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Requirement of Succinate Dehydrogenase Activity for Symbiotic Bacteroid Differentiation of *Rhizobium meliloti* in Alfalfa Nodules†

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Transmission electron microscopy was used to study the cellular morphologies of a wild-type *Rhizobium meliloti* strain (L5-30), a nitrogen fixation-ineffective (Fix⁻) succinate dehydrogenase mutant (Sdh⁻) strain, and a Fix⁺ Sdh⁺ revertant strain within alfalfa nodules and after free-living growth in a minimal medium containing 27 mM mannitol plus 20 mM succinate. The results showed a requirement of succinate dehydrogenase activity for symbiotic differentiation and maintenance of *R. meliloti* bacteroids within alfalfa nodules and for succinate-induced cellular pleomorphism in free-living cultures. Also, the Sdh⁻ strain had a 3.5-fold lower rate of oxygen consumption in the defined medium than did the wild type.

Succinate is an abundant organic acid within legume root nodules (7, 19), and this dicarboxylic acid effectively supports nitrogen fixation by both free-living *Rhizobium* species (6, 28) and nodule bacteroids (5, 20). Organic acids are transported (8, 11, 14) and metabolized (17, 18) by rhizobia and also support the highest rate of oxygen consumption by respiring bacteroids (22). Utilization of tricarboxylic acid intermediates is related to symbiotic effectiveness (1), and *Rhizobium* mutant strains defective in the uptake and metabolism of tricarboxylic acid intermediates are ineffective in nitrogen fixation (Fix⁻) within legume root nodules (2, 8, 9, 10, 16).

There is considerable interest in understanding bacteroid development from vegetative cells of rhizobia because of its importance to root nodule symbiosis and also as an example of procaryotic differentiation. A clue to understanding morphological transformation is available from studies which showed that succinate added to an exponentially growing culture of *Rhizobium trifolii* causes swelling of the bacteria and can induce each of the particular pleomorphic shapes of nodule bacteroids (23, 25). Recently, Urban et al. (24) demonstrated nitrogen-fixing activity (^{15}N incorporation and acetylene reduction) accompanying succinate-induced swelling of free-living *R. trifolii* cultures.

The purpose of this study was to determine if a previously described Fix⁻ succinate dehydrogenase (EC 1.3.99.1) mutant (Sdh⁻) strain of *R. meliloti* (10) was able to differentiate morphologically into bacteroids in alfalfa nodules and undergo succinate-induced pleomorphism in free-living cultures.

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R. meliloti L5-30 Str^r Fix⁺ (wild-type strain) was obtained from J. Denarie. UR6 is an Sdh⁻ strain of *R. meliloti* L5-30 which can transport succinate and grow on other C_4-dicarboxylates (e.g., fumarate or malate) but not on succinate (10). This mutant induces Fix⁻ nodules on alfalfa plants (10). UR7 is a spontaneous revertant strain of UR6 which has an Sdh⁺ Fix⁺ phenotype (10). Stock cultures were grown on agar slants of a minimal medium (MM) (26) containing streptomycin sulfate (100 μg/ml), nitrate as the nitrogen source (no amino acids), and 0.5% mannitol as the carbon source. This defined medium contains higher total concentrations of calcium and magnesium than did the complete medium used by Vincent and Colburn (27) in their study of cell pleomorphisms in *R. trifolii* to avoid cellular abnormalities caused by a deficiency of these cations. For nodulation studies, the inoculum was grown for 5 days at 30°C. For broth culture studies, a standardized inoculum of 0.5% glycerol-grown cells was inoculated into MM contain-

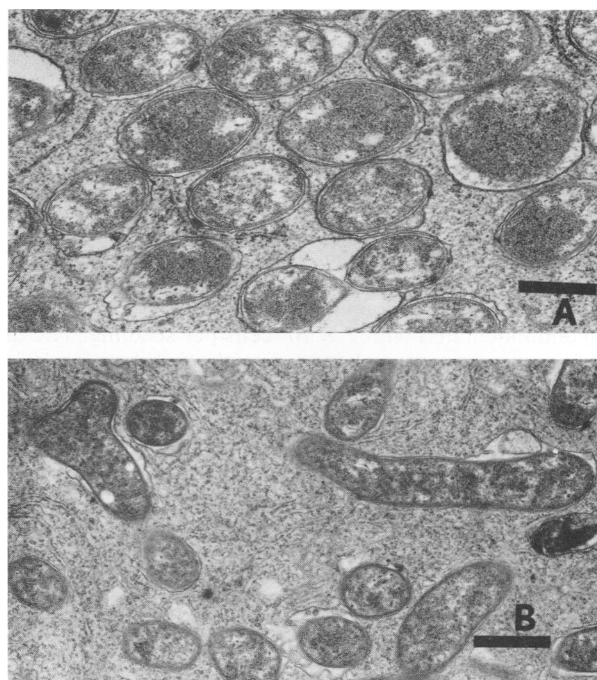

FIG. 1. Ultrastructure of alfalfa root nodules induced 6 weeks after inoculation with the UR7 revertant strain. Both panels show portions of a plant cell containing bacteroids in the central zone. Bars, 1 μm.

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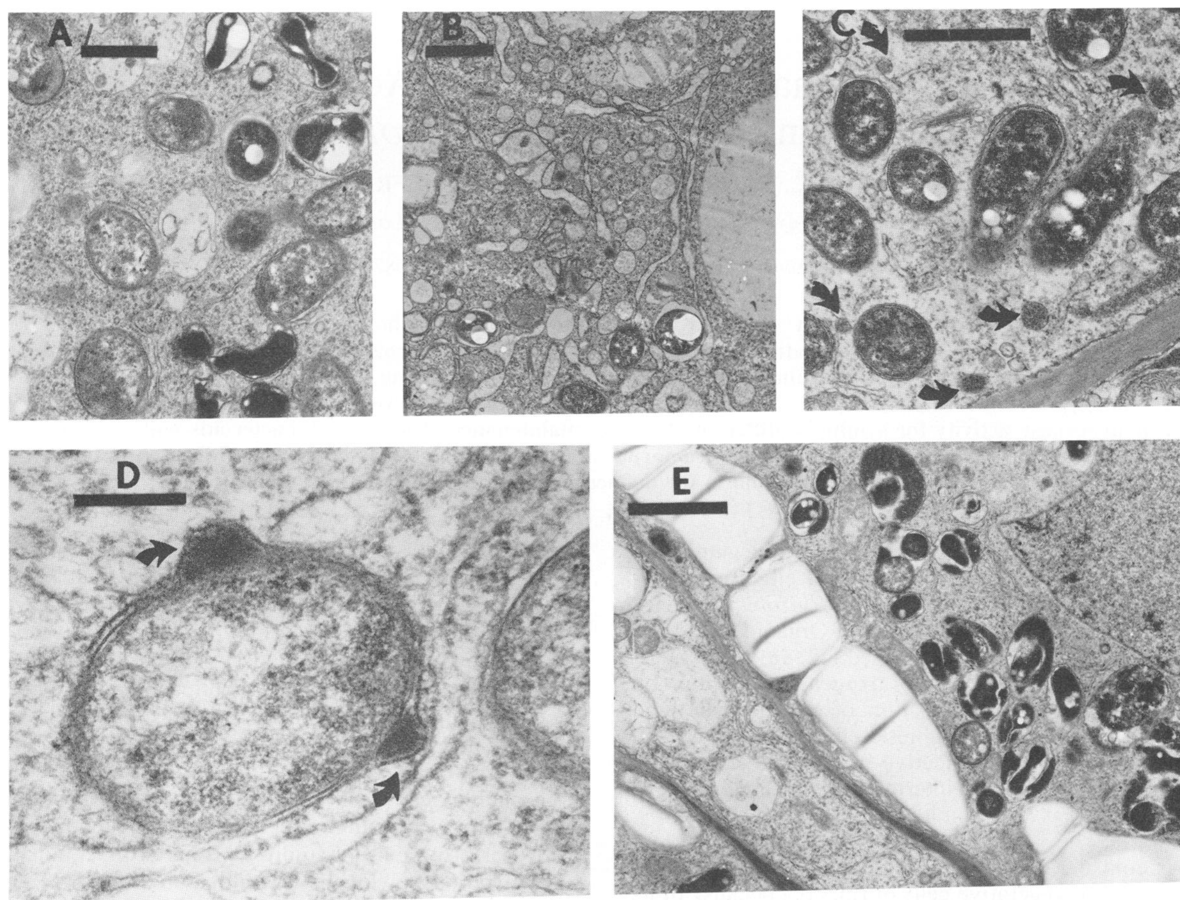

FIG. 2. Ultrastructure of alfalfa root nodules induced 3 weeks after inoculation with the Sdh$^-$ UR6 strain. (A) Degenerated bacteria in the proximal infection zone. (B) Enlarged profiles of rough endoplasmic reticulum in the distal infection zone. (C) Lysosomes (arrows) in the distal infection zone. (D) Electron-dense granules (arrows) commonly associated with bacteria in the distal infection zone. (E) Bacteria undergoing degeneration in host cells in the distal central zone. Note the ultrastructurally well-preserved host cytoplasm. Bars, 2 μm in panels A, B, C, and E and 0.5 μm in panel D.

ing the indicated carbon source but not streptomycin. Three replicate parallel cultures were examined. Growth was monitored turbidimetrically at 660 nm directly in sidearm flasks by using a Klett-Summerson colorimeter with the no 66 red filter or after dilution of samples with sterile broth medium.

Axenic cultures of alfalfa (*Medicago sativa* cv. Vernal) seedlings were grown on enclosed, nitrogen-free agar slopes (26) and inoculated with 5×10^6 cells per seedling. The tube cultures were incubated in a growth chamber for a 14-h photoperiod at 22°C (26,900 lx) and for 10 h in darkness at 20°C. Roots were examined weekly, and plants were assayed for nitrogen fixation by the acetylene reduction technique (10) after 6 weeks. The mutant strain recovered from surface-sterilized nodules had the original Sdh$^-$ Str^r phenotype. Root nodules were fixed in 4% glutaraldehyde in sodium cacodylate buffer (pH 7.2), postfixed in 1% OsO_4, dehydrated with an ethanol series, and embedded in Epon 812, and thin sections were stained by the uranyl acetate-lead citrate method (21). Transmission electron microscopy was performed with a Philips 300 instrument at 80 kV. Also, semithin sections were stained by the basic fuchsin-methylene blue method (13) for direct light microscopy.

Bacteria grown in free-living cultures were centrifuged at 12,000 × *g*, washed twice in phosphate-buffered saline (10 mM potassium phosphate, 140 mM NaCl [pH 7.2]), deposited on Formvar-coated grids, stained by the glutaraldehyde-ruthenium red-uranyl acetate technique (15), and observed by transmission electron microscopy. Oxygen consumption was measured for shaken cultures grown in 250-ml flasks containing 50 ml of MM with 27 mM mannitol plus 20 mM succinate. At the late exponential phase (100 Klett units), an oxygen electrode (New Brunswick Scientific Co., Inc., Edison, N.J.) was introduced into the cultures immediately after shaking was stopped, and the decrease in dissolved oxygen was recorded over a 30-min period. The oxygen electrode was calibrated with 5% sodium dithionite (0% dissolved oxygen) and distilled water saturated with air (100% dissolved oxygen) at 25°C, in accordance with the instructions of the manufacturer.

Meristematic nodules induced by strains L5-30 and UR7 were effective in nitrogen fixation (acetylene reduction). These nodules had typical histologies when observed by light microscopy (data not shown) and typical ultrastructures when observed by transmission electron microscopy, showing normal bacteroid differentiation and maintenance throughout a growth period of 6 weeks. Typical results are shown for the UR7 revertant in Fig. 1A and B. Nodules induced by strain UR6 were completely ineffective in acetylene reduction. At 3 weeks, transmission electron microscopy of the nodules showed that the UR6 cells were released

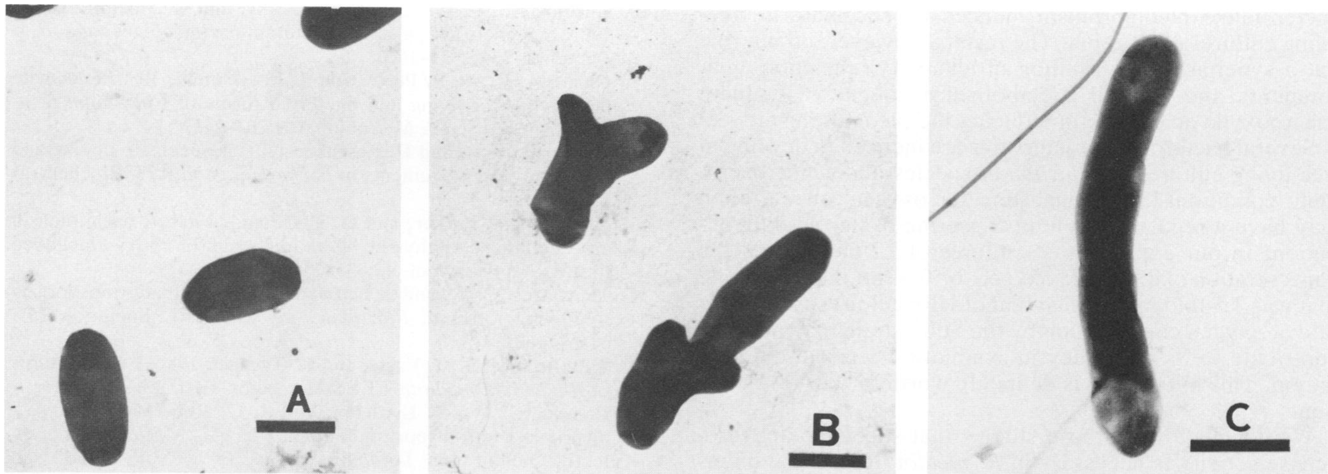

FIG. 3. Cell morphology of wild-type *R. meliloti* L5-30 grown with mannitol as the sole carbon source (A) and with mannitol plus succinate (B and C). Bars, 1 μm.

from infection threads and surrounded by peribacteroid membranes in the host cells. However, in contrast to L5-30 and UR7 cells, very few UR6 cells had morphologically differentiated into bacteriods. Also, a process of premature degeneration and lysis of the UR6 bacteria had occurred in the infection and central zones of the nodules. These processes were indicated by the presence of degenerated bacteria, an enlarged rough endoplasmic reticulum, and the proliferation of lysosomes and very electron-dense granules often associated with the bacteria (Fig. 2A to E). Thus, the root nodule symbiosis between alfalfa and strain UR6 was blocked at some stage in bacteroid differentiation following bacterial release from infection threads. At 6 weeks, the Fix$^-$ nodules induced by UR6 were fully senescent, and the bacteria were lysed in all zones. At this time, L5-30 and UR7 bacteroids were typically degenerated only in the normal senescent zone of the Fix$^+$ nodules.

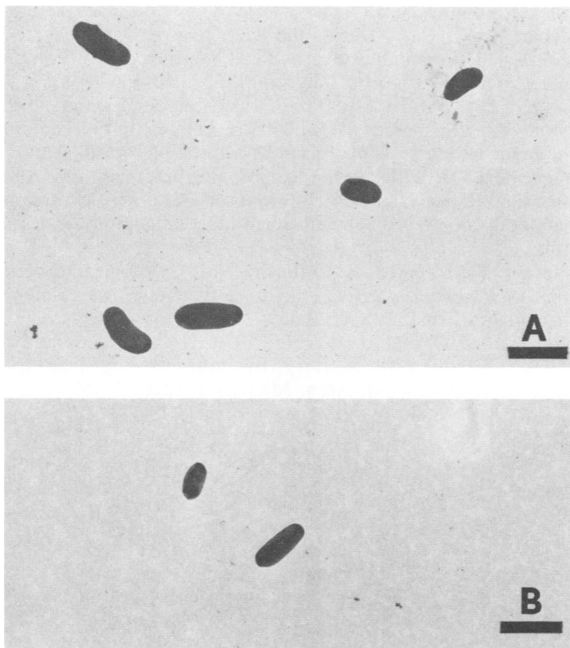

FIG. 4. Cell morphology of the Sdh$^-$ UR6 strain grown with mannitol as the sole carbon source (A) and with mannitol plus succinate (B). Bars, 2 μm.

Similar to our results with *R. meliloti* UR6, dicarboxylate transport mutants of *R. leguminosarum* and *R. trifolii* induced in their respective hosts Fix$^-$ nodules which have a less extensive bacteroid zone than do Fix$^+$ nodules and which display premature senescence and lysis (2, 9, 16). However, these mutants differ in symbiotic phenotype from UR6 in their ability to develop bacteroids (2, 9, 16), albeit they are smaller than the wild-type bacteroids within the nodule (16). This ability is probably the result of the metabolism of a residual level of C_4-dicarboxylates transported in the endosymbiotic state or of the metabolism of an internal pool of tricarboxylic acid cycle intermediates provided by some other carbon compounds which the mutants can transport.

Wild-type L5-30 can grow on either 20 mM succinate or 27 mM mannitol as the sole carbon source. The addition of 20 mM succinate affected the growth of L5-30 in MM containing 27 mM mannitol, lowering yields at the plateau of the stationary phase by approximately 40% (360 Klett units versus 214 Klett units). The wild-type cells were rod shaped when grown in MM containing mannitol alone but displayed elongation and branching when succinate was included. This cell pleomorphism was observed in the L5-30 culture at several growth phases, with the maximal proportion of pleomorphic cells occurring at the early stationary phase (150 Klett units). At this stage, approximately 15% of 100 cells examined had a bacteroidlike morphology (Fig. 3A to C). In contrast, the Sdh$^-$ strain neither was affected in cell yield at the stationary phase (387 Klett units verus 380 Klett units) nor displayed cell pleomorphism when grown in MM containing both succinate and mannitol (Fig. 4A and B).

A pH rise could be detected in the *R. meliloti* cultures when C_4-dicarboxylates (succinate or malate for the wild-type strain and malate for the Sdh$^-$ strain) were used as carbon sources. However, under these conditions, growth inhibition and bacteroidlike morphology were observed for the wild-type strain but not for the Sdh$^-$ strain. These results indicate that neither alkali production from symport of C_4-dicarboxylates, as occurs in *E. coli* (12), nor succinate-mediated chelation of divalent cations required for normal cell development is the major event responsible for the

bacteroidlike pleomorphism induced by succinate in free-living cultures of rhizobia. The results, however, do not rule out a synergistic contribution of alkalinity, chelation environment, and normal metabolism through a complete tricarboxylic acid cycle in inducing the pleomorphism.

Several bacteroid characteristics are induced to develop in free-living cultures of *Rhizobium* species under microaerophilic conditions (3, 4), suggesting that oxygen concentration may have a regulatory role in triggering bacteroid differentiation. In our experiments, cultures of L5-30 reduced the concentration of dissolved oxygen to 50% saturation at a rate that was 3.5-fold higher than that of UR6 cultures. The lower rate of oxygen consumption by the Sdh^- strain may possibly contribute to its deficiency in symbiotic bacteroid differentiation. This hypothesis is definitely worth further investigation.

We conclude from these studies that succinate dehydrogenase activity is necessary in *R. meliloti* for normal differentiation, maintenance, and function of alfalfa bacteroids.

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