Biotin and Other Water-Soluble Vitamins Are Key Growth Factors for Alfalfa Root Colonization by Rhizobium meliloti 1021

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Rhizosphere growth limitations imposed on Rhizobium meliloti by availability of biotin, thiamine, and riboflavin were overcome by adding nanomolar amounts of these vitamins. Studies done with R. meliloti 1021 showed that both synthesis and uptake of biotin promote colonization of alfalfa roots. Two lines of evidence indicated that plant-derived biotin normally promotes root colonization: (i) adding avidin significantly (P ≤ 0.01) reduced rhizosphere growth of R. meliloti 1021, and (ii) growth of Tn5-induced biotin auxotrophs still increased 10-fold in the rhizosphere. Synthesis, however, is the more important source of biotin for R. meliloti 1021 because in root colonization tests biotin auxotrophs competed very poorly with the parent strain. Mutations conferring biotin auxotrophy were closely linked on a single restriction fragment, and one was complemented with the Escherichia coli bio operon. Initial nucleotide sequencing and DNA-DNA hybridization tests showed the biotin synthesis genes in R. meliloti are quite different from those in E. coli.

Additional keyword: Medicago sativa.

Rhizobium meliloti, an agriculturally important bacterium that forms N₂-fixing root nodules on alfalfa (Medicago sativa L.), persists at low levels in soil and must compete with other bacteria to colonize host plant roots (Bottomley 1992). Details of complex plant-microbe signaling required for root nodule formation are well characterized (Dénarié et al. 1992), but less is known about interactions that favor root colonization (Beattie and Handelsman 1993; Bolton et al. 1993; Triplett and Sadowsky 1992). Limited data on root exudation of bacterial growth substrates (e.g., organic acids), growth stimulants (e.g., vitamins), and regulatory signals (e.g., flavonoids) remain to be assembled into a logical framework that explains both the rapidity and the specificity of root colonization (Phillips and Streit 1996).

The water-soluble vitamins biotin, thiamine, and riboflavin, which are released from alfalfa roots (Rovira and Harris 1961), promote Rhizobium growth under laboratory conditions (West and Wilson 1939). As yet, however, there is no evidence that these compounds in root exudates affect growth of bacteria in the rhizosphere, which normally is defined to include the root surface and closely adhering soil particles. Biotin, the primary subject of this study, is a cofactor for many carboxylases (Knowles 1989), and CO₂ is required for growth of R. meliloti (Lowe and Evans 1962). Thus, it is reasonable to hypothesize that plant-derived biotin may contribute toward root colonization through some mechanism involving CO₂. To measure directly whether R. meliloti uses plant-derived biotin to grow on alfalfa roots, several biotin auxotrophs were produced and biotin availability in the alfalfa rhizosphere was assessed. Data concerning biotin synthesis and/or uptake in a highly evolved symbiont like R. meliloti may lead to strategies that favor establishment of genetically altered bacteria in the rhizosphere.

RESULTS

Growth of R. meliloti 1021 under laboratory and rhizosphere conditions.

The response of R. meliloti 1021 (Rm1021) to biotin in GTS medium (Fig. 1) confirmed that this strain shows a classical biotin stimulation of growth. Maximum growth rate was produced with 400 pM biotin, but supplemental biotin concentrations as low as 4 pM stimulated growth on GTS medium. Availability of the biotin-binding reagent avidin allowed us to produce medium that contained <0.1 pg/ml biotin.

Fig. 1. Effects of biotin on growth of Rhizobium meliloti 1021. Cells were grown ± supplemental 4.0 nM biotin in GTS medium.
in enzyme-linked immunosorbent assay (ELISA). The term "bixin-free" is used for such avidin-treated media.

Rm1021 approached maximum colonization density on Moapa 69 alfalfa roots 6 days after inoculation (Fig. 2), and root nodule primordia appeared on day 7 or 8 under these experimental conditions. Consequently, day 6 was selected as a time for measuring root colonization. Average alfalfa root length (mean ± standard error) on day 6 was 9.4 ± 0.4 cm. Minimal numbers of bacterial cells (<100) were inoculated on the root of each at day 0, and 4.8 × 10⁶, 6.6 × 10⁶, and 7.4 × 10⁶ CFU per root were counted on day 6 for Rm1021 in three separate experiments. Thus, in this experimental rhizosphere Rm1021 typically doubled 10.5 times. Although the term "rhizosphere" is used in this context because traces of vermiculite adhered to harvested roots, most bacteria presumably were attached to the root. No extensive measurements of bacterial growth outside the harvested root-vermiculite complex were made, but plating tests showed that significant numbers of bacteria remained in Leonard jars after roots were removed (data not shown). Thus, all values reported here for root colonization underestimate true growth of the initial inoculum.

Adding trace amounts of biotin, thiamine, or riboflavin to alfalfa rhizospheres increased growth of Rm1021 to 3.5- to 6-fold higher titers than unsupplemented controls in the normal 6-day assay (Fig. 3). Several lines of evidence showed that bacterial cells normally had access to some biotin from the plant. First, direct ELISA measurements of biotin released by alfalfa seedlings showed that approximately 15 pg of biotin could be released from the root of a 4-day-old seedling. Second, adding small quantities of avidin decreased rhizosphere growth of Rm1021 in the normal rhizosphere (Fig. 4). When the biotin concentration available for rhizobial uptake was artificially lowered with avidin, Rm1021 was restricted to 7.5 doublings compared with the 10.5 doublings recorded for untreated control rhizospheres over the same 6-day period. In contrast, biotin-enriched rhizospheres supported 13.2 doublings. We conclude from these data that plant-derived biotin normally contributes to rhizobial growth and that the combination of plant and bacterial biotin is insufficient for optimum growth of Rm1021. An absence of complexing agents for thiamine and riboflavin prevented detailed studies with these compounds, and no ELISAs were developed to quantify these molecules directly.

Isolation and molecular characterization of R. meliloti biotin auxotrophs.

Initial tests established that Rm1021 is a capable biotin synthesizer. For example, ELISAs showed that stationary phase cultures of Rm1021 grown on GTS medium without supplemental biotin exuded 0.1 to 1 pg of biotin per ml. To

![Fig. 3. Effects of water-soluble vitamins on growth of Rhizobium meliloti 1021 in the alfalfa rhizosphere.](image)

![Fig. 4. Biotin limitations to growth of Rhizobium meliloti 1021 in the alfalfa rhizosphere. Bacteria were counted on roots 6 days after inoculating 52 cells per plant. Supplemental biotin (16 nmol) and avidin (2 μU) added on day 0, 2, and 4 had significant effects (P ≤ 0.01) on the mean number of cells recovered from four replicates.](image)
isolate genes responsible for this biotin synthesis, five mutants unable to grow in the absence of biotin were recovered from 3,000 Tn5-B30 recipients. While mutants Rm1021-B1, Rm1021-B3, and Rm1021-B6 failed to grow on GTS medium without supplemental biotin, mutants Rm1021-B2 and Rm1021-B4 showed biotin auxotrophy only when GTS medium was supplemented with aavid (0.04 units/ml). Because Rm1021-B1 and Rm1021-B2 were phenotypically identical to Rm1021-B3 and Rm1021-B4, respectively, and very closely linked in preliminary DNA mapping, they were not examined further.

Probing total genomic DNA from the mutants with known sequences from Tn5-B30 established that all insertions were located on a single 14-kb EcoRI DNA fragment (Fig. 5A). No EcoRI restriction sites are present in Tn5-B30. The presence of a single Tn5 insertion in each mutant was established by the additional fact that in every case XhoI DNA digests produced three bands in Southern analyses that hybridized with the probe and were consistent in size with published restriction sites in Tn5-B30 (Simon et al. 1989) (data not shown). Southern analyses of mutant lysates (Eckhardt 1978) showed no Tn5 insertions in plasmid DNA (data not shown), thus indicating the mutations are located on the bacterial chromosome.

A more detailed mapping of the different insertions was possible after a 2.9-kb XhoI overhanging DNA fragment from Rm1021-B4 was cloned into pBSK+. A standard reversed primer for pBSK+, as specified by the manufacturer, was used to sequence approximately 150 bp of the insert. This short DNA sequence was sufficient to make an internal polymerase chain reaction (PCR) primer (WS4063) reading toward the Tn5-B30 insertion. WS4063 and Tn5sout, a standard Tn5-outward reading primer, were used to amplify flanking regions of the putative biotin synthesis mutants. DNA fragments produced by this method from both Rm1021-B3 and Rm1021-B4 were approximately 1.4 kb (Fig. 5B). The same techniques yielded a 2.0-kb DNA fragment from Rm1021-B6; therefore, this insertion was approximately 600 bp distant from the Tn5 insertion of the other two mutants. Because this approach could not distinguish between Tn5 insertions in Rm1021-B3 and Rm1021-B4, these two mutations must be located very close together. The differing growth phenotypes in these two mutants, however, support a conclusion that they are not identical at the molecular level.

Mutant phenotypes of Rm1021-B3 and Rm1021-B6 were complemented by producing Rm1021-WS8 and Rm1021-WS9, respectively, with a cosmid from Rm1021 (Table 1). A 7.5-kb DNA probe for locating the corresponding region of Rm1021 DNA was made using long, inverse PCR with the Tn5sout primer and minicircles produced by self-ligation of an EcoRI DNA fragment that carried all the mutations. This 7.5-kb DNA fragment was isolated from an agarose gel, labeled with digoxigenin, and used to isolate corresponding wild-type

![Fig. 5. Analyses of three biotin auxotrophic mutants in Rhizobium meliloti 1021 (Rm1021). A, Southern analysis of total genomic DNA after EcoRI digestion. Gel blots were probed with Tn5-B30 sequences labeled with digoxigenin. Lane 1, Rm1021-B3; lane 2, Rm1021-B4; lane 3, Rm1021-B6. B, Polymerase chain reaction analysis of distances between Tn5 insertions in the three mutants. Lanes 1 to 3 as in A; lane M, 1-kb DNA marker ladder; lane 4, Rm1021. C, Restriction map of Rm1021 chromosomal DNA in pCosRmbio1 showing the location of the mutations. Arrows indicate insertion of Tn5-B30 in the different mutants. E: EcoRI; X: XhoI; H: HindIII; S: SalI.](image-url)
DNA from a cosmid bank containing Rm1021 DNA. One cosmid, pCosRm101, carrying the identified DNA fragment as an internal EcoRI DNA fragment was chosen and mated successfully into Rm1021-B3 and Rm1021-B6. Transconjugants grew on GTS medium in the absence of additional biotin and were Cm’ (50 mg/liter). Transconjugant Rm1021-W58 showed normal rhizosphere growth, while Rm1021-W59 grew better than the recipient parent Rm1021-B6 but significantly worse than the wild-type parent Rm1021 (Table 1). Various restriction enzymes were used to produce a map that located the mutations on pCosRm101 (Fig. 5C). A clone carrying the entire R. meliloti biotin synthesis operon from pCosRm101 is currently being sequenced.

Initial DNA sequence data obtained for Rm1021-B3 and Rm1021-B6 showed no significant homology at either the nucleotide or the deduced amino acid level with sequences reported for any genes in the databanks. Because only limited sequence data were available from our mutants, a direct test for homology to the Escherichia coli bio operon was conducted. A 7.5-kb digoxigenin-labeled probe for the E. coli locus was constructed with PCR and was used in DNA-DNA cross-hybridization tests to search for homologous sequences in total genomic DNA samples from E. coli K12 and Rm1021. The probe reacted strongly with E. coli K12 DNA and showed no significant homology to R. meliloti DNA under low stringency hybridization conditions (data not shown). The probe was used to locate a homologous 30-kb BamHI fragment in an E. coli K12 DNA cosmid bank; the fragment was given the name pCosEcbio1.

Complementation tests with pCosEcbio1 showed that the R. meliloti DNA locus mutated to produce biotin auxotrophy is functionally homologous to the E. coli bio operon (Table 1). This result, which is evident in the capacity of Rm1021-W510 to grow on GTS without additional biotin, was produced by mating pCosEcbio1 into Rm1021-B3. DNA analyses of one transconjugant clone using the E. coli bio operon as a DNA probe confirmed that transconjugant Rm1021-W510 carried both pCosEcbio1 and Tn5 from the original mutation (Fig. 6).

Supplementary experiments suggested that the R. meliloti biotin auxotrophs did not result from mutations in a homologue of the E. coli regulatory protein birA, which controls the bio operon (Cronan 1989). That inference was based on a failure to complement biotin auxotrophy in Rm1021-B3 with a birA-containing cosmid, pCosEcbirA, from the E. coli K12 cosmid bank.

### Rhizosphere growth of biotin auxotrophs.

Tests with biotin auxotrophs produced from Rm1021 (Table 2) supported the concept that biotin from both internal synthesis and plant exudation is important for rhizosphere growth. When 52 cells of Rm1021-B3 were inoculated into the experimental Moapa 69 alfalfa rhizosphere, the mutant persisted and doubled slightly more than three times during the first 6 days (Table 1). At that time, 520 bacteria were recovered from each root, which corresponded to less than 1% of the titer observed for the parent strain under the same conditions. Similar results were obtained for auxotrophs Rm1021-B4 and Rm1021-B6. Tests in which trace amounts of biotin were added to the alfalfa rhizosphere confirmed that these biotin auxotrophs retained an effective biotin uptake system because growth of the mutants under those conditions ranged from 15 to 88% of Rm1021. The crucial importance of internal biotin synthesis for rhizobial growth in the rhizosphere was confirmed by experiments in which nearly equal numbers (e.g., 65 vs. 66 CFU) of the parent and each mutant were coinoculated into the rhizosphere on day 0. In those trials the parent always outcompeted the mutant strains.

### DISCUSSION

Data from this study emphasize the ecological importance of water-soluble vitamins in the rhizosphere. Using an experimental rhizosphere, it was shown for the first time that colonization of plant roots can be limited by availability of biotin, thiamine, and riboflavin (Fig. 3). Interestingly, R. meliloti cells colonizing the alfalfa rhizosphere benefit from plant-derived biotin but are still limited by the availability of that vitamin (Fig. 4). While it has long been recognized that biotin promotes growth of R. meliloti (West and Wilson 1939) and that alfalfa roots release this vitamin (Rovira and Harris 1961), no direct contribution of plant vitamins to growth of rhizosphere bacteria has been documented. The importance of plant-derived biotin was supported by results from experiments with biotin auxotrophs generated by Tn5 mutagenesis (Table 1). Such auxotrophs face two extremes when introduced into the rhizosphere: (i) normal growth if the root releases optimum amounts of biotin, or (ii) no growth if plant-derived biotin is unavailable to rhizosphere cells. Our experiments showed an intermediate result. Thus, inocula containing fewer than 100 cells of the auxotroph Rm1021-B3 consis-

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### Table 1. Phenotypes of Rhizobium meliloti 1021 (Rm1021) biotin auxotrophs and complemented transconjugants

<table>
<thead>
<tr>
<th>R. meliloti strain</th>
<th>Growth on biotin-free GTS medium</th>
<th>Normal rhizospheres, single-strain inocula (%)</th>
<th>Biotin-enriched rhizospheres, single-strain inocula (%)</th>
<th>Normal rhizospheres, coinoculation with Rm1021 (%)</th>
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<tr>
<td>Rm1021</td>
<td>+</td>
<td>100 a</td>
<td>41 c</td>
<td>100 a</td>
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<tr>
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<td>88 a</td>
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<td>&lt;1 b</td>
<td>15 d</td>
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*Data are normalized to the titer observed for the different parent strain controls in the different experiments. Mean values followed by different letters varied significantly (P < 0.01) with bacterial treatments.
1 Supplemented with 0.04 U of avidin.
2 Rhizospheres were enriched with a total of 48 nmol of biotin in three additions.
3 Not determined.
tently doubled more than three times during 6 days in the alfalfa rhizosphere, but these cells competed very poorly with the parent strain Rm1021 in coinoculation experiments (Table 1). On the bacterial side of this interaction, these results imply that both synthesis and uptake of biotin contribute to successful root colonization.

These studies made no attempt to determine a biochemical basis for the vitamin-enhanced growth of rhizosphere bacteria (Fig. 3). However, any explanation of the biotin effect must consider the role of this vitamin as a cofactor for carboxylase enzymes. Acetyl-CoA carboxylase, a classic biotin-dependent enzyme, catalyzes the committed step in fatty acid synthesis, which is required for membrane formation by growing cells. In E. coli, growth rate is positively correlated with transcription of genes coding for acetyl-CoA carboxylase subunits (Li and Cronan 1993). If acetyl-CoA carboxylase activity in R. meliloti is limited by the availability of biotin, then any increase in that vitamin from external sources may support increased growth rate, assuming carbon energy substrates are available. Such population growth presumably would contribute to root colonization. This scenario is reasonable in both physiological and evolutionary terms because elevated levels of CO₂ are available in the rhizosphere (Cheng et al. 1993) and bacteria with a facility for using that resource to make membranes and divide could have a competitive advantage over cells that do not. Carbon dioxide is a required substrate for growth of R. meliloti (Lowe and Evans 1962). That need probably reflects the activity of carboxylase enzymes in Rhizobium, including propionyl-CoA carboxylase and acetyl-CoA carboxylase, which were detected in R. meliloti (De Hertough et al. 1964), and pyruvate carboxylase, which was studied in R. trifolii (Ronson and Primrose 1979). Molecular studies with these potentially important enzymes have not been pursued in detail for Rhizobium. Effects of thiamine and riboflavin on growth may also be related to membrane formation because both molecules function in the pyruvate dehydrogenase complex, which catalyzes the overall conversion of pyruvate and CoA to acetyl-CoA and CO₂.

Genetic details of biotin synthesis are perhaps best characterized in E. coli (Cronan 1989), and no studies of this type have been reported in Rhizobium or Bradyrhizobium. The fact that partially defined biotin auxotrophs in R. meliloti (Fig. 5) were complemented both by the bio operon from E. coli and by a corresponding wild-type R. meliloti DNA fragment (Table 1) indicates that the laboratory and rhizosphere phenotypes observed in Rm1021-B3 and Rm1021-B6 were caused by the mutated DNA fragment. For this reason we conclude that this region of the R. meliloti chromosomal DNA contains at least one functional homologue of genes in the E. coli bio operon. While the failure to complement these mutants with E. coli bira does not constitute definitive evidence, that negative result is consistent with our conclusion. Partial nucleotide sequences and DNA-DNA cross-hybridization experiments with the E. coli bio operon and the 7.5-kb EcoRI DNA fragment carrying the R. meliloti genes required for biotin synthesis suggest that these putative homologues of biotin synthesis

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**Fig. 6.** DNA analyses of a complemented biotin auxotrophic mutant in *Rhizobium meliloti* 1021 (Rm1021). Total genomic DNA was digested with EcoRI. Lane 1, auxotrophic Rm1021-B3; lane 2, Rm1021-WS10, a transconjugant of Rm1021-B3 carrying the *Escherichia coli* bio operon in pLAFR3; lane 3, *E. coli* K12; lane M, 1-kb DNA marker ladder. A, DNA in transmitted UV light. B, Southern analysis of DNA probed with the *E. coli* bio operon labeled with digoxigenin. C, Polymerase chain reaction analyses to detect the presence of Tn5 insertions.

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genes may be structurally quite different in these two bacteria, and detailed studies to explore this possibility are in progress.

Cell growth and survival are two related, but separable, traits that contribute to the competitive competence of rhizosphere bacteria under normal field conditions (Kloepper and Beauchamp 1992; Parke 1991; Schmidt 1979). Studies of root (Thomashow and Weller 1988) or leaf (Wilson et al. 1995) colonization that use large numbers of bacterial cells in the inoculum show a predictably brief period of active growth and a longer period in which cell populations survive or perhaps turn over slowly. We inoculated with fewer than 100 cells to maximize the growth component of root colonization. Such experiments may better represent the situation in which a few bacterial cells are present in soil at the tip of a growing root. Exploring those factors that strongly affect bacterial growth may define mechanisms favoring initial root colonization. We suggest that bacteria that colonize the root first will control the carbon energy resources that are sought by all soil microbes. Although our rhizosphere is an artificial system, it is experimentally manageable and fosters normal Rhizobium-legume interactions resulting in vigorous plant growth (DeJong et al. 1982; Phillips et al. 1985).

Under our experimental conditions *R. meliloti* grows and colonizes the root most rapidly between 3 and 6 days after inoculation (Fig. 2). Over that period, the doubling time of about 7 h is identical to that measured in the biotin-enriched GTS medium (Fig. 1). This fact clearly indicates that our experimental rhizosphere does not inhibit initial root colonization. The plateau in colonization measured on a whole root basis after day 9 is a characteristic of our system that suggests that lower parts of the root system are poorly colonized. That result probably is associated with the presence of the nutrient solution 20 cm below the upper surface of the vermiculite. For this reason, colonization phenotypes of bacterial mutants in this study were characterized during the first 6 days before this factor had an influence.

Results from this study have several implications. First, it should be possible to promote growth of beneficial bacteria in the alfalfa rhizosphere by increasing either biotin synthesis or uptake. While direct approaches to manipulating synthesis can be developed from nucleotide sequences for the *E. coli bio* operon (Otsuka et al. 1988), genes required for biotin uptake have not been defined in any bacteria. Second, the biotin enhancement of rhizosphere growth suggests that more information is needed to understand how bacteria use the CO_2 resources that are present in the rhizosphere. If additional biotin becomes available to a cell, then growth may be limited by other components of fatty acid synthesis required for membrane formation. Manipulation of biotin availability and membrane synthesis could complement other genetic traits being considered to enhance microbial colonization of plants, such as controlling the production and use of unique carbon energy sources (Murphy et al. 1995; Wilson et al. 1995) or production of antibiotics (Thomashow and Weller 1988; Triplett 1990).

### MATERIALS AND METHODS

#### Bacterial strains and plasmids.

Microbiological materials used in the present work are listed in Table 2. *E. coli* was grown at 37°C on Luria-Bertani (LB) medium (Sambrook et al. 1989) supplemented with the appropriate antibiotics. Rhizobia were cultured at 28°C on GTS (Kiss et al. 1979) or TY (Sambrook et al. 1989) medium.

#### Mutagenesis.

Mutants of Rm1021 were produced by mating with *E. coli* S17-1 containing Tn5-B30 in pSUP102 (Simon, et al. 1989) under standard conditions (Simon 1984). Tetracycline-resistant (2 mg/liter) transconjugants were selected on Vincent agar (Vincent 1970) containing streptomycin (500 mg/liter), biotin (2 mg/liter), thiamine (1 mg/liter), and riboflavin (1 mg/liter).

### Table 2. Microbiological materials

<table>
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<th>Relevant trait</th>
<th>Source or reference</th>
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<td>recA1, ΔlacZ</td>
<td>GibcoBRL (Gaithersburg, MD)</td>
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<td>VCS257</td>
<td>Host strain for cosmid banks</td>
<td>Stratagene (La Jolla, CA)</td>
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<td>HB101</td>
<td>pRK2013 helper strain for matings</td>
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<td>Reference strain containing <em>bio</em> operon</td>
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Mutants showing biotin-regulated growth were isolated by screening simultaneously on GTS agar with or without supplemental biotin (2 mg/liter). Putative biotin auxotrophs were confirmed by failure to grow in biotin-free GTS medium produced by the addition of avidin (0.04 U/ml).

**Cosmid banks.**

Cosmid banks containing DNA from Rm1021 and *E. coli* K12 were prepared in pSUP205 and pLAFR3, respectively, by means of standard protocols (Staskawicz et al. 1987). DNA fragments (20 to 30 kb) obtained after partial *Sau3A* digestion were ligated into BamHI restriction sites of the two different cosmid vectors. Phage packaging mixes were obtained from Stratagene (La Jolla, CA) and infection of *E. coli* WCS257 was performed according to the manufacturer's manual. Selected cosmids were conjugated into *R. meliloti* using *E. coli* HB101rpRK2013 as a helper strain in triparental matings. Transconjugants were selected in the presence of appropriate antibiotics on GTS, which does not support growth of the *E. coli* strains used in these studies.

**DNA manipulations and hybridization.**

DNA isolations and cloning steps were performed with standard methods (Sambrook et al. 1989). DNA modifying enzymes (Promega, Madison, WI) were used as specified by the manufacturer. DNA restriction enzymes were isolated for hybridizations by electrophoresis in 0.8% agarose gels, transferred onto nylon membranes (MSI, Westboro, MA) and cross-linked with UV light. Hybridizations were performed overnight using digoxigenin-labeled DNA probes (Boehringer, Mannheim, Germany) and high stringency conditions (68°C) or, when specifically noted, low stringency conditions (55°C). Hybridization signals were detected with colorimetric substances NBT and BCIP according to the manufacturer's instructions (Boehringer, Mannheim).

**Biotin ELISA.**

Biotin was measured with a competitive ELISA technique (Chang et al. 1994). The biotin standard was prepared either in GTS medium for the bacterial growth studies or in 0.25x Hoagland solution (Hoagland and Arnon 1951) for experiments with alfalfa seedlings. Biotin was measured in culture supernatants of *R. meliloti* after bacteria were pelleted by centrifugation. Biotin available from alfalfa seedlings was measured with 4-day-old seedlings germinated on 0.25x Hoagland-agar plates. Individual roots were rinsed repeatedly in 1 ml of sterile, double-deionized water and the eluate was centrifuged to remove cellular debris before biotin was determined in the supernatant.

**Laboratory growth studies.**

*R. meliloti* growth experiments were performed in 50-ml cultures of GTS medium. Trials were begun by inoculating from a 24-h-old culture grown without supplemental biotin in GTS medium. A 250-μl inoculum was prepared by washing cells twice with fresh GTS medium and adjusting optical density (600 nm) to 0.5. Bacteria were grown at 28°C with shaking (250 rpm, orbit shaker [Labline Instruments Inc., Melrose Park, IL]), and growth was monitored at OD660. To identify biotin synthesis phenotypes, mutants were grown in the presence of avidin (0.04 U/ml). Antibiotics were added as required.

**Rhizosphere colonization experiments.**

Scarified alfalfa seeds (*M. sativa* cv. Moapa 69) were treated 15 min in 70% ethanol and rinsed three times with sterile, double-deionized water. These sterilized seeds were plated on 0.5x TY agar (lacking CaCl2) and germinated at 28°C. After 24 h, five germinating seeds with no visible contamination were transferred into sterilized Leonard jars (DeJong and Phillips 1981) filled with vermiculite and 0.25x Hoagland solution. The 24-h-old seedlings were inoculated with 100 μl of bacterial suspension containing 30 to 150 CFU, covered with a layer of sterile perlite, and incubated under controlled conditions (24/19°C, 16/8 h, day/night, 50% relative humidity, and 400 μE s⁻¹ m⁻²). Day 0 was defined as the time of inoculation because the goal of these experiments was to study bacterial, not plant, growth. Inocula were prepared by pelleting 72-h-old cultures from GTS medium containing biotin (2 mg/liter), rinsing, and resuspending in sterile, distilled water before making further serial dilutions. Viable cell counts and uniformity were verified by plating on TY agar containing Congo red (10 mg/liter). Roots were harvested, shaken to remove vermiculite, cut into segments, and placed in 1.5-ml Eppendorf tubes containing 1 ml of extraction buffer (distilled water containing 0.01% Tween 20). Rootless older than 6 days were placed in 5 ml of extraction buffer in a 20-ml tube to recover rhizobia. To remove more than 95% of the viable bacteria, roots were vortexed 30 s, incubated 30 min in a sonicating bath (Branson 1200R-1), and revortexed for 30 s. Appropriate dilutions were plated on TY agar containing the required antibiotics. CFU data recorded from roots were subjected to a log₁₀ transformation to produce normal distributions (Loper et al. 1984), which were then tested for significant treatment effects by calculating appropriate least significant difference values for each experiment. Every treatment consisted of at least three replicate Leonard jars, each of which contained five plants. All experiments were repeated at least once.

In those cases in which vitamins were added to the rhizosphere, nanomole quantities were supplied on days 0, 2, and 4 to the top of the primary root as 2-ml aliquots of solutions containing biotin (2 mg/liter), riboflavin (1 mg/liter), or thiamine (1 mg/liter). In some cases avidin (1 U/liter) was added in 2-ml aliquots according to the same schedule.

**PCR conditions and sequencing.**

Distances between Tn5 insertions in various mutants were estimated by PCR analyses. Short DNA flanking regions between a 5'-Xhol restriction site and the Tn5-B30 insertion were amplified with primers WS4063 (5'CGT CGA CCT CGA GAG CCG TCT TC3') and TnSout (5'GAA AGG TTC CGT TCA GGA CGC TAC3') in a 35-cycle PCR reaction. Reactions were started by incubating the sample for 1 min at 94°C before normal cycling began. Each cycle consisted of denaturing (1 min, 94°C), annealing (1 min, 63°C), and extension (2 min at 72°C) steps. A 10-μl sample of the 100-μl reaction mixture was analyzed on a 1.5% agarose gel. Sizes were estimated with a 1-kb DNA marker ladder (Gibco:BRL, Gaithersburg, MD). PCR products were sequenced with a nonradioactive DNA sequencing kit (Boehringer, Mannheim) and sequences were compared with standard databases by BLAST software (NCBI, USNIH).

A wild-type cosmid clone containing *R. meliloti* genes required for biotin synthesis was located with a 7.5-kb EcoRI
fragment prepared from mutant Rm1021-B3. For this purpose, 5 μg of total genomic DNA was digested with EcoRI, extracted twice with chloroform, and ligated overnight in a 200-μl reaction mixture (Silver 1991). Resulting minicircles for inverse PCR (Silver 1991) containing the Tn5-EcoRI flanking regions were then amplified with a long PCR kit (Perkin Elmer, Branchburg, NJ) using primer Tn5out according to the manufacturer’s protocol. PCR conditions were 1 min at 94°C and 12 min at 68°C. After 45 PCR cycles, DNA was separated on 0.8% agarose gel, and a single 7.5-kb EcoRI DNA band was recovered with a sephaglass kit (Pharmacia, Alameda, CA). This fragment was labeled with digoxigenin and used as a DNA probe.

A DNA probe for the E. coli bio operon was produced by digoxigenin labeling after amplifying the complete bio operon with the long PCR technique and previously reported sequences (Otsuka et al. 1988) to develop primers W54954 (5′GGC CCA TCT GGA AGA ATT AAC T3′) and W54955 (5′TAC AGA ATG GCT ACA ACA AGG CAA3′). The DNA probe for e. coli birA was produced by digoxigenin labeling after using previously reported sequences (Howard et al. 1985) to develop primers W55002 (5′GAA GGA GCG ATT AAG AGT GCC ATG3′) and W55003 (5′CTA CAA ACT CCT CCT GTC GTA ATA3′), which were used to amplify birA with the standard PCR technique.

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LITERATURE CITED


