Roles for Riboflavin in the *Sinorhizobium*–Alfalfa Association

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Genes contributing to riboflavin production in *Sinorhizobium meliloti* were identified, and bacterial strains that overproduce this vitamin were constructed to characterize how additional riboflavin affects interactions between alfalfa (*Medicago sativa*) and *S. meliloti*. Riboflavin-synthesis genes in *S. meliloti* were found in three separate linkage groups and designated as *ribBA*, *ribDribC*, and *ribH* for their similarities to *Escherichia coli* genes. The *ribBA* and *ribC* loci complemented corresponding *E. coli* *rib* mutants. *S. meliloti* cells containing extra copies of *ribBA* released 10 to 20% more riboflavin than a control strain but grew at similar rates in a defined medium lacking riboflavin. Cells carrying extra copies of *ribBA* colonized roots to densities that were 55% higher than that of a control strain. No effect of extra *rib* genes was detected on alfalfa grown in the absence or presence of combined N. These results support the importance of extracellular riboflavin for alfalfa root colonization by *S. meliloti* and are consistent with the hypothesis that this molecule benefits bacteria indirectly through an effect on the plant.

Additional keywords: lumichrome, nitrogen fixation.

Riboflavin is a necessary component of the flavin coenzymes FMN and FAD in both bacteria and higher organisms (McCormick 1989). Less recognized is the fact that, although bacteria contain numerous flavoenzymes, synthesis of riboflavin can far exceed the amount required by those proteins. Thus, in *Escherichia coli*, the ratio of extracellular to intracellular metabolite is only 0.01 for amino acids and 0.05 for nucleic acid bases, but the corresponding value for riboflavin varies from 0.8 to 8.0 (Wilson and Pardee 1962). Riboflavin from *E. coli* cells presumably benefits human hosts, and the release of riboflavin by most root-colonizing bacteria (Strzelczyk and Rozycki 1985) suggests that this molecule may have evolved as a significant ecological factor in plant–bacteria interactions.

Riboflavin clearly contributes to symbiotic associations between legumes and members of the family *Rhizobiaceae*. Rhizobia showing riboflavin auxotrophy form root nodules

but reduce little N₂ (Schwinghamer 1970). Presumably, riboflavin present in root exudates (Rovira and Harris 1961) supports growth and root colonization by such auxotrophs, but riboflavin availability in the root zone can limit plant–microbe interactions, because adding trace amounts of this vitamin to the rhizosphere increases alfalfa root colonization by *Sinorhizobium meliloti* 1021 (Sm1021) (Streit et al. 1996). The nature of that limitation is not clear because rhizobia, in general, do not respond with additional growth when riboflavin is supplied (Vincent 1977) and Sm1021, in particular, releases riboflavin in medium lacking riboflavin (Phillips et al. 1999). Some rhizobial riboflavin in the root zone is degraded to lumichrome, which stimulates root respiration (Phillips et al. 1999) and may, therefore, contribute to the exogenous CO₂ required for growth by this organism (Lowe and Evans 1962). The question, therefore, is whether adding riboflavin to the rhizosphere promotes root colonization through direct effects on bacterial growth or through indirect promotion of bacterial growth, for example, by increasing root respiration or exudation. One approach to answering this question is to construct rhizobia that overproduce riboflavin. Unfortunately, no published information on riboflavin synthesis in the family *Rhizobiaceae* is available, and thus a series of preliminary studies was required.

Biochemical pathways for riboflavin synthesis and the *rib* genes required for those reactions are well characterized in both *E. coli* (Fig. 1) and *Bacillus subtilis* (Perkins and Pero 1993). Homologous genes have been identified by sequencing and/or complementation of defined *E. coli* mutants in *Actinobacillus pleuropneumoniae* (Fuller and Mulks 1995) and *Haemophilus influenzae* (Fleischmann et al. 1995). Riboflavin overproducers have been developed for pharmaceutical purposes (Demain 1972), and *E. coli* cells containing *Helicobacter pylori* *ribBA* excrete additional riboflavin (Worst et al. 1998), which facilitates iron acquisition and ferric-iron reduction in the transconjugants. This study was initiated to develop riboflavin-overproducing constructs of *S. meliloti* that could help clarify the role of riboflavin in *S. meliloti*–alfalfa interactions.

RESULTS

S. meliloti *rib* genes.

Using microbiological materials specified in Table 1, two separate *S. meliloti* genomic cosmids that complemented *E. coli* *rib* mutants were found. One restored growth in the absence of riboflavin to *E. coli* strains BSV18 and BSV11, which have mutations in *ribA* and *ribB*, respectively, while another complemented *E. coli* strain BSV13, which is mutated in *ribC*. Restriction mapping, subcloning, and sequencing of the two cosmids established physical maps (Fig. 2A and B) and identi-

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fied open reading frames that code for amino acid sequences in *S. meliloti* that are 48 to 60% similar to corresponding *E. coli* and *H. influenzae* genes (Table 2). The corresponding complementation of *E. coli* strains BSV18 and BSV11 by the probable *ribBA* gene and of *E. coli* strain BSV13 by the putative *ribC* gene supports the conclusion that these *S. meliloti* genes are involved in riboflavin synthesis. The 62% homology of the *S. meliloti* RibBA protein to *E. coli* RibB at the N-terminal end and the 48% homology to *E. coli* RibA at the C-terminal end (Table 2; Fig. 3) and the capacity of *S. meliloti* *ribBA* to complement both the *ribA* and *ribB* mutants of *E. coli* support the suggestion that *S. meliloti* produces a single, multifunctional RibBA protein, as occurs in *Helicobacter pylori* (Worst et al. 1998). The capacity of the complete *ribBA* sequence amplified by polymerase chain reaction (PCR) primers GY50 and GY51 to complement both the *ribA* and the *ribB* mutation in *E. coli* differs from the results obtained with *ribBA* in *Helicobacter pylori*, in which riboflavin synthesis was restored only in the *ribB* mutation in *E. coli* (Fassbinder et al. 2000). The presence in *S. meliloti* *ribBA* of two putative, in-frame translational stop sites, which are shown in Figure 3 as asterisks, was confirmed by sequencing three times and subsequently by 100% identity to data reported by the *S. meliloti* genome project (available online through Institut National de la Recherche Agronomique [INRA]).

Thus, the simplest conclusion is that *S. meliloti* *ribBA* produces a protein with the activities of both 3,4-dihydroxy-2-butanone 4-phosphate synthase (Richter et al. 1992) and GTP cyclohydrolase (Richter et al. 1993), while *ribC* codes for riboflavin synthase (Eberhardt et al. 1996) (Fig. 1). Sequence analysis identified an open reading frame immediately upstream from *ribC* (Fig. 2B) with a predicted protein product similar to the RibD protein in *E. coli* (Taura et al. 1992) and *H. influenzae* (Fleischmann et al. 1995) (Table 2). The putative *ribH* gene amplified from *S. meliloti* genomic DNA (Fig. 2C), likewise, was identified by its 50% similarity to the amino acid sequence of the RibH proteins in *E. coli* (Taura et al. 1992) and *H. influenzae* (Fleischmann et al. 1995) (Table 2).

Effects of extra *rib* genes on *S. meliloti*.

S. meliloti cells containing plasmid-borne copies of extra *ribBA*, Sm1021-GY1, consistently released 10 to 20% more ri-

boflavin than the control strain Sm1021-WS12, which carries the empty plasmid vector (Fig. 4). Liquid cultures of Sm1021-GY1 could be visually separated from control cells by an obvious yellow color. In some tests, the presence of extra *ribDribC* genes, Sm1021-GY2, produced additional riboflavin (Fig. 4, day 3), but that result was not consistent (Fig. 4, day 4). Sm1021-GY4 containing extra *ribH* genes produced no additional riboflavin during initial pure-culture tests and was not examined further for this capacity. High-performance liquid chromatography (HPLC) analyses of riboflavin and lumichrome released from bacteria incubated 3 days in a C- and N-deficient "root-exudate" medium showed similar increases in total flavins (mean $\pm$ standard error [SE], nmol/ml) associated with extra copies of *ribBA*: Sm1021, 4.07 ± 0.08 ; Sm1021-GY5, 5.08 ± 0.72 ; and Sm1021-TVB1, 5.00 ± 1.34 .

Cells containing extra *ribBA* genes showed highly significant increases in N content when grown in Vincent's defined medium (Table 3), and the liquid supernatant was visibly yellow in comparisons with control strains. In tests with both Vincent's defined medium lacking riboflavin (Fig. 4) and the "root-exudate" medium (data not shown), extra *rib* genes had no effect on cell growth, measured either by 600-nm absorbance or by direct counts of CFU. Thus, there was no evidence to support the concept that *S. meliloti* strains with a normal complement of *rib* genes examined in this study were deficient in the riboflavin production required for growth in pure culture.

The most striking result of this study was that a strain containing extra copies of *ribBA*, which produced slightly elevated levels of riboflavin, colonized alfalfa roots to a much greater extent than the control (Fig. 5), even though the strain exhibited no corresponding increase in growth in pure culture (Fig. 4). In a representative experiment, the presence of extra *ribBA* (Sm1021-GY1) increased root colonization by 55% (Fig. 5). Sm1021-GY2 containing extra copies of *ribDribC* never colonized roots better than the control strain, despite an occasional transitory increase in riboflavin production (Fig. 4). This colonization test was a direct measure of cell growth in the root zone, because after 6 days, 40,000 to 60,000 CFU were recovered on the root from an inoculum of 400 ± 70 cells (mean $\pm$

Fig. 1. Riboflavin biosynthesis and the *Escherichia coli* genes involved, based on Richter and colleagues (1992, 1997).

SE). The plasmid pLAFR3 was maintained stably in these tests, as reported previously (Streit and Phillips 1996).

Plant experiments.

Symbiosis tests showed no effects of extra *ribBA* genes in Sm1021 on either plant growth or N₂ fixation. In 5-week plant-growth experiments using Sm1021-GY1, Sm1021-GY5, or Sm1021-TVB1 with Sm1021-WS12 or Sm1021 as the appropriate control strain, bacterial genotypes had no significant effect on plant dry weight in the presence or absence of mineral N (data not shown).

Experiments designed to test whether extra riboflavin production by rhizosphere bacteria could promote bacterial growth

on roots by increasing exudation of amino acids also gave negative results. In those experiments, 20 or 200 nM riboflavin was supplied to roots of sterile, hydroponically grown alfalfa seedlings for 5 days, and picomolar amounts of amino acids in the root solution were separated by HPLC and quantified with a fluorescence assay on day 0 and day 5. No significant increase in any amino acid in the exudates was detected with either riboflavin treatment (data not shown).

DISCUSSION

Results from this study establish that *S. meliloti* genes involved in riboflavin synthesis are functionally and structurally

Table 1. Strains and plasmids used in this study

Strain	Relevant traits ^z	Source or reference
<i>Escherichia coli</i>		
DH5 α	<i>recA1</i> , Δ <i>lacZ</i>	Invitrogen, Carlsbad, CA, U.S.A.
HB101	pRK2013 helper strain for matings	Stratagene, La Jolla, CA, U.S.A.
S17-1	Modified RP4 integrated into genome	Simon et al. (1983)
BSV18	<i>ribA</i> :Tn5 mutant, Km ^r	Bandrin et al. (1983)
BSV11	<i>ribB</i> :Tn5 mutant, Km ^r	Bandrin et al. (1983)
BSV13	<i>ribC</i> :Tn5 mutant, Km ^r	Bandrin et al. (1983)
<i>Sinorhizobium meliloti</i>		
Sm1021	Sm2011, Sm ^r	Meade et al. (1982)
Sm102F51	Field isolate	Nitragin Co., Milwaukee, WI, U.S.A.
Sm1021-WS12	Sm1021pLAFR3	Streit and Phillips (1996)
Sm1021-GY1	Sm1021pLAFR3 containing Sm1021 <i>ribBA</i>	This work
Sm1021-GY2	Sm1021pLAFR3 containing Sm1021 <i>ribDribC</i>	This work
Sm1021-GY4	Sm1021pLAFR3 containing Sm1021 <i>ribH</i>	This work
Sm1021-GY5	Sm1021pSUP205 <i>ribBA</i> recombinant, fast growth on 5 μ g of Tc per ml	This work
Sm102F51-GY6	Sm102F51pSUP205 <i>ribBA</i> recombinant, fast growth on 5 μ g of Tc per ml	This work
Sm1021-TVB1	Sm1021pSUP205 <i>ribBA</i> recombinant, slow growth on 2.5 μ g of Tc per ml	This work
Plasmids/transposons		
pBSK+	Multicopy cloning vector, Ap ^r	Stratagene, La Jolla, CA, U.S.A.
pCR2.1	Multicopy cloning vector, Ap ^r , Km ^r	Invitrogen, Carlsbad, CA, U.S.A.
pLAFR3	pLAFR1 derivative, <i>mob</i> , λ <i>cos</i> , Tc ^r , low copy in both <i>E. coli</i> and <i>S. meliloti</i>	Staskawicz et al. (1987)
pJQ200mp18	pACYC184 <i>ori</i> , <i>mob</i> , <i>sacB</i> , Gm ^r	Quandt and Hynes (1993)
pRK2013	pRK212.2 conjugation helper plasmid	Figurski and Helinski (1979)
pSUP205	pBR325 derivative, <i>mob</i> , λ <i>cos</i> , Cm ^r , Tc ^r , multicopy in <i>E. coli</i> , does not replicate in <i>S. meliloti</i>	Simon et al. (1983)
Ω -Tc	pUT::mini-Tn5Tc, Tc ^r	De Lornezo et al. (1990)

^z Ap = ampicillin, Cm = chloramphenicol, Gm = gentamicin, Km = kanamycin, Sm = streptomycin, and Tc = tetracycline.

Fig. 2. Riboflavin biosynthesis genes from *Sinorhizobium meliloti*. **A**, *ribBA*; **B**, *ribDribC*; and **C**, *ribH*. Restriction sites are represented as E, *Eco*RI; H, *Hind*III; K, *Kpn*I; P, *Pst*I; and S, *Sma*I. Arrows below the map indicate open reading frames predicted from nucleotide sequence analysis. Polymerase chain reaction primers denoted by GY50 to GY56 are specified in text.

related to *E. coli* homologues and that increased riboflavin production enhances alfalfa root colonization. Although a role for riboflavin in symbiotic performance of rhizobia was indicated previously by comparing auxotrophic and prototrophic strains (Schwinghamer 1970), our data show that a small increase (10 to 20%) in the capacity to release riboflavin in *S. meliloti* (Fig. 4) enhances root colonization (Fig. 5) without altering

Table 2. Similarity in amino acid sequences of riboflavin-synthesis proteins

^x Similarity is expressed as conserved residues calculated by GenBank analysis (Altschul et al. 1990) using sequences reported for *E. coli* RibA (Richter et al. 1993), RibB (Richter et al. 1992), RibC (Eberhardt et al. 1996), RibD (Taura et al. 1992), and RibH (Taura et al. 1992) or for *H. influenzae* (Fleischmann et al. 1995).

S. meliloti RibBA: MSYDQKRVVEATRAFEAGEIVVVTDDGGRENEGDLIVA AVHCTPEKMAFI
E. coli RibA: |||||
E. coli RibB: ERVENAL AALREGRGVMVLDDEDRENEGDMIFPAETMTVEOMALT

Fig. 3. Comparison of the *Sinorhizobium meliloti* strain Sm1021 RibBA (395 amino acids) with the RibA and RibB homologues in *Escherichia coli*. Identical amino acids in the homologues are linked by lines; comparable amino acids are enclosed with borders. Asterisks (*) indicate apparent stop codons.

tion that adding nanomolar amounts of riboflavin to the alfalfa rhizosphere increased *S. meliloti* root colonization (Streit et al. 1996). Data reported here offer the first genetic evidence for a link between riboflavin production and root colonization (Fig. 5) and show that riboflavin effects in the rhizosphere are not simply explained by a direct effect on growth of the bacterial cells producing it. Our interpretation of the evidence, which is discussed below, suggests that beneficial effects of extracellular riboflavin on rhizosphere bacteria probably are mediated by the plant.

The amounts of riboflavin overproduction measured in this study were small compared with those observed in microorganisms modified for the pharmaceutical synthesis of this vitamin (Demain 1972). For this reason, the overproducing strains described here can be viewed as practical tools for testing ecologically relevant hypotheses. Strains characterized in Table 3 might be used, for example, to test the effects of riboflavin production on predation by nematodes.

The mechanism or mechanisms by which our strains produce additional riboflavin probably involve an increased conversion of GTP at the beginning of the pathway (Fig. 1). This suggestion is based on the fact that the RibA and RibB proteins in *E. coli*, for which Sm1021 *ribBA* substituted in the complementation tests, interact with GTP at the first committed step toward riboflavin biosynthesis. Extra copies of *ribBA* clearly increased riboflavin production (Fig. 4), and this fact suggests that, in Sm1021, the reaction of RibBA with GTP normally limits riboflavin synthesis.

The exact mechanism by which riboflavin contributes to root colonization by *S. meliloti* remains to be clarified. The simple explanation would be that extracellular riboflavin, either added to the rhizosphere by the investigator (Streit et al. 1996) or supplied through genetic modification of the bacteria (Fig. 5), increases root colonization by direct effects on bacterial cells that are deficient in the vitamin. This explanation, however, is not consistent with several facts. First, Sm1021 is not a riboflavin auxotroph because, even without extra *rib* genes, it grows and releases riboflavin into a medium lacking riboflavin (Phillips et al. 1999) (Fig. 4). Second, there is no evidence that Sm1021 cells are physiologically deficient in riboflavin under

any growing conditions tested here, because extra copies of the *ribBA* gene had no effect on growth in pure culture, even though the bacterial population was exposed to higher levels of riboflavin (Fig. 4). (One can note in passing that extracellular riboflavin is not used easily by all bacteria. *E. coli* riboflavin auxotrophs studied here, for example, grew only when riboflavin was supplied at levels that created a visibly yellow medium.) Rhizobial cells containing extra copies of *ribBA* genes clearly had higher N contents (Table 3), but the only physiological effect associated with that additional internal N in these studies was an increase in root colonization (Fig. 5). This analysis suggests that one can look outside the bacterial cell to understand how riboflavin is functioning in this plant-bacteria interaction. One possibility is that riboflavin produced by bacteria increases their growth on roots indirectly through an effect on the plant.

Rhizosphere bacteria benefit greatly from carbon supplied by the root, including both reduced-carbon compounds and, possibly, CO₂. In the case of rhizobia, exogenous CO₂, which they require for growth (Lowe and Evans 1962), may come partially from the increase in root respiration that occurs in the presence of lumichrome, a degradation product of riboflavin (Phillips et al. 1999). Results from the current study are consistent with that mechanism, if extra riboflavin from the *ribBA* transconjugants was degraded to lumichrome and increased root respiration. Unfortunately, such parameters cannot be measured without disturbing a root-colonization test. It is also possible that bacterial riboflavin can affect net exudation of reduced-carbon compounds from the root. We found no evidence for such an effect on amino acids, but a new, highly sensitive fluorescence assay for carbohydrates (Honda et al. 2000) should permit more detailed analyses in the future.

MATERIALS AND METHODS

Bacterial strains, media, and chemicals.

Strains and plasmids used in this study are listed in Table 1. Sm1021 and its derivatives were grown at 28°C in complex TY (Beringer 1974); in Vincent's defined minimal, riboflavin-free medium (Vincent 1970) containing glucose (10 mg/ml); or in alfalfa (*Medicago sativa* L.) "root exudate," which is described under plant tests.

E. coli was grown at 37°C in Luria-Bertani (LB) medium (Sambrook et al. 1989). Antibiotics, unless otherwise stated, were used at the following concentrations: kanamycin, 25 µg/ml for *E. coli*, 200 µg/ml for *S. meliloti*; chloramphenicol, 50 µg/ml; tetracycline, 5 µg/ml for *S. meliloti*, 10 µg/ml for *E. coli*; ampicillin, 50 µg/ml; and streptomycin, 400 µg/ml. *E. coli* mutants *ribA*:Tn5 (BSV18), *ribB*:Tn5 (BSV11), and *ribC*:Tn5 (BSV13) (Bandrin et al. 1983), which were generously supplied by B. Bachmann from the *E. coli* Genetic Stock Center, Yale University, New Haven, CT, U.S.A., were maintained on a high-riboflavin medium (K₂HPO₄, 2 mg/ml; KH₂PO₄, 1 mg/ml; NH₄Cl, 2.5 mg/ml; MgSO₄, 250 µg/ml; glucose, 10 mg/ml; casein hydrolysate, 10 mg/ml; and riboflavin, 2.5 µg/ml). Stan-

Fig. 4. Growth and riboflavin production in Vincent's defined medium lacking riboflavin by recombinant *Sinorhizobium meliloti* strains containing extra copies of riboflavin biosynthesis genes in pLAFR3. Sm1021-GY1 and Sm1021-GY2 were constructed with the self-replicating plasmid pLAFR3; the control strain Sm1021-WS12 contained an empty pLAFR3. Mean cell numbers ($\pm$ standard error) did not differ significantly among genotypes on days 3 and 4, when they were $1.18 \times 10^9 \pm 1.52 \times 10^7$ and $1.22 \times 10^9 \pm 5.76 \times 10^6$, respectively. Gene effects were significant at $P \leq 0.05$ (*).

Table 3. Effects of extra *ribBA* genes on C and N content of *Sinorhizobium meliloti* grown to stationary phase in Vincent's defined medium lacking riboflavin²

Strain	Extra genes	C %	N %	C/N
Sm1021	None	47.6 a	4.59 a	10.4 a
Sm1021-GY5	<i>ribBA</i>	38.9 b	8.43 c	4.6 b
Sm102F51	None	41.3 b	6.96 b	5.9 b
Sm102F51-GY6	<i>ribBA</i>	40.6 b	8.47 c	4.8 b

² Mean values in a column followed by different letters (a,b,c) show significant ($P \leq 0.05$) genotype effects.

dard chemicals, reagents, and antibiotics were purchased from Fisher Scientific (Santa Clara, CA, U.S.A.) and Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Isolation of *S. meliloti* genes.

Three major *rib* genes were located in Sm1021 by complementing defined *E. coli* mutants. Genomic Sm1021 DNA was partially digested with *Sau*3AI, and the 5- to 8-kilobase fragments were collected after electrophoresis in 0.8% agarose gels. Those DNA fragments were ligated into pBSK+ and electroporated into the *E. coli rib* mutants. Cells were allowed to recover in SOC medium (Sambrook et al. 1989) with riboflavin (2.5 µg/ml) for 5 h at 37°C, and then riboflavin-synthesizing transformants were selected by several passages on LB medium containing kanamycin and ampicillin. Plasmids complementing the *rib* mutants were isolated for further study. The unannotated nucleotide sequence for the Sm1021 genome, which became available during the final stages of this work (*S. meliloti* genome project [available online through INRA]), was searched for homologies to *E. coli ribH* (Taura et al. 1992), and a putative *ribH* gene was amplified by PCR from genomic Sm1021 DNA using primers *GY55*, 5'-ATG CCG ATA TCC TTG AGA CCG-3', and *GY56*, 5'-GTC TGC CCG GAG ATA GGT GTC-3'. The resulting product (Fig. 2C) was ligated into pLAFR3.

S. meliloti DNA complementing the *E. coli ribA*, *ribB*, and *ribC* mutants was subcloned into pCR2.1 with a TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA, U.S.A.), and both strands of the complementing DNA were sequenced automatically (ABI 377; Applied Biosystems Perkin-Elmer, Foster City, CA, U.S.A.) at the Division of Biological Sciences DNA Sequencing Facility (University of California, Davis, U.S.A.) using primers specified by the manufacturer. Assembled sequences were analyzed for similarities to known sequences using the BLAST tool (Altschul et al. 1990).

Construction of riboflavin-overproducing strains.

To construct Sm1021 strains containing extra *rib* genes, DNA was amplified by PCR using the following primers (Fig. 2): *GY50*, 5'-GGC GGC TGA AGT AAC TCC ACT-3'; *GY51*, 5'-AAG ACG AGC TTC TCA AGA CCT-3'; *GY52*, 5'-CCG AAG AGG TCC CGG TCT ACT-3'; *GY53*, 5'-GTC TTC GGA ACC GCA ATA GGG-3'; *GY54*, 5'-GAC GTT ATT CCT GCT TGG GAG-3'; and *GY55* and *GY56* specified above. The ends of these DNA fragments were modified as required and inserted into the multiple-cloning sites of pLAFR3 and pSUP205. Sm1021-GY5, Sm102F51-GY6, and Sm1021-TVB1 were constructed by mating the nonreplicating vector pSUP205 with *ribBA* into either Sm1021 or Sm102F51. This technique produces an integration by recombination into the *ribBA* region of the chromosome, and selection on tetracycline gives rise to a nontandem direct duplication of *ribBA* and the associated vector (Mavingui et al. 1997). Sm1021-TVB1 and Sm1021-GY5 were selected for growth on 2.5 and 5 µg of tetracycline per ml, respectively, to yield strains with different copy numbers of *ribBA*. Sm102F51-GY6 was selected on 5 µg of tetracycline per ml.

Riboflavin production tests.

Tests for riboflavin production (i.e., synthesis and release) in pure culture were conducted with bacteria growing in Vincent's minimal medium. Bacteria were removed by centrifugation, and absorbance at 444 nm was measured in a Perkin-Elmer Lambda 20 spectrophotometer using fresh medium as a control and standard curves determined from known concentrations of authentic riboflavin.

Plant tests.

Alfalfa seedlings used for producing hydroponic root exudates were generated by a procedure verified to give sterile plants when tested on TY medium. This method involves treating unscarified seeds (500 mg) 8 min with concentrated H₂SO₄, rinsing with six volumes of sterile water, and then soaking for 25 to 30 min in commercial bleach solution (5.25% NaClO, wt/vol) and rinsing again with six volumes of sterile water. Finally, the seeds are soaked overnight at 4°C in a solution containing ampicillin (100 µg/ml), kanamycin (100 µg/ml), and rifampicin (10 µg/ml). Seeds treated with these procedures were rinsed extensively with sterile water before being placed in a sterile hydroponic chamber consisting of a petri dish (90 × 25 mm, diameter × height) containing 25 ml of sterile N-free mineral solution (Fähraeus 1957) with a cheesecloth seed bed supported by wire mesh. The 130 resulting seedlings were grown without aeration for 5 days before collecting the root-exudate-containing solution. Sterility of the solution from every container was verified on TY medium before it was stored at -20°C. Seedlings also were verified for external sterility of roots by streaking on TY.

Assays for bacterial colonization of alfalfa cv. Moapa 69 roots were performed in vermiculite as described previously (Streit et al. 1996). In these tests, the roots of sterile, 24-h-old seedlings were inoculated with small numbers of cells (250 to 550 CFU per plant), and bacterial growth was assessed by isolating cells from roots after 6 days. In cases in which symbiotic properties of bacteria were assessed, larger numbers of cells (e.g., 10⁸ CFU per plant) were inoculated onto roots of sterile, 24-h-old seedlings growing in vermiculite Leonard jars containing N-free nutrient solution (DeJong and Phillips 1981). Plants were harvested after 28 to 35 days to record fresh weight and dry weight of shoots and roots.

Chemical analyses.

Amino acids were separated by HPLC and quantified with a fluorescent tag (Cohen and Michaud 1993) using the AccQ Tag system (Waters, Milford, MA, U.S.A.) according to the manufacturer's protocol. Total C and N content were analyzed by combustion (Pella 1990a, 1990b).

Fig. 5. Alfalfa root colonization by recombinant *Sinorhizobium meliloti* strains containing extra copies of riboflavin biosynthesis genes. Sm1021-GY1, Sm1021-GY2, and Sm1021-GY4 were constructed with the self-replicating plasmid pLAFR3; the control strain Sm1021-WS12 contained an empty pLAFR3. The effect of *ribBA* on root colonization (+55%) was significant at $P \leq 0.05$ (*), as shown on the log scale. Error bars indicate 1 standard error (SE). An average ($\pm$ SE) of 400 $\pm$ 70 cells was inoculated on each root 6 days before colonization was measured.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

Sinorhizobium meliloti genome database: <http://sequence.toulouse.inra.fr/meliloti.html>