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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 ($P \leq 0.01$) reduced rhizosphere growth of *R. meliloti* 1021, and (ii) growth of Tn*5*-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 N_2-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 CO_2 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 CO_2. 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.

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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

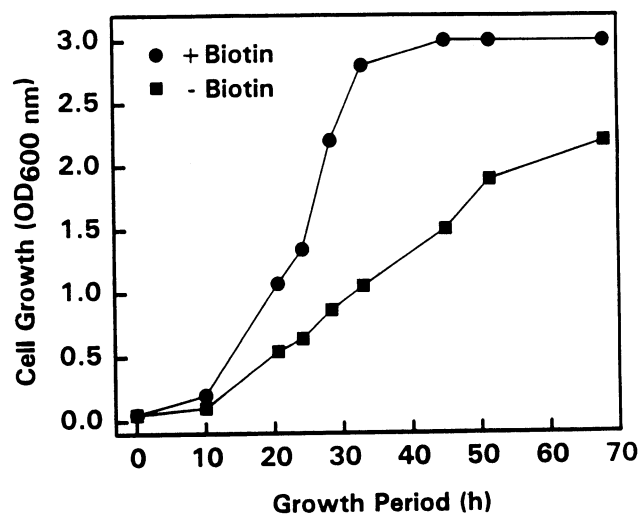

Fig. 1. Effects of biotin on growth of *Rhizobium meliloti* 1021. Cells were grown ± supplemental 4.0 nM biotin in GTS medium.

in enzyme-linked immunosorbent assay (ELISA). The term "biotin-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 4.8×10^4, 6.6×10^4, and 7.4×10^4 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 sixfold 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 rinsed 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.

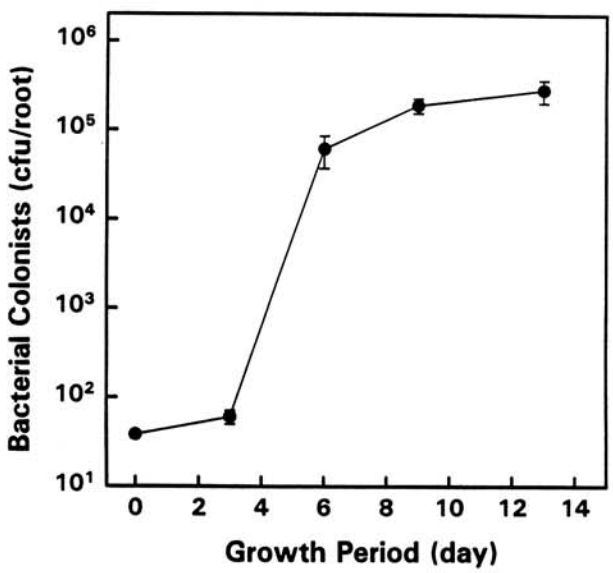

Fig. 2. Growth of *Rhizobium meliloti* 1021 in the alfalfa rhizosphere. Bacteria inoculated on day 0 were quantified in the inoculum; on subsequent days bacteria were recovered from roots. Data represent mean numbers of bacteria ± standard error recovered from three replicates.

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

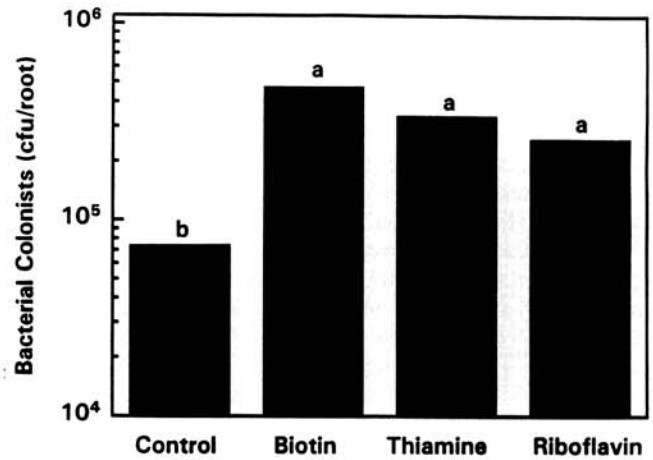

Fig. 3. Effects of water-soluble vitamins on growth of *Rhizobium meliloti* 1021 in the alfalfa rhizosphere. Nanomolar amounts of the indicated vitamins were supplied on day 0, 2, and 4; bacteria were counted on day 6. Mean value bars labeled with the same letter were not significantly ($P \leq 0.01$) different.

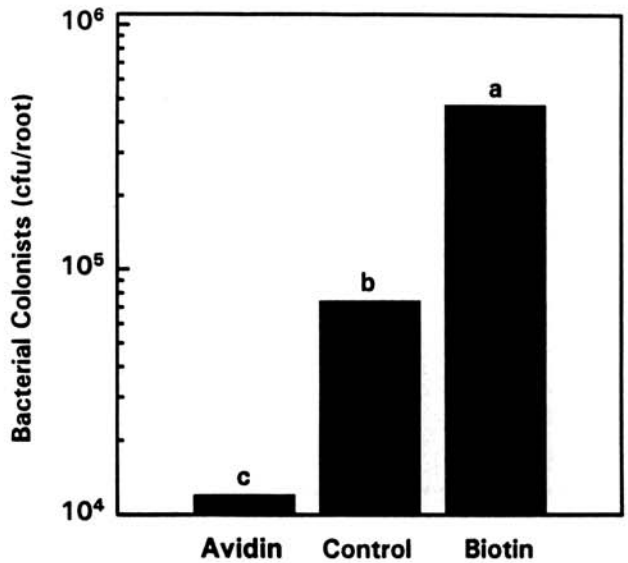

Fig. 4. Biotin limitations to growth of *Rhizobium meliloti* 1021 in the alfalfa rhizosphere. Bacteria were counted on roots 6 days after inoculating 52 cells per plant. Supplemental biotin (16 nmol) and avidin (2 μU) added on day 0, 2, and 4 had significant effects ($P \leq 0.01$) on the mean number of cells recovered from four replicates.

isolate genes responsible for this biotin synthesis, five mutants unable to grow in the absence of biotin were recovered from 3,000 Tn*5*-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 avidin (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 Tn*5*-B30 established that all insertions were located on a single 14-kb *Eco*RI DNA fragment (Fig. 5A). No *Eco*RI restriction sites are present in Tn*5*-B30. The presence of a single Tn*5* insertion in each mutant was established by the additional fact that in every case *Xho*I DNA digests produced three bands in Southern analyses that hybridized with the probe and were consistent in size with published restriction sites in Tn*5*-B30 (Simon et al. 1989) (data not shown). Southern analyses of mutant lysates (Eckhardt 1978) showed no Tn*5* 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 *Xho*I 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 Tn*5*-B30 insertion. *WS4063* and *Tn5out*, a standard Tn*5*-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 Tn*5* insertion of the other two mutants. Because this approach could not distinguish between Tn*5* 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 *Tn5out* primer and minicircles produced by self-ligation of an *Eco*RI 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

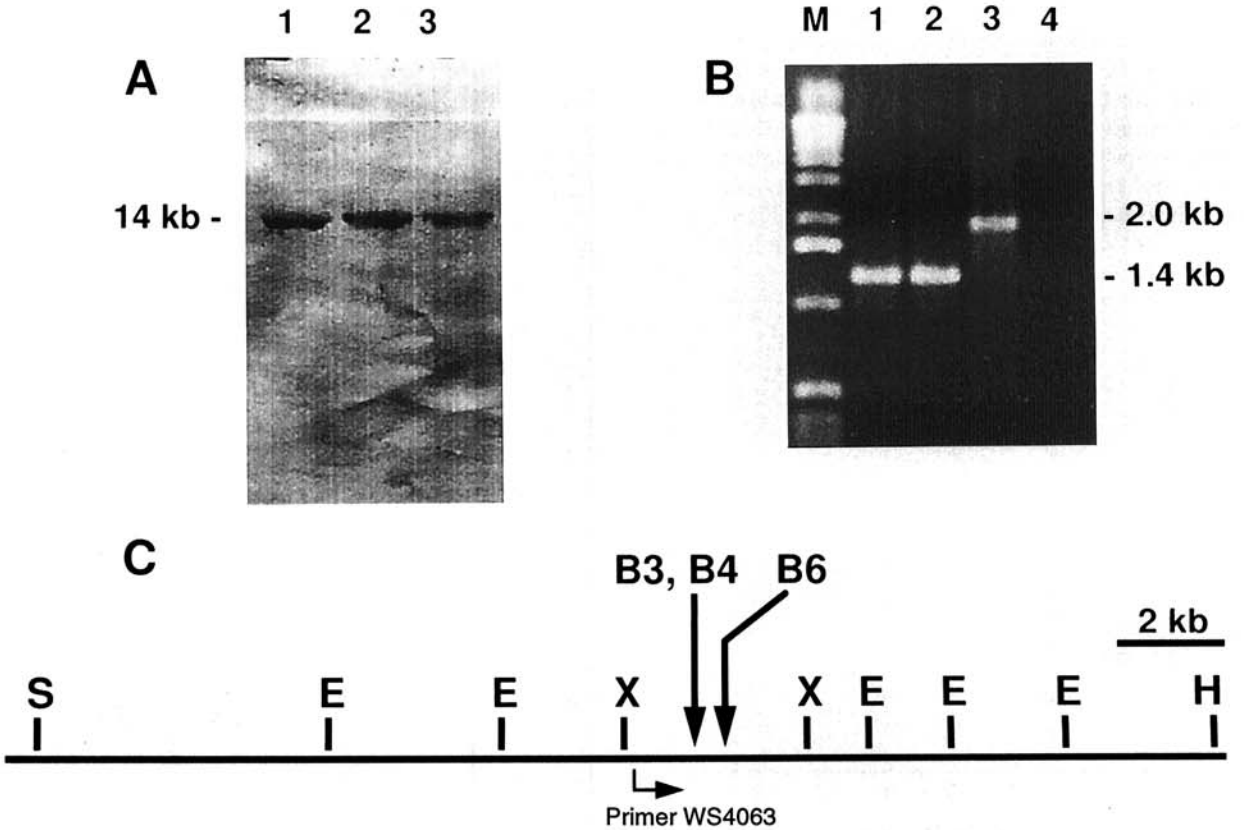

Fig. 5. Analyses of three biotin auxotrophic mutants in *Rhizobium meliloti* 1021 (Rm1021). **A,** Southern analysis of total genomic DNA after *Eco*RI digestion. Gel blots were probed with Tn*5*-B30 sequences labeled with digoxigenin. Lane 1, Rm1021-B3; lane 2, Rm1021-B4; lane 3, Rm1021-B6. **B,** Polymerase chain reaction analysis of distances between Tn*5* 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 Tn*5*-B30 in the different mutants. E: *Eco*RI; X: *Xho*I; H: *Hin*dIII; S: *Sal*I.

DNA from a cosmid bank containing Rm1021 DNA. One cosmid, pCosRmbio1, carrying the identified DNA fragment as an internal *Eco*RI 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^r (50 mg/liter). Transconjugant Rm1021-WS8 showed normal rhizosphere growth, while Rm1021-WS9 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 pCosRmbio1 (Fig. 5C). A clone carrying the complete *R. meliloti* biotin synthesis operon from pCosRmbio1 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 but 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 *Bam*HI 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-WS10 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-WS10 carried both pCosEcbio1 and Tn*5* 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 Tn*5* 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-

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

R. meliloti strain	Growth on biotin-free GTS medium[x]	Relative root colonization in alfalfa rhizospheres[w]		
		Normal rhizospheres, single-strain inocula (%)	Biotin-enriched rhizospheres, single-strain inocula (%)[y]	Normal rhizospheres, coinoculation with Rm1021 (%)
Rm1021	+	100 a	100 a	100 a
Rm1021-B3	–	<1 b	41 c	<1 b
Rm1021-B4	–	<1 b	88 b	<1 b
Rm1021-B6	–	<1 b	15 d	<1 b
Rm1021-WS8	+	100 a	ND[z]	ND
Rm1021-WS9	+	76 c	ND	ND
Rm1021-WS10	+	ND	ND	ND

[w] 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.
[x] Supplemented with 0.04 U of avidin.
[y] Rhizospheres were enriched with a total of 48 nmol of biotin in three additions.
[z] 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 CO_2 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 CO_2.

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 *Eco*RI DNA fragment carrying the *R. meliloti* genes required for biotin synthesis suggest that these putative homologues of biotin synthesis

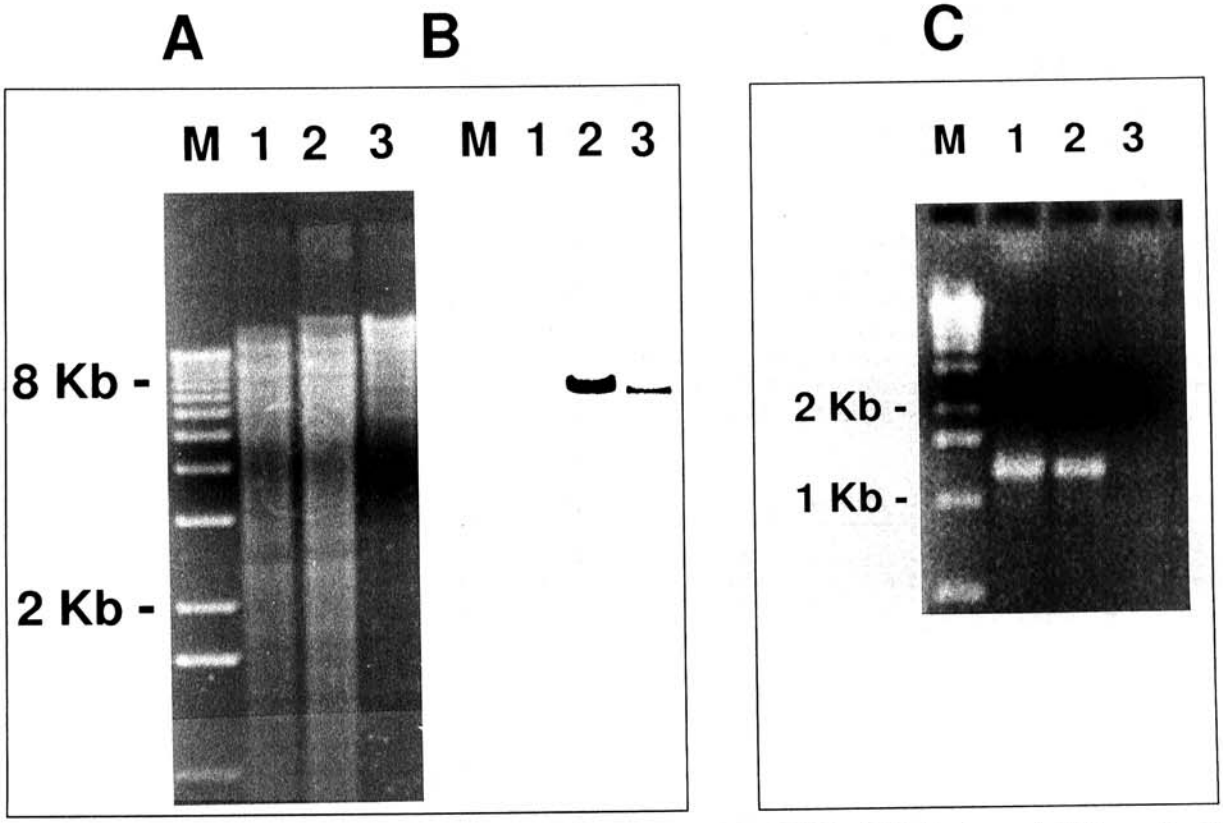

Fig. 6. DNA analyses of a complemented biotin auxotrophic mutant in *Rhizobium meliloti* 1021 (Rm1021). Total genomic DNA was digested with *Eco*RI. Lane 1, auxotrophic Rm1021-B3; lane 2, Rm1021-WS10, a transconjugant of Rm1021-B3 carrying the *Escherichia coli bio* operon in pLAFR3; lane 3, *E. coli* 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 Tn*5* insertions.

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 Tn*5*-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

Material	Relevant trait	Source or reference
Escherichia coli		
DH5α	*rec*A1, Δ*lacZ*	GibcoBRL (Gaithersburg, MD)
VCS257	Host strain for cosmid banks	Stratagene (La Jolla, CA)
HB101	pRK2013 helper strain for matings	Stratagene
K12	Reference strain containing *bio* operon	ATCC
S17-1	Modified RP4 plasmid integrated into genome	Simon et al. 1983
Rhizobium meliloti		
Rm1021	2011, Sm^r	Meade et al. 1982
Rm1021-B3	Tn*5*B30::*bio* mutant of Rm1021	This work
Rm1021-B4	Tn*5*B30::*bio* mutant of Rm1021	This work
Rm1021-B6	Tn*5*B30::*bio* mutant of Rm1021	This work
Rm1021-WS8	Rm1021-B3 containing pCosRmbio1	This work
Rm1021-WS9	Rm1021-B6 containing pCosRmbio1	This work
Rm1021-WS10	Rm1021-B3 containing pCosEcbio1	This work
pBSK+	pBluescriptSK+ multicopy cloning vector	Stratagene
pCosEcbio1	pLAFR3 with 30-kb *Bam*HI fragment containing the *E. coli bio* operon	This work
pCosEcbirA	pLAFR3 with 30-kb *Bam*HI fragment containing *E. coli birA*	This work
pCosRmbio1	pSUP205 with 28-kb *Bam*HI fragment containing the *R. meliloti bio* operon	This work
pLAFR3	pLAFR1 derivative, *mob*, λ *cos* site, *tra*, Tc^r, low copy cosmid vector	Staskawicz et al. 1987
pRK2013	pRK212.2 derivative for matings	Figurski and Helinski 1979
pSUP205	pBR325 derivative, *mob*, λ *cos* site, *tra*, Cm^r, Tc^r, multicopy cosmid vector in *E. coli*	Simon et al. 1983
pWS3	2.9-kb *Xho*I fragment of 1021-B4	This work
Tn*5*-B30	pSUP102 with transposable *nptII* promoter probe, Tc^r	Simon et al. 1989

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 *Sau*3A digestion were ligated into *Bam*HI restriction sites of the two different cosmid vectors. Phage packaging mixes were obtained from Stratagene (La Jolla, CA) and infection of *E. coli* VCS257 was performed according to the manufacturer's manual. Selected cosmids were conjugated into *R. meliloti* using *E. coli* HB101pRK2013 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 fragments were separated 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 0.25× 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 0.25× 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 OD_{600}. 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 0.5× TY agar (lacking $CaCl_2$) 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 0.25× 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). Roots 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 (Bransonic 1200R-1), and revortexed for 30 s. Appropriate dilutions were plated on TY agar containing the required antibiotics. CFU data recovered from roots were subjected to a $\log_{10}$ 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 Tn*5* insertions in various mutants were estimated by PCR analyses. Short DNA flanking regions between a 5′-*Xho*I restriction site and the Tn*5*-B30 insertion were amplified with primers *WS4063* (5′CGT CGA CCT CGA GAG CCG TCT TC3′) and *Tn5out* (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 (GibcoBRL, 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 *Eco*RI

fragment prepared from mutant Rm1021-B3. For this purpose, 5 μg of total genomic DNA was digested with *Eco*RI, extracted twice with chloroform, and ligated overnight in a 200-μl reaction mixture (Silver 1991). Resulting minicircles for inverse PCR (Silver 1991) containing the Tn*5*-*Eco*RI 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 *Eco*RI 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 *WS4954* (5′GCG CCA TCT GGC AGA GAG ATT AAC T3′) and *WS4955* (5′TAC AGA ATG GCT ACA ACA AGG CAA3′). The DNA probe for *E. coli bir*A was produced by digoxigenin labeling after using previously reported sequences (Howard et al. 1985) to develop primers *WS5002* (5′GAA GAA GCG ATT AAG AGT GCC ATG3′) and *WS5003* (5′CTA CAA ACT CTT CCT GTC GTC ATA3′), which were used to amplify *birA* with the standard PCR technique.

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