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Recombinant *Rhizobium meliloti* Strains with Extra Biotin Synthesis Capability

WOLFGANG R. STREIT AND DONALD A. PHILLIPS*

Department of Agronomy and Range Science, University of California, Davis, California 95616

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The growth of *Rhizobium meliloti* 1021 in an experimental alfalfa (*Medicago sativa* L.) rhizosphere was stimulated by adding nanomolar amounts of biotin. To overcome this biotin limitation, *R. meliloti* strains were constructed by conjugating the *Escherichia coli* biotin synthesis operon into biotin auxotroph *R. meliloti* 1021-B3. Transconjugant strains Rm1021-WS10 and Rm1021-WS11 grew faster in vitro and achieved a higher cell density than did *R. meliloti* 1021 and overproduced biotin on a defined medium. The increase in cell yield was associated with as much as a 99% loss in viability for Rm1021-WS11, but data suggested that a separate stabilizing factor in the *E. coli* DNA reduced cell death in Rm1021-WS10. In rhizosphere tests, the recombinant strains showed delayed growth and competed poorly against Rm1021.

Bacteria that associate with plants often survive for long periods in soil and begin growing only when a root develops nearby (5). Such indigenous bacteria frequently prevent the successful introduction of genetically improved strains for agricultural purposes (23, 31, 33, 36). Factors affecting microbial competition for root colonization have been examined in many bacteria, including *Bradyrhizobium* and *Rhizobium* symbionts (1, 35), *Agrobacterium* pathogens (12), and *Pseudomonas* biocontrol organisms (20). Strain comparisons have identified individual genetic traits, such as antibiotic production (9, 16, 34), that contribute to the rhizosphere success of certain bacteria, but no genes that promote root colonization by a wide spectrum of recipient strains have been reported.

Several research groups have enhanced particular physiological functions of rhizosphere bacteria by recombinant DNA technology. Potentially important bacteria produced by this approach often show expected improvements under controlled conditions, but responses under field conditions can be more variable. For example, recombinant strains of *Rhizobium meliloti*, an N_2-fixing bacterium that associates with alfalfa (*Medicago sativa* L.), were produced with extra copies of *nifA* and *dctABD* genes to enhance symbiotic performance, but field tests failed to show in all cases the benefits that had been measured under controlled conditions (4). Other examples of promising genetic constructions, such as insertion of *cryIII* (3) or a chitinase gene (29) in *R. meliloti*, also might benefit from increased competitiveness. Indeed, the problem is not restricted to recombinant organisms, because many natural variants or undefined mutants with desirable traits would be more useful under field conditions if their competitiveness were enhanced, e.g., *Bradyrhizobium japonicum* mutants with superior N_2 fixation capabilities (22, 38). The long-term goal of this project is to develop a genetic cassette that increases the growth rate of any recipient strain on alfalfa roots and thereby promotes competitive root colonization.

The work reported here centers on enhancing biotin synthesis. Biotin is a classical vitamin cofactor that increases the growth rate of *Rhizobium trifolii* in vitro (37). Although biotin is released from alfalfa roots (24), recent work with biotin auxotrophs of *R. meliloti* 1021 established that (i) growth of this bacterium in an alfalfa rhizosphere was limited by the availability of biotin and (ii) both synthesis and uptake of biotin by this bacterium contribute to rhizosphere growth (32). The demonstration in that study that a 30-kb cosmid containing the *Escherichia coli* biotin synthesis operon complemented the auxotrophic mutants in vitro suggested that more detailed studies of recombinant strains would be informative. The immediate objective of this study was to test whether extra copies of genes coding for biotin synthesis increase the growth rate and competitiveness of *R. meliloti.*

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this work are listed in Table 1. Published information (2) on the *E. coli* DNA fragments used to construct pCosEcbio1 and pCosEcbio2 is shown in Fig. 1. *E. coli* was grown at 37°C on Luria-Bertani medium (27), and rhizobia were cultured at 28°C on GTS (14) or TY (27) medium. Antibiotics were added as required. Cosmid banks were prepared with DNA from *R. meliloti* Rm1021 or *E. coli* K-12 in pSUP205 or pLAFR3 as described previously (32). Cosmids were maintained in *E. coli* VCS257 and conjugated into *R. meliloti* with *E. coli* HB101(pRK2013) as a helper strain in triparental matings. Transconjugants were selected with appropriate antibiotics (tetracycline, 3 μg/ml; chloramphenicol, 50 μg/ml; streptomycin, 500 μg/ml) on GTS, which does not support the growth of the auxotrophic *E. coli* strains used in these studies.

DNA manipulations and hybridizations. DNA isolation and cloning steps were performed by standard methods (27). DNA-modifying enzymes (Promega, Madison, Wis.) were used as specified by the manufacturer. DNA hybridizations were done with DNA probes labeled by random priming with digoxigenin (Boehringer, Mannheim, Germany). Hybridizations were performed under high-stringency conditions (68°C), and hybridization signals were detected with colorimetric substances Nitro Blue Tetrazolium (NBT) and 5-bromo-4-chloro-3-indolylphosphate (BCIP) as specified by the manufacturer.

A DNA probe for the *E. coli* biotin synthesis operon was produced by amplifying the complete *E. coli* operon with a kit for long PCR (Perkin-Elmer, Branchburg, N.J.). Primers for this purpose, WS4954 (5′GCG CCA TCT GGC AGA GAG ATT AAC T3′) and WS4955 (5′TAC AGA ATG GCT ACA ACA AGG CAA3′), were made from published sequences (21). DNA hybridizations with the *R. meliloti* biotin synthesis operon involved a digoxigenin-labeled DNA probe derived from a 7.5-kb *Eco*RI internal DNA fragment carried in pCosRmbio1 (32). To detect the *E. coli* biotin synthesis operon in recombinant *R. meliloti* strains, the following PCR conditions were used: 1 min at 94°C, 1 min at 64°C, and 2 min at 72°C for 35 cycles with primers WS4954 and WS4953 (5′ATG ACA ACG GAC GAT CTT GCC TTT GAC3′).

Biotin ELISA. Biotin was measured by a competitive enzyme-linked immunosorbent assay (ELISA) technique (7) modified for these experiments (32). Biotin standards were prepared in GTS medium, and biotin was measured in culture supernatants after removal of bacteria by centrifugation.

Rhizosphere experiments. Bacterial growth in the rhizosphere of alfalfa (*M.*

* Corresponding author. Mailing address: Department of Agronomy & Range Science, University of California, Davis, CA 95616. Phone: (916) 752-1891. Fax: (916) 752-4361. Electronic mail address: DAPhillips@UCDavis.edu.

TABLE 1. Microbiological materials used in this study

Material	Relevant trait	Source or reference
E. coli		
VCS257	Host strain for cosmid banks	Stratagene
HB101	Helper strain for matings	Stratagene
K-12	Reference strain with biotin synthesis operon	ATCC[a]
S17-1	Modified RP4 plasmid integrated into genome	28
R. meliloti		
Rm1021	*R. meliloti* 2011, Sm^r	17
Rm1021-B3	Tn*5*B30::*bio* mutant of Rm1021	32
Rm1021-WS10	Rm1021-B3 containing pCosEcbio1	32
Rm1021-WS11	Rm1021-B3 containing pCosEcbio2	This work
Rm1021-WS12	Rm1021pLAFR3	This work
Plasmids		
pCosEcbio1	pLAFR3 with 30-kb *Bam*HI fragment containing *E. coli* biotin synthesis operon	32
pCosEcbio2	pLAFR3 with 7.5-kb *Eco*RI fragment containing *E. coli* biotin synthesis operon	This work
pCosRmbio1	pSUP205 with 28-kb *Bam*HI fragment containing *R. meliloti* biotin synthesis operon	32
pCosRmbio2	pLAFR3 with 15-kb *Eco*RI fragment containing *R. meliloti* biotin synthesis operon	This work
pCosRmbio3	pLAFR3 with 22-kb *Eco*RI fragment containing *R. meliloti* biotin synthesis operon	This work
pLAFR3	pLAFR1 derivative, *mob*, λ *cos* site, *tra*, Tc^r, low-copy cosmid vector	30
pRK2013	pRK212.2 derivative for matings	10
pSUP205	pBR325 derivative, *mob*, λ *cos* site, *tra*, Cm^r, Tc^r, multicopy cosmid vector in *E. coli*	28

[a] ATCC, American Type Culture Collection.

sativa L. cv. Moapa 69) was measured by a standard root colonization assay (32). In this assay, alfalfa seeds were germinated 24 h before being planted in vermiculite and inoculated with fewer than 200 CFU per plant on day 0. In competition tests, two bacterial strains were combined in the normal 100-μl inoculum, and day 0 CFU values for each were determined separately. "Rhizosphere growth" and "root colonization" are used interchangeably to describe the results of this test, because some vermiculite adhered to harvested roots. The number of viable bacterial cells recovered from each root system was subjected to a $\log_{10}$ transformation to produce normal distributions (15), and the mean values were then tested for significant treatment effects by calculating appropriate least-significant-difference values for each experiment. Each treatment contained at least three replicate Leonard jars with a minimum of 15 plants (5 plants per jar). All experiments were repeated at least once. Biotin augmentation experiments were conducted by adding 2-ml aliquots of various biotin solutions (0.0, 0.2, 2.0, or 10 mg/liter) to the germinating root on day 0 or the bottom of the stem on days 2 and 4 to supply a total of 0, 4.8, 48, or 240 nmol of biotin per plant.

Laboratory growth studies. *R. meliloti* growth studies were performed in 50-ml cultures of GTS with a 250-μl inoculum and appropriate antibiotics. Prior to inoculation, the cells were pelleted and washed twice with fresh GTS, and the optical density at 600 nm (OD_{600}) was adjusted to 0.5 unit. Bacteria were grown

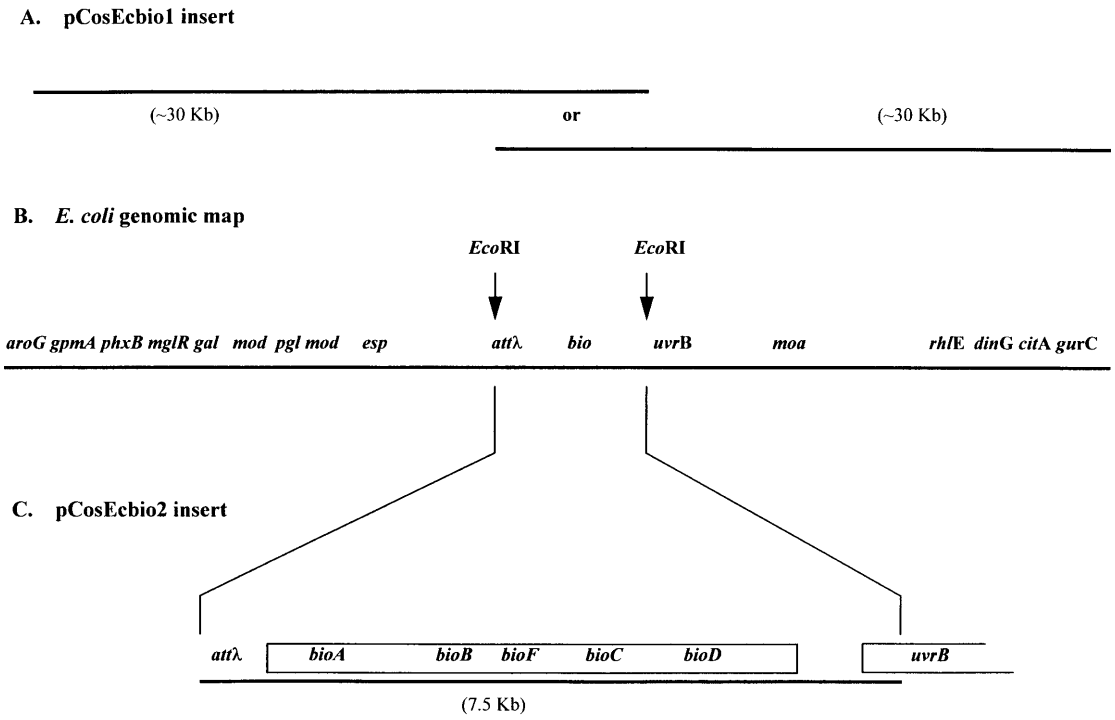

FIG. 1. Previously identified genetic traits (2) on *E. coli* DNA fragments used in this study. The DNA insert in pCosEcbio1 (A) contains most, but not all, of the indicated genes (B). The absolute location of the subclone used to construct pCosEcbio2 (C) has not yet been determined.

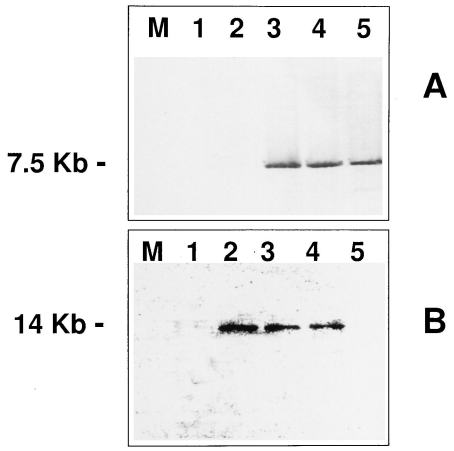

FIG. 2. Southern analysis of *R. meliloti* strains containing *E. coli* biotin synthesis genes. Total genomic DNA was digested with *Eco*RI and separated in agarose. Lanes: M, 1-kb DNA marker ladder; 1, Rm1021; 2, Rm1021-B3; 3, Rm1021-WS10; 4, Rm1021-WS11; 5, *E. coli* K-12. (A) Hybridization with a probe for the *E. coli* biotin synthesis operon. (B) Hybridization with a probe for Tn*5*.

at 28°C with shaking at 250 rpm, and growth was monitored by measuring the OD_{600}.

To select for any mutation in Rm1021-WS11 that increases viability, 5-ml GTS cultures without added biotin were inoculated with 100 μl of the original glycerol stock, and then 8×10^5 viable cells were transferred into fresh medium after 72 h. The process was repeated through five cycles, and total cell counts and viable cell counts on GTS without added biotin were determined after each cycle.

RESULTS

Biotin limitations in the rhizosphere. The growth of *R. meliloti* Rm1021 in the experimental alfalfa rhizosphere studied in these experiments was limited by the availability of biotin. A mere 4.8 nmol of biotin supplied in three equal amounts on days 0, 2, and 4 enhanced bacterial growth significantly ($P < 0.05$), and 48 nmol of biotin increased cell titers 5.5-fold ($P < 0.001$). The specific mean values of log CFU per root ± standard error for supplements (nanomoles of biotin per plant) of 0, 4.8, 48, and 240 were 4.18 ± 0.16, 4.59 ± 0.08, 4.92 ± 0.18, and 5.06 ± 0.18, respectively.

Production of recombinant strains. Attempts to overcome biotin limitations in the alfalfa rhizosphere produced two different *R. meliloti* strains carrying extra copies of the biotin synthesis operon from *E. coli*. Because previous data showed that some portion of a 30-kb *Bam*HI fragment of *E. coli* K-12 DNA in pCosEcbio1 cured biotin auxotrophy in Rm1021-B3 (32), a 7.5-kb *Eco*RI fragment of the cosmid which hybridized to the *E. coli* biotin synthesis operon was incorporated into pCosEcbio2 (Table 1). Plasmid pCosEcbio2 was mated successfully into Rm1021-B3 to produce recombinant strain Rm1021-WS11, which was used to test the hypothesis that a smaller piece of foreign DNA would increase the fitness of *R. meliloti* more than the larger pCosEcbio1 construct would.

Southern analyses of Rm1021-WS10 and Rm1021-WS11 with the *E. coli* biotin synthesis operon as a probe showed a single 7.5-kb band which corresponded to the *Eco*RI fragment in pCosEcbio1 and pCosEcbio2 (Fig. 2A). No hybridization was detected with that probe under either high- or low-stringency conditions on gels containing total genomic digests of the parent, Rm1021-B3. When the same DNA gels were analyzed with a probe specific for Tn*5*, a separate 14-kb DNA-hybridizing band was found in Rm1021-WS10, Rm1021-WS11, and Rm1021-B3 (Fig. 2B). These results indicated that both recombinant strains contained the *E. coli* biotin synthesis operon as well as Tn*5*-B30, which caused the biotin auxotrophic phenotype in the original mutant Rm1021-B3.

Several other attempts to mobilize biotin synthesis genes were unsuccessful. First, no stable transconjugants were produced by mating pCosEcbio1 and pCosEcbio2 into the biotin prototroph Rm1021. Also, 15- and 22-kb subclones of the 28-kb cosmid in pCosRmbio1 were captured in pCosRmbio2 and pCosRmbio3, but they could not be recovered in either Rm1021 or Rm1021-B3 transconjugants. Indeed, both plasmids were highly unstable in *E. coli* VCS257. In some experiments, adding 4 μM biotin to the growth medium slightly increased the stability of these constructs in *E. coli*, but still no transconjugants were recovered after triparental matings with Rm1021-B3. Southern analyses of pCosRmbio2 and pCosRmbio3 in these *E. coli* strains showed a pattern of DNA digestion that suggested a general plasmid instability (data not shown).

Two stable recombinant strains, Rm1021-WS10 and Rm1021-WS11, were thus available to test whether *R. meliloti* cells containing extra copies of genes required for biotin synthesis showed superior physiological and/or ecological traits. Each of these strains contained small numbers of the *E. coli* biotin synthesis operon on the self-replicating plasmid pLAFR3.

Characteristics of recombinant *R. meliloti* strains. Trials showed that recombinant strains Rm1021-WS10 and Rm1021-WS11 each produced several orders of magnitude more biotin than did parent strain Rm1021 during a 70-h growth period on GTS medium when no biotin was added to the growth medium. Values (nanomoles of biotin per liter) for Rm1021, Rm1021-WS10, and Rm1021-WS11 were <0.004, 2.6, and 3.3, respectively. The exceedingly slow growth of Rm1021 in that experiment ($T_D \approx 100$ h) was typical of this strain after it had been grown for several cycles on GTS medium in the absence of biotin. One must conclude that cells synthesized biotin under these conditions, because the total amount of biotin in the culture medium increased from exudation at the same time as the cell mass grew (Fig. 3) and because the cells could be repeatedly diluted into fresh, biotin-free medium and grown with similar results. Cell dry weight measured under such conditions increased 10-fold for both recombinant *R. meliloti* strains compared with the control Rm1021-WS12 (Table 2). As a result of the biotin overproduction in those strains, doubling times in the absence of added biotin during exponential growth were greatly reduced compared with the 105 h recorded in Rm1021. Doubling times observed under those conditions for

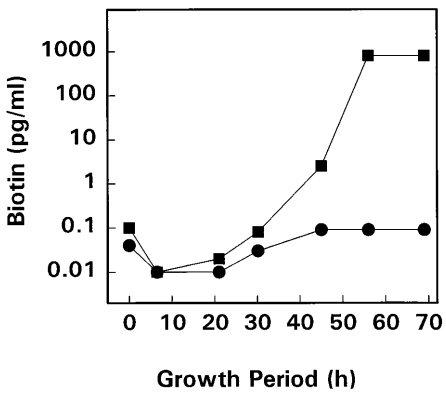

FIG. 3. Effect of *E. coli* biotin synthesis genes on extracellular biotin production by *R. meliloti*. Biotin in culture supernatants of wild-type Rm1021 (●) and of recombinant Rm1021-WS11 (■) growing on GTS medium without added biotin was detected by an ELISA.

TABLE 2. Effects of extra copies of the *E. coli* biotin synthesis operon on *R. meliloti* after 72 h of growth[a]

R. meliloti strain	Viable counts (10^8 CFU/ml)	Total counts (10^8 cells/ml)	Viability (%)	Cell yield (g/liter)	T_d[b] (h)
No biotin added					
Rm1021-WS12	0.7 a	0.99 a	71 a	0.15 a	101 a
Rm1021-WS10	36 b	63 b	56 b	1.67 b	4.9 b
Rm1021-WS11	0.8 a	78 b	1 c	1.64 b	4.9 b
4 μM biotin added					
Rm1021-WS12	30 a	34 a	88 a	1.79 a	5.1 a
Rm1021-WS10	20 a	45 a	45 b	1.63 b	5.1 a
Rm1021-WS11	0.5 b	34 a	1.5 c	1.64 b	5.1 a

[a] Values in a column and for specific biotin treatment followed by the same letter are not significantly different ($P \leq 0.05$).
[b] Calculated from OD_{600} data.

TABLE 3. Root colonization by *R. meliloti* after 6 days in experimental alfalfa rhizospheres

R. meliloti strains	No. of cells inoculated (CFU/root)[a]	No. of bacterial colonists (CFU/root)[a]
Rm1021[b]	65 a	7.0×10^4 a
Rm1021-WS10[b]	80 a	5.6×10^3 b
Rm1021-B3[b]	190 a	585 d
Rm1021 and Rm1021-WS10[c]	47, 82 a	9×10^4 a, <500 d
Rm1021-B3 and Rm1021-WS10[c]	117, 44 a	3.0×10^3 c, 3.1×10^3 c

[a] Values in a column followed by the same letter are not significantly different ($P \leq 0.05$).
[b] Single strain inoculated on day 0.
[c] Two competing strains inoculated together on day 0.

Rm1021-WS10 and Rm1021-WS11 equaled those measured for Rm1021-WS12 in the presence of added biotin (Table 2).

Rm1021-WS10 and Rm1021-WS11 cells grew to quite high OD_{600} values compared with Rm1021 or Rm1021-WS12 (e.g., 6.0 versus 0.6 U), and analyses of total cell yields supported the concept that in the absence of supplemental biotin, the biotin overproducers generated more bacterial cells (Table 2). Plating tests proved, however, that higher OD_{600} measurements in stationary growth phase in the absence of supplemental biotin were associated with increases in the number of viable cells only for Rm1021-WS10 (Table 2). Total cell counts indicated that the percent viability relative to the control strain was reduced significantly in Rm1021-WS10 and dramatically in Rm1021-WS11 in both the presence and absence of supplemental biotin (Table 2).

The growth of both Rm1021-WS10 and Rm1021-WS11 was impaired in the experimental alfalfa rhizosphere relative to Rm1021 (Fig. 4). Although Rm1021-WS10 reached the same titer as Rm1021 after 13 days, the titer of Rm1021-WS11 was significantly lower at that time and showed essentially no growth during the first 9 days. On day 6, there was no significant difference between root colonization by Rm1021 and Rm1021-WS12, which carries the empty pLAFR3 (data not shown). PCR assays for the presence of the *E. coli* biotin synthesis operon in 50 isolates taken on day 13 from roots inoculated with Rm1021-WS11 showed that 43 isolates still contained those genes. Analyses with two other primers, Tn*5*out and WS4063, which are specific for the Tn*5* and a DNA sequence 1.3 kb upstream from the Tn*5* insertion in the biotin synthesis operon of *R. meliloti*, respectively (32), showed that all isolates still carried Tn*5*-B30 in the same position as in the original mutant Rm1021-B3. Five cycles of selection in vitro for any mutations that increased viability in Rm1021-WS11 showed that viability never increased above 1% (data not shown). PCR analyses of cells from the fifth cycle of selection showed that 15 of 51 cells had lost the *E. coli* biotin synthesis operon.

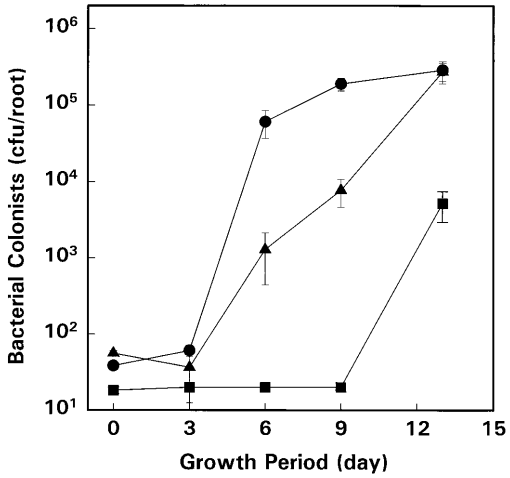

FIG. 4. Effect of *E. coli* biotin synthesis genes on rhizosphere growth of *R. meliloti*. Cells of Rm1021 (●), Rm1021-WS10 (▲), or Rm1021-WS11 (■) were inoculated on day 0 and recovered from alfalfa rhizospheres at the times indicated.

In competitive colonization tests, the biotin-overproducing strain Rm1021-WS10 was outcompeted by Rm1021 and was impaired significantly relative to its growth in the absence of the wild-type parent (Table 3). There was no evidence that biotin produced from Rm1021-WS10 increased the growth of Rm1021 under those conditions, but parallel tests with the biotin auxotroph Rm1021-B3 grown in the presence and absence of Rm1021-WS10 suggested that the latter strain released biotin in the rhizosphere.

DISCUSSION

Recombinant strains of *E. coli* (6, 25), *Bacillus sphaericus* (11, 19), and *Serratia marcescens* (26) that overproduce biotin were constructed previously by various groups interested in alternatives to the chemical synthesis of this vitamin. The *R. meliloti* strains constructed here represent the first attempt to use biotin synthesis genes to manipulate plant-microbe interactions. The strains produced thus far grow faster than does the control strain Rm1021-WS12, release larger amounts of biotin, and yield more cell matter in the absence of added biotin (Table 2). At the same time, however, these recombinant strains have reduced viability in pure culture (Table 2) as well as impaired growth (Fig. 4) and competitiveness (Table 3) in the alfalfa rhizosphere.

Reducing the size of the *E. coli* DNA insert in pLAFR3 to produce pCosEcbio2 from pCosEcbio1 did not improve the performance of strain Rm1021-WS11 over that of Rm1021-WS10. Surprisingly, only 1% of Rm1021-WS11 cells were viable when cultures reached stationary phase after 72 h of growth in the absence of added biotin (Table 2). This result suggested that biotin overproduction associated with an enhanced growth rate in *R. meliloti* may have led to accumulation of a product which increased cell death directly or upset normal metabolic or regulatory functions. The fact that decreased viability in Rm1021-WS10 and Rm1021-WS11 was found also in the pres-

ence of high levels of biotin (4 μM), which normally shut off biotin synthesis in *E. coli* (8), suggests that the expression of the *E. coli* biotin synthesis genes in *R. meliloti* is poorly regulated. Biotin itself cannot be blamed for the decreased viability, because the 4 μM treatment did not kill Rm1021-WS12 cells (Table 2).

The decreased viability associated with overexpression of the biotin synthesis genes in Rm1021-WS10 and Rm1021-WS11 has not been reported previously for cells with additional biotin synthesis capacity. This phenomenon probably differs from the negative effect of these genes on the growth rate of *S. marcescens* (26) and *E. coli* (13), because no decline in growth rate was detected for the *R. meliloti* recombinant strains. Growth inhibition in the *E. coli* study (13) was overcome by a mutation in *bioB*, which is contained in the 7.5-kb DNA fragment present in both Rm1021-WS10 and Rm1021-WS11 (Table 1; Fig. 1). The *bioB* gene codes for a poorly understood enzyme involved in converting dethiobiotin to biotin (13). If mutating *bioB* could overcome the 99% loss in viability measured in Rm1021-WS11 (Table 2), our unproductive attempts to isolate such a mutant through five cycles of selection should, in statistical terms, have succeeded. These facts lead to the conclusion that the significantly greater viability of Rm1021-WS10 than of Rm1021-WS11 (Table 2) is probably associated with the additional 22.5 kb of DNA in Rm1021-WS10. Studies looking for a stabilizing factor in the extra 22.5 kb of DNA in pCosEcbio1 by complementation tests in Rm1021-WS11 are under way. A second possible solution might be to decrease biotin overproduction slightly by inserting an extra copy of the regulatory gene *birA* (8).

It is not clear that the decreased viability of Rm1021-WS10 and Rm1021-WS11 in pure culture (Table 2) explains the impaired root colonization measured for these recombinant strains (Fig. 4). Day 0 CFU values for both strains were determined by plating at the time of inoculation onto the root, and wild-type Rm1021 cells clearly grew exponentially until day 6 in this experimental system. In pure cultures with or without supplemental biotin, these recombinant strains grew better than Rm1021 cells lacking extra biotin synthesis capability (Table 2), so one would expect that the small number of recombinant cells present on day 0 in the rhizosphere would have grown as well as Rm1021. Thus, there may be some additional problems slowing the growth of recombinant strains on the root. If simple mutations can improve rhizosphere growth of these strains, the common strategy of enriching for that phenotype by several cycles of growth on plant roots may select improved strains (1). Whether it will be possible to characterize such changes at the molecular level must be determined in every case.

The use of recombinant strains produced in this study offers a preliminary view of both the difficulties and the potential facing microbial ecologists who consider using biotin as a tool for enhancing the rhizosphere growth of beneficial bacteria. One of the greatest advantages in using either pCosEcbio1 or pCosEcbio2 with the biotin auxotroph Rm1021-B3 is that these plasmids are maintained in the strain by internal, rather than external, selection pressures. This type of autoselective system was described recently in terms of using a foreign *thyA* gene to maintain a plasmid in *R. meliloti* lacking thymidylate synthase (18). Theoretically, genes that contribute to root colonization or some useful rhizosphere phenotype could be placed in pCosEcbio1 or pCosEcbio2 and maintained by such a mechanism. When the negative effects of biotin overproduction on the viability of *R. meliloti* are overcome, such genetic constructs should be quite useful.

ACKNOWLEDGMENTS

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