting ex planta nitrogen fixation by isolated bacteroids (McDermott et al., 1989; Day and Copeland, 1991). In fast-growing rhizobia that form indeterminate nodules, such as *Rhizobium meliloti*, *Rhizobium trifolii*, and *Rhizobium leguminosarum* bv *viciae*, mutants defective in dicarboxylic acid uptake fail to fix nitrogen (Fix^- phenotype), whereas hexose uptake mutants are normal (Fix^+; Streeter, 1991). The dicarboxylic acid uptake mutants successfully invade the host plant and form bacteroids (Nod^+ phenotype), indicating that these rhizobia do not become exclusively dependent on dicarboxylic acids until the late stages of differentiation (McDermott et al., 1989). For rhizobia forming determinate nodules, such as *Bradyrhizobium japonicum* and *Rhizobium leguminosarum* bv *phaseoli*, mutations that effect dicarboxylic acid uptake and impair nitrogen fixation have also been described (Humbeck and Werner, 1989; Lafontaine et al., 1989; El-Din, 1992).

The most straightforward way for bacteroids to catabolize dicarboxylic acids imported from the plant is via the TCA cycle. Indeed, extensive radiorespirometric and enzyme activity studies have yielded data consistent with the operation of this pathway in nitrogen-fixing bacteroids (McDermott et al., 1989; Streeter, 1991). In *R. meliloti* mutations in succinate dehydrogenase (Gardiol et al., 1982) or isocitrate dehydrogenase (McDermott and Kahn, 1992) lead to a Nod^+Fix^- symbiotic phenotype, providing genetic evidence that these steps of the TCA cycle are essential for nitrogen fixation. However, because of the dual catabolic/anabolic function of the TCA cycle, it is not clear whether the symbiotic defect of these mutants arose out of an

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Abbreviations: DAI, days after inoculation; TCA, tricarboxylic acid.

inability to harvest sufficient energy from substrates supplied by the host plant or from a more specific auxotrophy manifested in the mature bacteroids (e.g. a need for glutamate).

Mutants demonstrating the importance of the other steps of the TCA cycle in *R. meliloti* or of any of the TCA cycle enzymes in other rhizobia have so far been lacking. An α-ketoglutarate dehydrogenase-deficient mutant of *R. meliloti* has been reported to be Fix^- (Duncan and Fraenkel, 1979), and this result has been widely cited as proof that this portion of the TCA cycle is required for nitrogen fixation. However, the Fix^- nature of the symbiotic phenotype of this mutant was only stated in a single sentence in the discussion and no data were shown. Acuña et al. (1991) inactivated a gene for fumarase in *B. japonicum*, but because there was substantial residual fumarase activity in this strain the importance of the enzyme during symbiosis could not be tested. Likewise, the existence of more than one aconitase prevented Thöny-Meyer and Künzler (1996) from constructing a *B. japonicum* strain entirely free of this enzyme. *B. japonicum* strains that were partially deficient in either fumarase or aconitase (reduced to about 60 and 30% of wild-type activity, respectively) were completely Fix^+ (Acuña et al., 1991; Thöny-Meyer and Künzler, 1996).

Although it is clear that bacteroids have all of the enzymes of the TCA cycle, some studies of *B. japonicum* indicate that the pathway may not operate at full capacity during nitrogen fixation (McDermott et al., 1989; Day and Copeland, 1991). For example, the TCA cycle enzymes α-ketoglutarate dehydrogenase, malate dehydrogenase, and isocitrate dehydrogenase are all subject to inhibition by high NAD(P)H/NAD(P) ratios in vitro (Emerich et al., 1988; Salminen and Streeter, 1990; D.B. Karr and D.W. Emerich, unpublished data). Because of such metabolic regulation, it has been argued that the flux of carbon through these steps of the TCA cycle might be blocked if oxidative phosphorylation and the regeneration of oxidized pyridine nucleotides become oxygen-limited during nitrogen fixation (Tajima et al., 1988; McDermott et al., 1989; Salminen and Streeter, 1990). Indeed, *B. japonicum* bacteroids accumulate large pools of glutamate, which is consistent with the inhibition of α-ketoglutarate dehydrogenase (Salminen and Streeter, 1987, 1990, 1992).

Because of the potential for inhibition of the TCA cycle in *B. japonicum*, some workers have postulated that dicarboxylic acids may be catabolized, at least partially, by other routes during nitrogen fixation (Wong and Evans, 1971; Stovall and Cole, 1978; Kouchi and Fukai, 1988; Kouchi et al., 1988; McDermott et al., 1989; Day and Copeland, 1991). However, attempts to obtain biochemical evidence for alternate metabolic pathways in bacteroids, such as the glyoxylate cycle or the γ-aminobutyrate shunt, have so far been unsuccessful (Johnson et al., 1966; Stovall and Cole, 1978; McDermott et al., 1989; Salminen and Streeter, 1990). Does *B. japonicum* have a way to bypass portions of the TCA cycle that might be inhibited during symbiosis?

To answer this question we have constructed, through site-directed mutagenesis, a *B. japonicum* mutant strain (LSG184) that is missing α-ketoglutarate dehydrogenase activity (Green and Emerich, 1997). In this strain a kanamycin resistance marker was inserted into the amino-terminal portion of *sucA*, one of the structural genes for the α-ketoglutarate dehydrogenase enzyme complex, thus eliminating transcription of the gene and all detectable α-ketoglutarate dehydrogenase activity. Unlike any other bacterial α-ketoglutarate dehydrogenase mutant so far described, the *B. japonicum sucA* mutant was able to grow on malate, succinate, and β-hydroxybutyrate, substrates that normally require a complete TCA cycle for their catabolism. This surprising result implied either that *B. japonicum* can compensate for the loss of α-ketoglutarate dehydrogenase by using alternate routes for the complete catabolism of these substrates or that it has a unique capacity to harvest sufficient energy for growth from their incomplete oxidation.

To test whether α-ketoglutarate dehydrogenase or, by extension, a complete TCA cycle is necessary during nitrogen fixation we examined the symbiotic performance of the *sucA* mutant. We demonstrate here that the interruption of *sucA* in *B. japonicum* causes an array of defects in early symbiotic development but that the mutant eventually forms normal, Fix^+ bacteroids in the absence of α-ketoglutarate dehydrogenase. This result argues strongly for the existence of alternatives to the conventional TCA cycle for the catabolism of dicarboxylic acids in *B. japonicum* bacteroids.

MATERIALS AND METHODS

Plant Material and Bacterial Strains

Soybean (*Glycine max* L. cv Williams 82) plants were grown under microbiologically controlled conditions in modified Leonard jars (Vincent, 1970) in a 3:3:1 mixture of vermiculite:perlite:sand (v/v) and a nitrogen-free plant nutrient solution (Ahmed and Evans, 1960). Soybean seeds were surface-sterilized and inoculated at the time of planting with either *Bradyrhizobium japonicum* USDA110 or its α-ketoglutarate dehydrogenase-deficient derivative LSG184 (*sucA* mutant; Green and Emerich, 1997). In some experiments plants were inoculated with the same *B. japonicum* strains carrying a complementing cosmid, pLAFR1-*mdh* (110/*mdh*, 184/*mdh*; Green and Emerich, 1997). This cosmid carries a 20-kb insert encoding a cluster of TCA cycle structural genes, including those for malate dehydrogenase, succinyl-CoA thiolase, and α-ketoglutarate dehydrogenase. Plants were grown in a growth chamber with a day/night temperature of 27°C (16 h)/24°C (8 h).

Light Microscopy

Whole nodules were collected 19 and 32 DAI and prepared for light microscopy (Pueppke and Payne, 1987). Tissue was fixed in a 2.5% (v/v) glutaraldehyde and 0.1 M sodium phosphate buffer, pH 7.0, dehydrated through an ethanol and chloroform series, and embedded in paraffin. Sections were mounted on glass slides and stained with safranin and fast green (Sass, 1958). For the higher-magnification light micrographs, semithin sections of samples prepared for transmission electron microscopy (see

below) were stained with toluidine blue (1%, w/v, in 1% sodium borate).

Transmission Electron Microscopy

Nodule samples were collected 19 and 32 DAI and prepared for transmission electron microscopy. Samples were fixed overnight in 2% (v/v) paraformaldehyde, 2% (v/v) glutaraldehyde, and 0.1 M sodium cacodylate, pH 7, at 4°C. After being postfixed in 1% (w/v) OsO_4 and 0.1 M sodium cacodylate, the samples were dehydrated and embedded in resin (Epon-Araldite, Electron Microscopy Sciences, Fort Washington, PA). Ultrathin sections were stained with 1% (w/v) aqueous uranyl acetate and Reynold's lead citrate (Venable and Coggeshall, 1965).

Acetylene Reduction Assays

Nitrogenase activity of intact root crowns was estimated using the acetylene reduction method (Schwinghamer et al., 1970). Ethylene production rate was determined using three plants in each sample. Values were normalized to the total fresh weight of the nodules in each assay.

Bacteroid Isolation

Nodules were picked from plants harvested either 29 (for USDA110) or 32 (for LSG184) d after planting. Bacteroids were purified from the nodules by a sequential Suc gradient method as previously described (Karr et al., 1984), except that Tes was used instead of phosphate as the buffer.

Enzyme Assays

For enzyme assays bacteroids were broken in a French pressure cell at 16,000 p.s.i., and the extracts were clarified and desalted before assaying (Green and Emerich, 1997). The activity of α-ketoglutarate dehydrogenase (EC 1.2.4.2) was measured by the method of Reed and Mukherjee (1969; Green and Emerich, 1997), and malate dehydrogenase activity (EC 1.1.1.37) was assayed as previously described (Karr et al., 1984). The protein content of the extracts was determined by the method of Bradford (1976), using BSA as the standard.

Protein Gel Electrophoresis and Immunoblotting

Protein samples were dissolved by boiling in 2% (w/v) SDS, 20 mM EDTA, 10% (v/v) glycerol, 0.05% (v/v) β-mercaptoethanol, 0.003% (w/v) bromphenol blue, and 60 mM Tris, pH 6.8. Protein samples were resolved on 1% (w/v) SDS and 12.5% polyacrylamide gels using a Laemmli buffer system (Laemmli, 1970) and visualized by staining with a solution of 0.04% Coomassie R-250, 0.05% crocein scarlet, 10% acetic acid, 20% methanol, and 0.5% $CuSO_4$.

For immunodetection of the Fe protein of nitrogenase, bacteroid soluble proteins resolved by denaturing gel electrophoresis were transferred to nitrocellulose (Towbin et al., 1979) and incubated with rabbit polyclonal antibody raised against nitrogenase Fe protein purified from *Rhodospirillum rubrum* (Kanemoto and Ludden, 1984). Reacting bands were detected with alkaline phosphatase conjugated to goat anti-rabbit IgG according to the manufacturer's instructions (Bio-Rad).

Pouch Nodulation Assays

Soybeans were surface-sterilized and sprouted on sterile 1% agar/water at 28°C in covered trays. After 2 d, when roots were about 2 to 4 cm long, the seedlings were dipped in the inoculum and transplanted to sterile growth pouches (Mega International, Minneapolis, MN) containing nitrogen-free plant nutrient solution. The inoculum was a suspension of *B. japonicum* USDA110 or LSG184 at 10^5 or 10^8 cfu/mL. The position of the root tip was marked and the pouches were placed in a growth chamber. Roots were checked each day for the presence of nodules, and 18 to 21 DAI the total number of nodules on each tap root and the position of the topmost nodule relative to the root tip mark were recorded.

RESULTS

Nodulation of Soybean by *B. japonicum* LSG184

Soybean plants grown in Leonard jars (Vincent, 1970) and inoculated with the α-ketoglutarate dehydrogenase-deficient mutant strain LSG184 were visibly yellowed by 20 DAI, indicating a deficiency in symbiotic nitrogen fixation. The plants inoculated with LSG184 formed nodules, but there were fewer than normal on the main root of each plant at the earliest time checked, 15 DAI (13.5 ± 1.6 for USDA110, 4.2 ± 0.5 for LSG184). By 19 DAI and thereafter (up to 39 DAI) there was no significant difference between the average number of nodules formed on the tap root by the wild type versus the mutant strain. The mutant appeared to form more nodules than the wild type on lateral roots, but this was not systematically studied.

The average fresh weight of nodules formed by LSG184 lagged behind that of nodules formed by the wild type throughout most of the symbiosis (Fig. 1). However, the nodules formed by the mutant continued to grow and by 39 DAI had reached a normal size. Therefore, the *sucA* mutant LSG184 had a Nod^+ phenotype but delayed nodule development. Because we tested the *sucA* mutant on only Williams 82 cv, we do not know whether its performance would be the same on other soybean cultivars or on other legumes nodulated by *B. japonicum*.

Structure of Nodules Formed by LSG184

Nodules formed by LSG184 were pale inside, ranging in color from off-white to tan, indicating a deficiency in leghemoglobin content. To determine whether nodules formed by the mutant strain were of normal morphology, we examined cross-sections of paraffin- and resin-embedded nodules by light microscopy. At 19 DAI the nodules formed by the wild type showed features typical of mature soybean nodules (Fig. 2, A and B). The nodules formed by the mutant also appeared to contain all of the tissues found

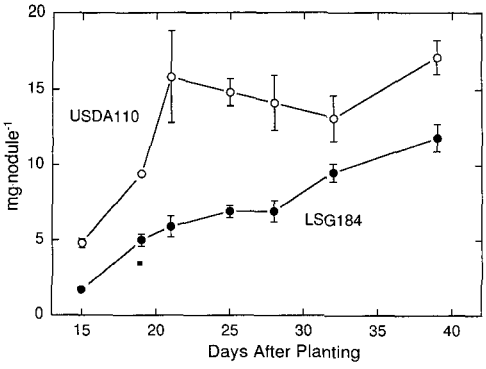

Figure 1. Fresh weight of nodules formed by the wild-type or *sucA* mutant of *B. japonicum*. Soybeans were grown in modified Leonard jars and inoculated with either USDA110 (wild type, ○) or LSG184 (*sucA* mutant, ●) at the time of planting. At the indicated times, plants were harvested, and the nodules were picked and weighed. The total nodule fresh weight was divided by the number of nodules to obtain an average nodule fresh weight value for each plant. Each point represents the mean of the average nodule fresh weights for three or four plants. Error bars represent SEs.

in normal soybean nodules (Newcomb, 1981), including peripheral vascular bundles, a layer of schlerenchyma cells, and an infected region in the center of the nodule (Fig. 2, C and E). However, at 19 DAI, the nodules formed by LSG184 contained many fewer infected cells than those formed by the wild-type strain, and the cytoplasm of the infected cells contained fewer bacteroids (Fig. 2, C and D). At this stage, the infected cells were apparently still in the process of being invaded, with the bacteroids concentrated near the infection foci (Fig. 2D). Both infected and uninfected cells showed large accumulations of starch in 19-d-old nodules formed by the mutant (Fig. 2D). By 32 DAI nodule cells infected by the mutant had attained a normal appearance, but there were still far fewer of them in each nodule than is typical (Fig. 2, E and F). The infected cells were filled with bacteroids and no longer contained large starch grains, although the uninfected cells still had more starch than those in nodules formed by the wild type (Fig. 2, B and F). Throughout symbiosis, the nodule cells infected with the mutant strain were present only as small patches surrounded by many layers of uninfected cells (Fig. 2, C–F).

To examine the appearance of the bacteroids in nodules formed by LSG184, we used transmission electron microscopy. At 19 and 32 DAI wild-type nodules contained bacteroids that appeared typical for these stages of development (Fig. 3, A, C, E, and G). At 19 DAI nodules formed by the mutant also contained bacteroids released into the cytoplasm of infected cells, but the bacteroids were often few in number and confined to a small portion of the cell (Fig. 3B). Several infection threads could be seen (not shown), suggesting that infection of the host cells was still taking place at this stage of nodule development. Here, again, large accumulations of starch were evident (Fig. 3B).

In contrast, by 32 DAI nodule cells infected by LSG184 had a normal appearance in that bacteroids filled the cytoplasm and there were no longer large starch granules present (Fig. 3D). At 19 DAI in the mutant, symbiosomes usually contained only one or two bacteroids (Fig. 3, B and F); however, in the older nodules symbiosomes containing four to six bacteroids were common (Fig. 3, D and H), indicating that mutant bacteroids had undergone cell division after their release into the plant cytoplasm. The overall structural appearance of the mutant bacteroids was indistinguishable from the wild type, showing intact membranes and numerous polyhydroxybutyrate inclusion bodies (Fig. 3, E–H).

Acetylene Reduction Activity of LSG184 Nodules

The nitrogen-starved appearance of soybeans inoculated with LSG184 suggested that the strain was Fix$^-$. To test this hypothesis we measured the acetylene reduction activity of plants nodulated with LSG184. As expected, plants inoculated with the mutant had little or no acetylene reduction activity through 25 DAI (Fig. 4). However, at 28 DAI plants inoculated with LSG184 began to show acetylene reduction activity, and this activity was maintained at later times. The maximum rate of acetylene reduction measured for LSG184 (at 28 and 39 DAI) on a nodule fresh weight basis was about 20% of that attained by the wild type (at 25 DAI). Plants inoculated with 184/*mdh*, a derivative of LSG184 carrying a complementing cosmid, pLAFR1-*mdh* (Green and Emerich, 1997), did not yellow and had normal acetylene reduction activities (Table I).

Several controls were performed to determine whether the bacteroids in older nitrogen-fixing nodules formed by LSG184 still retained the characteristics of the original mutant. First, bacteroids were recovered from a random sampling of 32-d-old nodules formed on plants inoculated with LSG184 and were plated under different selective conditions. All of the recovered bacteroids were resistant to kanamycin, indicating the retention of the transposon used to construct LSG184, and none were able to grow on 20 mM acetate, a phenotype associated with the loss of α-ketoglutarate dehydrogenase (Green and Emerich, 1997). One colony was picked at random, returned to culture, and inoculated onto fresh soybean plants. This strain exhibited the same symbiotic phenotype as the original mutant, indicating that it had not reverted or become suppressed by a second mutation. Southern hybridization of DNA isolated from this recovered strain also showed that it retained the original transposon insertion in *sucA*.

The above controls could not eliminate the possibility that a small subset of revertant bacteroids that was missed in our sample was responsible for the acetylene reduction activity observed in plants nodulated by LSG184. Therefore, we harvested all of the nodules from 60 plants inoculated with the wild type and from 92 plants inoculated with the mutant strain at 29 and 32 DAI, respectively. Acetylene reduction assays of a sampling of these plants showed that the mutant nodules were active in nitrogen fixation. Bacteroids were purified from these nodules and disrupted, and the extracts were assayed for α-ketoglutarate dehydrogenase activity. The bacteroids recovered from plants inoculated with LSG184 had less than 1% of the α-ketoglutarate dehydrogenase activity found in wild-type bacteroids (Table II). In contrast, the specific activity of malate dehydrogenase was

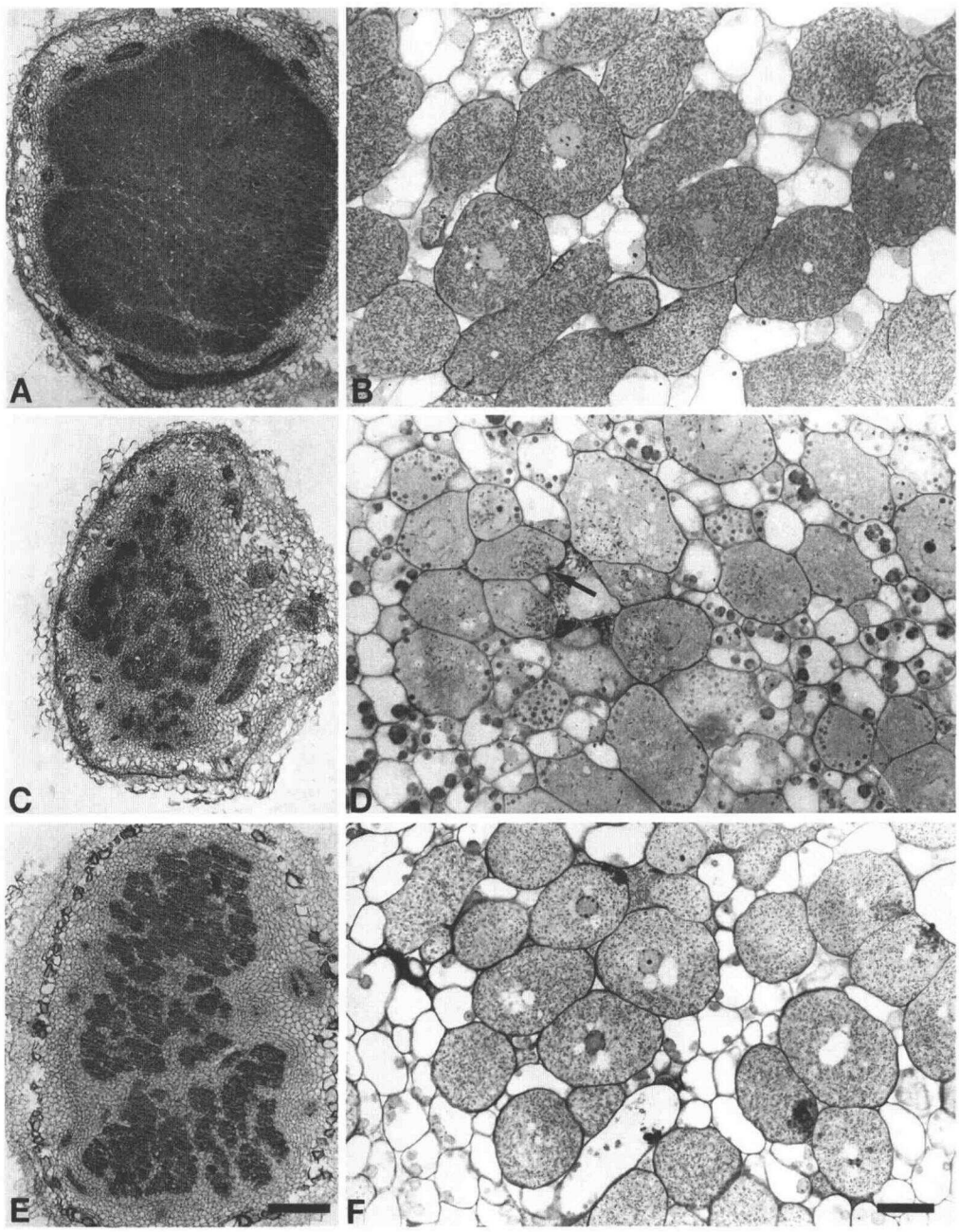

Figure 2. Light micrographs of nodules formed by the wild-type or *sucA* mutant of *B. japonicum*. Nodules were inoculated with either USDA110 (wild type) or LSG184 (*sucA* mutant) and harvested 19 or 32 DAI. For A, C, and E, nodules were fixed and embedded in paraffin, and the sections were stained with fast green and safranin. For B, D, and F, nodules were embedded in resin and the sections were stained with toluidine blue. D, Arrow points to a nodule cell apparently in the process of being invaded. Micrographs are of 19-d-old nodules formed by USDA110 (A and B), 19-d-old nodules formed by LSG184 (C and D), and 32-d-old nodules formed by LSG184 (E and F). A, C, and E, Magnification = ×38; scale bar = 300 μm. B, D, and F, Magnification = ×530; scale bar = 20 μm.

essentially the same in the two extracts. This result provided further evidence that the α-ketoglutarate dehydrogenase-deficient phenotype of the mutant strain was stable during symbiosis.

Nitrogenase Content of LSG184 Bacteroids

Consistent with the low number of infected cells observed microscopically within nodules formed by LSG184, the yield of mutant bacteroids from a given weight of nodules was only about 20% of that of the wild type (Table II). This result indicated that there were many fewer bacteroids in each nodule formed by the mutant and, therefore, that the acetylene reduction activity displayed by the mutant at 28 DAI was similar to the wild type at 25 DAI, when normalized to the number of bacteroids rather than to nodule fresh weight. As one test of this possibility, and

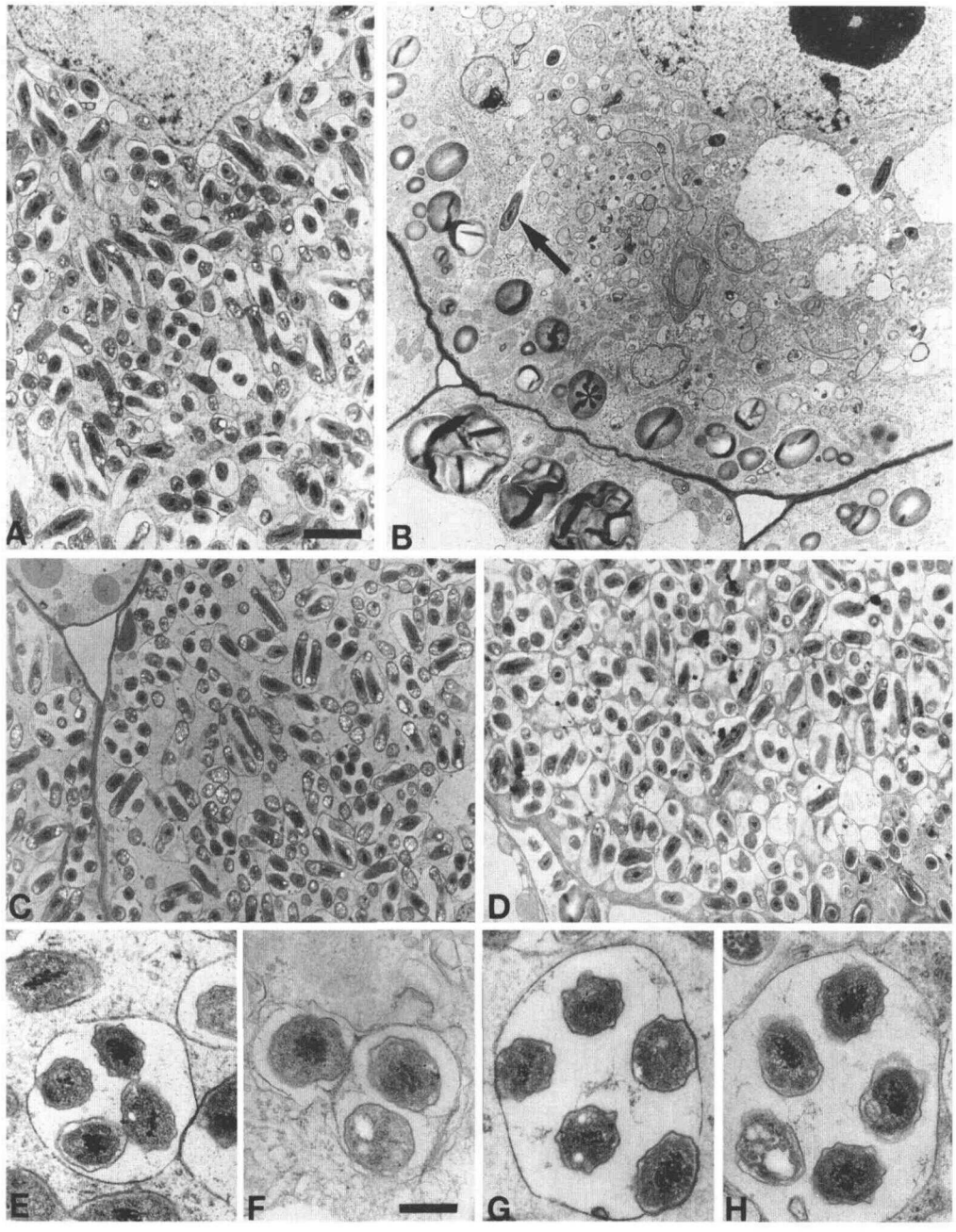

Figure 3. Transmission electron micrographs of wild-type and *sucA* mutant bacteroids. Nodules formed by USDA110 (wild type) or LSG184 (*sucA* mutant) were picked and prepared for electron microscopy as described in "Materials and Methods." Micrographs are of nodules formed by USDA110 at 19 DAI (A and E) and 32 DAI (C and G) and for LSG184 at 19 DAI (B and F) and 32 DAI (D and H). In B, a bacteroid (arrow) and starch grain (asterisk) are indicated. A to D, Magnification = ×5,300; scale bar = 2 μm. E to H, Magnification = ×26,000; scale bar = 0.4 μm.

as an additional measure of nitrogenase activity in the mutant strain, we examined the amount of nitrogenase Fe protein in LSG184 bacteroids. Extracts were made from 32-d-old nodules formed by the wild type or mutant, and equivalent amounts of soluble protein were resolved by SDS-PAGE and transferred to nitrocellulose. When probed with antibody against nitrogenase Fe protein, the extracts from the mutant cells showed just as strong a reaction as those from the wild type (Fig. 5). The stained protein profiles of the mutant and the wild type were also indistinguishable (data not shown), a further indication that the LSG184 inoculum had differentiated into normal bacteroids.

Nodulation Kinetics of LSG184

The acetylene reduction activity and morphology of nodules formed by LSG184 indicated an overall delay in sym-

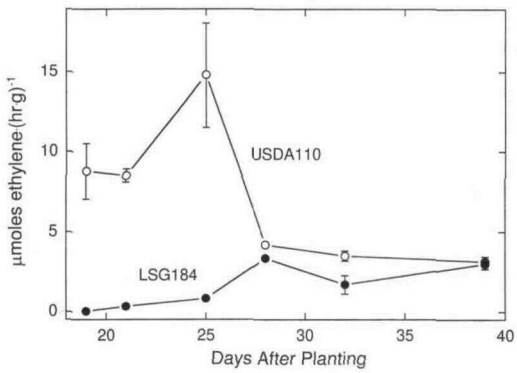

Figure 4. Acetylene reduction activity of soybean root crowns nodulated by the wild-type or *sucA* mutant of *B. japonicum*. Plants were grown in modified Leonard jars and inoculated with either USDA110 (wild type, ○) or LSG184 (*sucA* mutant, ●) at the time of planting. At each time point, three plants in each sample were assayed for acetylene reduction activity. Error bars indicate SES.

biotic development. To see how early in symbiosis this delay was manifested, we looked at the kinetics of nodulation by LSG184 on pouch-grown plants (Pueppke and Payne, 1987). Plants inoculated with the mutant formed visible nodules about 5 d later than those inoculated with USDA110 (Fig. 6). At the lower inoculum density (10^5 cfu/mL) only about 70% of the plants inoculated with LSG184 had nodulated by the end of the experiment. Plants inoculated with LSG184 also formed fewer nodules per plant and the nodules tended to form further down on the root relative to the wild type (Table III). The presence of a complementing cosmid (pLAFR1/*mdh*; Green and Emerich, 1997) in the mutant returned the nodulation phenotype to normal (Table III; nodulation kinetics not shown).

DISCUSSION

We have described here the symbiotic phenotype of an α-ketoglutarate dehydrogenase-deficient mutant of *B. japonicum* USDA110. Although this mutant, LSG184, grows well under most free-living culture conditions (Green and Emerich, 1997), during symbiosis it showed a persistent developmental delay relative to the wild type, including a lag in nodule initiation and expansion and in the onset of nitrogen fixation. Despite this defect, and in the continuing absence of α-ketoglutarate dehydrogenase activity, the mutant was eventually able to form effective, nitrogen-fixing bacteroids.

Because the α-ketoglutarate dehydrogenase mutant was made by inserting a Kan^r marker into *sucA*, a polar effect on the transcription of downstream genes in this strain could contribute to its phenotype. Indeed, northern hybridizations show that the interruption of *sucA* also eliminates expression of *sucB*, which lies just downstream of *sucA* and encodes the E2 component of α-ketoglutarate dehydrogenase (Green and Emerich, 1997). However, elimination of *sucB* expression is not expected to have any phenotypic effect beyond the loss of α-ketoglutarate dehydrogenase already caused by the inactivation of *sucA*.

We do not know what genes lie downstream of *sucB* and might also form part of the *sucAB* operon, although, based on the organization of these genes in other bacteria, it is unlikely that there are any additional cotranscribed genes.

Table I. *Average nodule fresh weight and acetylene reduction activity of soybean plants inoculated with various strains of B. japonicum*

Soybeans were grown in Leonard jars and inoculated with the wild-type (USDA110), the *sucA* mutant (LSG184), or the same strains carrying a complementing cosmid (pLAFR1-*mdh*) with a wild-type copy of *sucAB* (110/*mdh* and 184/*mdh*). After 25 d, the acetylene reduction activity of the root crowns was assayed and the average fresh weight of the nodules was determined as described in "Materials and Methods." Values represent the means ± SE of data collected from three plants in each treatment.

Inoculum	Nodule Fresh Wt	Acetylene Reduction Activity
	mg	*µmol g^{-1} nodules h^{-1}*
USDA110	11.8 ± 1.4	4.0 ± 0.9
LSG184	6.6 ± 0.5	0.25 ± 0.01
110/*mdh*	12.1 ± 0.8	6.7 ± 1.8
184/*mdh*	12.8 ± 2.6	8.6 ± 1.8

Table II. *Yield and enzyme activities of bacteroids isolated from nodules formed by the wild-type (USDA110) and sucA mutant (LSG184) strains of B. japonicum*

Soybean plants were grown in modified Leonard jars until 29 DAI with USDA110 or 32 DAI with LSG184. Bacteroids were purified from nodules as described in "Materials and Methods," pelleted, and weighed. Extracts were made from the purified bacteroids, desalted, and assayed for α-ketoglutarate dehydrogenase (KGDH) and malate dehydrogenase (MDH) activities. Values represent the results from one experiment.

Inoculum	Bacteroid Yield	KGDH	MDH
	mg cells g^{-1} nodule	*nmol mg^{-1} protein min^{-1}*	
USDA110	121	61.7	1508
LSG184	26	0.6	1760

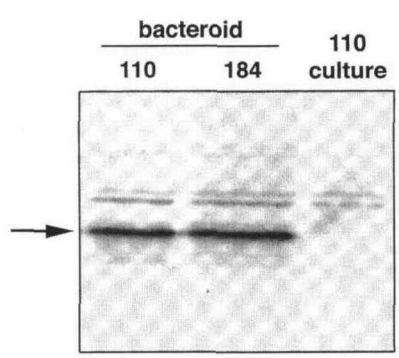

Figure 5. Immunodetection of nitrogenase protein in bacteroid extracts. Soluble protein extracts from isolated 29-d-old USDA110 (wild type) or 32-d-old LSG184 (*sucA* mutant) bacteroids were prepared as described in "Materials and Methods." Proteins were resolved by SDS-PAGE, transferred to nitrocellulose, and probed with antisera raised against the Fe protein of nitrogenase. Bacteroid samples contained 10 µg of protein per lane and the sample from cultured cells contained 5 µg of protein. The arrow indicates the band corresponding to the Fe protein of nitrogenase.

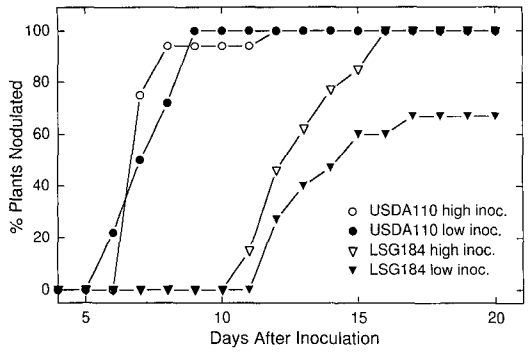

Figure 6. Pouch nodulation test of the wild-type and *sucA* mutant of *B. japonicum*. Plants were grown in pouches and inoculated with either USDA110 (wild type; 10^8 and 10^5 cfu/mL) or LSG184 (*sucA* mutant; 10^8 and 10^5 cfu/mL). Roots were visually inspected each day for the presence of nodules. There were 13 to 18 plants in each treatment.

The *sucA* mutant was successfully complemented with a cosmid containing a wild-type copy of *sucAB* (Green and Emerich, 1997), but because the cosmid also carries an extensive stretch of uncharacterized downstream DNA, the possibility still remains that polarity of the insertion in *sucA* contributes to the phenotype of LSG184. Nevertheless, this uncertainty does not in any way undermine the conclusion that *B. japonicum* can form effective bacteroids in the absence of α-ketoglutarate dehydrogenase.

The delay in nodule initiation by the *sucA* mutant suggests that it may have some deficiency in the very earliest stages of symbiosis, either in the stimulation of nodule primordia or in the colonization or invasion of the root. Many different mutations have been found to cause a delayed nodulation phenotype in *B. japonicum*. For example, defects in extracellular polysaccharide production (*exoB*; Parniske et al., 1993, 1994), as well as in unknown functions involved in host specificity (Deshmane and Stacey, 1989), nodulation (*nod-1*, *nod-2*; Hahn and Hennecke, 1988), or competitiveness (*nfeC*; Chun and Stacey, 1994), can lead to a 2- to 6-d delay in nodulation of soybean plants.

There are several possible mechanisms that could explain the delayed nodulation phenotype of LSG184. The loss of α-ketoglutarate dehydrogenase activity could lead to a growth deficiency in the rhizosphere that could impede the infection process (Streit et al., 1996). It is also possible that the loss of this enzyme has a pleiotropic effect on the production of cell-surface polysaccharides or of the Nod factor, both of which are required for efficient nodulation (Stacey et al., 1991, 1995; Parniske et al., 1993, 1994; Eggleston et al., 1996). Exogenous dicarboxylic acids, which as TCA cycle intermediates might accumulate in LSG184, are known to have a dampening effect on the induction of *nod* genes in *B. japonicum* (Yuen and Stacey, 1996), revealing a potential regulatory link between intermediary carbon metabolism and Nod factor production. Finally, the insertion in *sucA* used to construct LSG184 could have a polar effect on downstream genes involved in nodulation, which results in the delayed nodulation phenotype.

The extreme delay in the onset of nitrogen fixation by LSG184 suggests that this strain is also deficient at symbiotic steps beyond nodule initiation and invasion of the root. Furthermore, the morphology of nodules formed by LSG184 indicates that fewer host cells than normal were infected by the invading bacteria. A strikingly similar infection pattern was seen by Müller et al. (1995) for a signal peptidase mutant of *B. japonicum*, and they proposed that this resulted from the plant cells in the nodule primordium dividing faster than they could be infected by the mutant bacteria. In the developmental program of the determinate soybean nodule there may also be a specific phase during which nodule cells can be infected and, once that window is passed, no new infection events can take place. If this is the case, any bacterial lesion causing a delay in infection threads reaching the appropriate cells of the nodule primordium, or in the release of bacteroids into those cells, could give rise to a patchy infection pattern.

Transmission electron microscopy of nodules formed by LSG184 indicated that the mutant may also take longer to fill the cytoplasm of successfully infected host cells. Both of these phenotypes could be explained if the loss of α-ketoglutarate dehydrogenase leads to slower proliferation of *B. japonicum* within both the infection thread and the symbiosome. Although the mutant grew well under most culture conditions (Green and Emerich, 1997), it showed a significantly longer doubling time than the wild type on several carbon substrates. Even a small deficiency in growth rate during the infection process could lead to many fewer bacterial cells during the course of several days of symbiotic development. Little is known about plant functions controlling the final number of infected cells in the determinate nodule or how they might be influenced by the invading bacteria.

The total amount of acetylene reduction activity displayed by LSG184 on a per nodule fresh weight basis was extremely delayed and low. However, because nodules formed by the mutant contained only about 20% of the normal number of bacteroids, it can be inferred that the mutant bacteroids, on an individual basis, are capable of fixing nitrogen at a rate near that of the wild type. Furthermore, purified mutant bacteroids contained normal levels of the Fe protein of nitrogenase. These results argue against an absolute requirement for α-ketoglutarate dehydrogenase in the differentiation of *B. japonicum* bacteroids, and they further imply that alternate routes for catabolizing malate and succinate are sufficient to meet the energy demands of nitrogen fixation. This surprising conclusion is consistent with the unusual ability of LSG184, in contrast to α-ketoglutarate dehydrogenase mutants of other bacteria, to grow on malate or succinate as its sole carbon source (Green and Emerich, 1997).

The symbiotic phenotype of LSG184, assuming there is no polarity on nodulation genes in this strain, implies that the early stages of symbiosis are more affected by the loss of α-ketoglutarate dehydrogenase than is the functioning of mature bacteroids. There are several possible reasons why this might be so. First, the metabolic rates required to

Table III. *Number and position of nodules formed on pouch-grown plants inoculated with various strains of B. japonicum*

Presprouted soybean plants were transferred to pouches as described in "Materials and Methods" and inoculated with the wild type (USDA110) or *sucA* mutant (LSG184) of *B. japonicum*. 110/*mdh* and 184/*mdh B. japonicum* strains contained a complementing cosmid clone carrying a wild-type copy of *sucA*. After 21 d (for USDA110 and LSG184) or 16 d (for 110/*mdh* and 184/*mdh*) the total number of nodules on each plant and the position of the topmost nodule were recorded. Negative values for the position measurement indicate that the topmost nodules were above the root tip mark. Results are means ± SE. The number of plants in each sample is indicated in parentheses.

Inoculum		Nodule/Plant	Highest Nodule Position
cfu mL^{-1}		*no.*	*mm below tip mark*
USDA110	10^5 (18)	7.3 ± 0.9	3.9 ± 2.0
	10^8 (16)	12.4 ± 1.6	1.6 ± 1.3
LSG184	10^5 (15)	0.7 ± 0.2	25.5 ± 11.6
	10^8 (13)	3.4 ± 0.8	26.0 ± 7.8
110/*mdh*	10^8 (8)	10.6 ± 1.0	−3.5 ± 3.0
184/*mdh*	10^8 (8)	9.5 ± 1.0	−0.1 ± 2.6

maintain nitrogen fixation in mature bacteroids may be lower than those needed early in symbiosis, when the invading bacteria are actively proliferating. Thus, alternate catabolic pathways that might be adequate later in symbiosis could prove to be limiting, either to energy production or the supply of a necessary metabolite, during growth and/or differentiation. Second, the nutrients available to the invading bacteria probably vary during the course of symbiotic development, and this may influence the performance of the mutant cells at different stages. Third, alternate metabolic pathway(s) that can compensate for the loss of α-ketoglutarate dehydrogenase may be regulated in such a way as to be more available to the mutant later in symbiotic development.

Our data show that LSG184, despite its TCA cycle defect, is able to form effective bacteroids, albeit in reduced numbers. This result is in contrast to the Fix$^-$ phenotype of a previously described α-ketoglutarate dehydrogenase mutant of *R. meliloti* (Duncan and Fraenkel, 1979). There are several possible explanations for this difference. First, because the data used to classify the *R. meliloti* mutant as Fix$^-$ were not presented, their conclusiveness cannot be evaluated. For example, a delayed nitrogen fixation phenotype similar to that of LSG184 would have appeared to be completely Fix$^-$ if acetylene reduction activity was measured only at a relatively early time or assessed on the basis of plant yellowing. Second, although the *R. meliloti* mutant was deficient in α-ketoglutarate dehydrogenase activity, the nature of the genetic lesion was unknown and may have differed from a simple inactivation of one of the structural genes for the enzyme. Third, *R. meliloti* and *B. japonicum* are not closely related taxonomically and may differ in their ability to compensate for the loss of α-ketoglutarate dehydrogenase activity during symbiosis; for example, *B. japonicum* may be more able than *R. meliloti* to activate an alternate pathway for metabolizing dicarboxylic acids. Fourth, nodules formed by *R. meliloti* and *B. japonicum*, being indeterminate and determinate, respectively, might be affected differently by defects in bacteroid carbon metabolism. For example, the development of soybean nodules may be less dependent on ongoing infection thread growth than is the development of alfalfa nodules and, therefore, may be less sensitive to bacterial mutations affecting in this process.

In the past there has been considerable uncertainty about the true in vivo activity of bacteroid enzymes such as α-ketoglutarate dehydrogenase, which are subject to inhibition by highly reduced pyridine nucleotide pools. We have shown here that, if bacteroid α-ketoglutarate dehydrogenase is indeed significantly inhibited during nitrogen fixation, then *B. japonicum* appears to have the metabolic flexibility to compensate for this inhibition. In the future, it will be important to determine what metabolic pathways are being used by the α-ketoglutarate dehydrogenase mutant to catabolize malate and succinate and to then determine whether these pathways play a role during symbiotic nitrogen fixation by wild-type bacteroids.

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