Sinorhizobium meliloti Flavin Secretion and Bacteria-Host Interaction: Role of the Bifunctional RibBA Protein

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Sinorhizobium meliloti, the nitrogen-fixing bacterial symbiont of *Medicago* spp. and other legumes, secretes a considerable amount of riboflavin. This precursor of the cofactors flavin mononucleotide and flavin adenine dinucleotide is a bioactive molecule that has a beneficial effect on plant growth. The *ribBA* gene of *S. meliloti* codes for a putative bifunctional enzyme with dihydroxybutanone phosphate synthase and guanosine triphosphate (GTP) cyclohydrolase II activities, catalyzing the initial steps of the riboflavin biosynthesis pathway. We show here that an in-frame deletion of *ribBA* does not cause riboflavin auxotrophy or affect the ability of *S. meliloti* to establish an effective symbiosis with the host plant but does affect the ability of the bacteria to secrete flavins, colonize host-plant roots, and compete for nodulation. A strain missing the RibBA protein retains considerable GTP cyclohydrolase II activity. Based on these results, we hypothesize that *S. meliloti* has two partly interchangeable modules for biosynthesis of riboflavin, one fulfilling the internal need for flavins in bacterial metabolism and the other producing riboflavin for secretion. Our data also indicate that bacteria-derived flavins play a role in communication between rhizobia and the legume host and that the RibBA protein is important in this communication process even though it is not essential for riboflavin biosynthesis and symbiosis.

Riboflavin (7,8-dimethyl-10-ribitylisoalloxazine; vitamin B₂) is a water-soluble vitamin and the direct precursor of the flavin cofactors flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD). FMN and FAD play important roles in energy metabolism in aerobic cells by accepting electrons during oxidation of amino acids, carbohydrates, and fatty acids, and by donating electrons to complex II of the electron transport chain. These flavin nucleotides are also required in redox reac-

tions during DNA repair, bioluminescence, and light sensing. Moreover, flavins are essential for the biosynthesis of secondary metabolites and the metabolism of vitamins B₆ and B₁₂ as well as folates. Flavin metabolism likely plays a significant role in the interaction of bacteria with eukaryotic host cells. Lumichrome, a riboflavin derivative produced by the unicellular green alga *Chlamydomonas reinhardtii*, has been shown to bind the bacterial quorum-sensing receptor of *Pseudomonas aeruginosa* (Rajamani et al. 2008). Bacterial flavins were shown to be important in *Brucella* virulence (Bonomi et al. 2010; Swartz et al. 2007).

Bacteria, yeast, and plants have functional pathways for the biosynthesis of riboflavin (Bacher et al. 2000, 2001; Roje 2007) whereas animals do not and, thus, require the compound as a vitamin in the diet. In bacteria, the enzymes encoded by the *ribBA*, *ribD*, *ribH*, and *ribE/C* genes convert one molecule of guanosine triphosphate (GTP) and two molecules of ribulose-5-phosphate into riboflavin. Yeasts and plants synthesize flavins through a similar pathway, although reduction of the ribosyl moiety follows deamination of the pyrimidine moiety in bacteria and plants and precedes it in yeast (Roje 2007).

Sinorhizobium meliloti is an α -proteobacterium belonging to the order Rhizobiales. It invades the root cells of the host *Medicago* plants and triggers the development of symbiotic organs, called root nodules, which contain specialized plant cells occupied by the bacteria (Saeki 2011). Inside these nodules, *S. meliloti* reduces atmospheric di-nitrogen (N₂) to ammonium and provides it to the host plant for growth (Den Herder and Parniske 2009). Like many other root-colonizing bacteria (Dahm et al. 1993; Kanu 2009; Strzelczyk and Rozycki 1985), *S. meliloti* secretes a considerable amount of riboflavin and lumichrome (Phillips et al. 1999; Yang et al. 2002). The extracellular riboflavin and lumichrome play an important role in host-plant root colonization by *S. meliloti* and enhance root respiration and shoot growth in the host plant (Phillips et al. 1999; Yang et al. 2002). It has been proposed that riboflavin as well as FMN and FAD mediate this effect by facilitating electron transfer from the cell surface to the extracellular respiratory chain in plants and bacteria (Brutel and Gralnick 2012; Higa et al. 2010). Moreover, application of biologically active levels of lumichrome can improve photosynthetic rates in soybean and several other plant species (Khan et al. 2008; Matiru and Dakora 2005a and b).

Previous studies have shown *S. meliloti* has close sequence homologs of the *ribD* (SMc01772) and *ribH* (SMc01777) genes of *Escherichia coli*, and that it has functional homologs of the

In memory of Dietz Bauer.

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ribA, *ribB*, and *ribC* (*ribE*/SMc01773) genes that can complement the corresponding mutants in *E. coli* (Yang et al. 2002). With the exception of *ribH1* (SMc01777) and *ribH2* (SMc01609), only one copy of each gene of the riboflavin biosynthetic pathway is found in the *S. meliloti* Rm1021 genome (Galibert et al. 2001). With the exception of RibH2, all other enzymes comprising the riboflavin biosynthetic pathway in *S. meliloti* appear to be well conserved within the order Rhizobiales, even in the pathogenic bacteria genera such as *Brucella* and *Bartonella*, the opportunistic pathogen *Ochrobactrum*, and the plant pathogen *Liberibacter*.

In this study, we investigated the role of RibBA protein (SMc00008) in *S. meliloti* flavin metabolism and secretion. We show that, although the RibBA protein exhibits GTP cyclohydrolase II activity, there are other enzymes that can catalyze this reaction and prevent riboflavin starvation in the absence of RibBA. We also show that the presence of RibBA is necessary for *S. meliloti* to secrete flavins efficiently. Our data suggest that *S. meliloti* has two partly interchangeable modules for making riboflavin, one fulfilling the internal need for flavins for bacterial metabolism and the other producing flavins for secretion.

RESULTS

The *S. meliloti* *ribBA* deletion mutant does not require riboflavin for growth.

Previous work showed that introducing a second copy of *ribBA* onto the chromosome increased riboflavin and lumichrome secretion in *S. meliloti* Rm1021 and supported the proposed biosynthetic function of the RibBA protein in riboflavin metabolism (Yang et al. 2002). To further investigate the role of *ribBA* in riboflavin biosynthesis and secretion, we generated in-frame deletions of the predicted full-length bifunctional dihydroxybutanone phosphate synthase and GTP cyclohydrolase II (Δ *ribBA*) or of the GTP cyclohydrolase II domain (Δ *ribA*). Three independent mutants per gene were constructed that were missing either the *ribA* domain or the full-length *ribBA* gene. They all exhibited identical phenotypes. Both Rm1021 Δ *ribBA*

and Rm1021 Δ *ribA* mutants were able to grow on the defined solid media (Min-mannitol-NH₄ [MMNH₄]) without riboflavin supplementation and both strains had similar growth rate in MMNH₄ broth (Fig. 1; Supplementary Fig. S1).

On the other hand, riboflavin auxotrophy was established when the Rm1021 strain was mutated by deleting *ribD*, which codes for a putative bifunctional 5-amino-6-(5-phosphoribosyl) uracil reductase diaminohydroxy-phosphoribosyl aminopyrimidine deaminase (Δ *ribD*) (Fig. 1). This indicated that the riboflavin-supplemented medium, which was used to maintain Rm1021 during construction and verification of the deletion of *rib* genes, supported the growth of riboflavin auxotrophs and prevented a selective advantage for genetic rearrangements allowing *rib* mutants to grow with insufficient exogenous riboflavin. The inability of the *ribD* mutant to grow also indicates that the media did not have sufficient levels of riboflavin to support mutants unable to make riboflavin and indicates that the growth of the *ribA* and *ribBA* mutants was not due to efficient scavenging.

The lack of riboflavin auxotrophy in strains missing *ribA* and *ribBA* was surprising because i) RibB and RibA are the only known enzymes catalyzing the dihydroxybutanone phosphate synthase and GTP cyclohydrolase II reactions, respectively, that are essential for riboflavin biosynthesis (Bacher et al. 2000, 2001; Roje 2007) and ii) only one putative *ribBA* gene was found in the Rm1021 genome (Galibert et al. 2001). Furthermore, TBLASTN analysis identified no putative homologs to *ribA* or *ribB* in Rm1021. This finding suggests that *S. meliloti* has alternative enzymes that catalyze the initial steps of the riboflavin biosynthetic pathway. One possibility is that these alternative enzymes catalyze reactions identical to those catalyzed by RibA and RibB.

The *S. meliloti* *ribBA* deletion mutant retains GTP cyclohydrolase II activity.

To test whether the *ribBA* mutant lost the catalytic activity attributed to the RibA domain, GTP cyclohydrolase II activity was measured in Rm1021 Δ *ribBA* missing the whole *ribBA*

Fig. 1. Growth of the *Sinorhizobium meliloti* *rib* mutants. *S. meliloti* strains were grown in Min-mannitol-NH₄ (MMNH₄) media (Rm1021, Rm1021 Δ *ribBA*, Rm1021 Δ *ribA*, and Rm1021 Δ *ribD*) or in MMNH₄ media supplemented with 250 μ M riboflavin (Rm1021 Δ *ribD*(RF)). OD₆₀₀ = optical density at 600 nm.

gene. This strain retained approximately 70% of the GTP cyclohydrolase II activity compared with the wild-type bacteria (Table 1). This indicates that *S. meliloti* produces an alternative enzyme that can convert GTP to 2,5-diamino-6-hydroxy-4-(5-phosphoribosylamino)pyrimidine in the absence of RibBA.

RibBA protein exhibits GTP cyclohydrolase II activity.

A similar level of GTP cyclohydrolase II activity in the wild-type strain and the *ribBA* mutant suggested that the RibBA protein might be inactive in *S. meliloti*. Examples of RibBA

Table 1. Symbiotic phenotype of *rib* mutants and guanosine triphosphate (GTP) cyclohydrolase activity of crude protein extracts from *Sinorhizobium meliloti* strains and recombinant RibBA^a

Sample	Description	Specific activity (pmol min ⁻¹ mg ⁻¹)	Shoot dry weight (mg/plant)
No inoculation	No bacteria	...	4.2 ± 1.26
Rm1021	Wild type	31.1 ± 5.17*	9.1 ± 2.53
Rm1021Δ <i>ribBA</i>	<i>ribBA</i> mutant	22.3 ± 7.31*	8.2 ± 2.56
Recombinant RibBA	Purified protein	103 ± 6.2	...

^a Asterisk (*) = specific activity of GTP cyclohydrolase II in Rm1021Δ*ribBA* was not significantly different from Rm1021 (significance was analyzed by Student's *t* distribution).

sequence homologs lacking GTP cyclohydrolase II activity have been found previously. For example, the riboflavin biosynthetic gene (*ribBA*) from the γ -proteobacterium *Shewanella oneidensis*, annotated as bifunctional dihydroxybutanone phosphate synthase and GTP cyclohydrolase II, lacks the second activity (Brutinel et al. 2013). However, RibBA from *Sinorhizobium meliloti* can functionally substitute for its *E. coli* homologs. In addition, the introduction of a second copy of the *ribBA* gene into the *S. meliloti* genome stimulated riboflavin secretion (Yang et al. 2002), indicating that the RibBA protein is probably functional. To clarify this matter, RibBA was cloned into the pET-30 Ek/LIC vector, overexpressed in *E. coli* and purified using Ni-NTA His_Bind resin (Supplementary Fig. S2). Activity assays of the recombinant RibBA protein showed that it does have the GTP cyclohydrolase II activity (Table 1).

S. meliloti Rm1021 efficiently secretes flavins.

In addition to secreting riboflavin, as reported previously (Phillips et al. 1999), Rm1021 released even higher amounts of FMN and FAD (Fig. 2). However, in contrast to the previous report (Phillips et al. 1999), lumichrome was not detected in the growth medium for Rm1021. Lumichrome is the major product of flavin photolysis (Sikorski et al. 2001). To prevent flavin degradation, we routinely grew samples for measurements

Fig. 2. Flavin secretion and accumulation by the *Sinorhizobium meliloti* strains. *S. meliloti* strains were grown in Min-mannitol-NH₄ media. Cell culture (1 ml) was taken after 1 or 3 days of growth. Cells were spun down and the mass of the pellets was measured. Lumichrome (LMC), flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD), and riboflavin (RF) concentration in the supernatants and the pellets were measured using high-performance liquid chromatography. The flavin concentration was normalized against the protein concentration. No significant differences were detected between the masses of the pellets formed by *rib* mutants and parental strain.

of flavin secretion in foil-covered flasks, and we found a significant amount of lumichrome in the medium when we grew Rm1021 in a flask that was not covered with foil (Fig. 2). Simultaneously, the concentration of other flavins in the same medium was significantly decreased, suggesting that the presence of lumichrome in Rm1021 growth medium was due to photolysis of other flavins.

To verify that the presence of lumichrome is due to flavin photolysis, we filter sterilized the growth medium of the Rm1021 strain after growing the bacteria in foil-covered flasks for 3 days. We then further incubated the medium with or without covering the flasks for an additional 3 days. The medium that was additionally incubated in foil-covered flasks had a flavin profile similar to the original medium, whereas the medium incubated in the flasks without the foil cover contained a significant amount of lumichrome and a decreased concentration of other flavins (Supplementary Fig. S3). These data strongly indicate that the presence of lumichrome is due to photolysis and not due to the enzymatic activity by bacterial proteins, as was suggested previously (Phillips et al. 1999).

Flavin secretion is reduced in the RibBA deletion strain.

To better understand the role of RibBA in *S. meliloti* Rm1021, we tested the ability of the *ribA* and *ribBA* mutants to secrete flavins. After 1 and 3 days of growth, *S. meliloti* cells missing the entire *ribBA* gene or the predicted *ribA* domain released a much smaller amount of flavins compared with the wild-type strain or the *ribBA* mutant transformed with a RibBA-overexpressing plasmid (Fig. 2), confirming that RibBA was important for riboflavin secretion (Yang et al. 2002).

To verify that the flavins detected in the growth medium did not result from cell lysis, control experiments were performed to measure the intracellular flavins (Fig. 2). After 1 day of growth, FMN and FAD but not riboflavin were detected inside the Rm1021 cells, whereas FMN and riboflavin but not FAD were detected in the Rm1021 growth medium. After 3 days of growth, FAD and FMN were detected inside Rm1021 cells and in the Rm1021 growth medium, whereas riboflavin was only detected in the growth medium. Interestingly, the *rib* mutant contained more but secreted less FMN and FAD than the parental strain. These differences indicate that flavins were actively secreted rather than released from porous or lysed dead cells.

Expression of *ribBA* in free-living cells.

To better understand the mechanism regulating the *ribBA* expression in *S. meliloti*, we measured the activity of the *ribBA* promoter in response to endogenous riboflavin using a translational β -glucuronidase *ribBA-gusA* (pJ1700-pRibBA) fusion.

The *ribBA* promoter was active in the exponential culture of Rm1021 grown in MMNH₄ medium with or without riboflavin (5.9 ± 1.14 and 5.6 ± 0.45 Miller units, respectively). The level of *ribBA* expression in the stationary phase was similar to that in the exponential phase (data not shown).

Symbiotic phenotype of the Rm1021 Δ *ribBA* mutant.

Deletion of the *ribA* region did not affect the ability of *S. meliloti* to establish an effective symbiosis with alfalfa. The plants nodulated with Rm1021 Δ *ribBA* were green and healthy. The shoot dry weight of the plants nodulated with the *ribBA* mutant did not differ significantly from the mass of the plants nodulated with the parental strain (Table 1), and the nodules formed by the wild-type strain and the mutant had similar rates of acetylene reduction (data not shown). Additionally, the *ribBA* mutant did not show any delay in the rate of nodule formation compared with the parental strain (Supplementary Fig. S4). In contrast, Rm1021 Δ *ribD* failed to form nodules on alfalfa roots (data not shown), indicating that the host-plant root rhizosphere does not contain a sufficient amount of riboflavin to complement the riboflavin auxotrophy and to ensure infection and nodule formation by the mutant.

The expression of *ribBA* was also examined in mature nodules. Four weeks after inoculation, the GUS activity resulting from the expression of β -glucuronidase from the *ribBA-gusA* (pJ1700-pRibBA) fusion was visualized in the nodules formed by Rm1021(pJ1700-pRibBA) by staining with β -D-glucuronide (X-Glu). A high level of *ribBA-gusA* expression was detected in infection zone II, where bacteria are released from infection threads, and within nitrogen fixing zone III containing mature bacteroids (Supplementary Fig. S5). These data suggest that the RibBA protein plays a role in both free-living and symbiotic cells.

The *ribBA* mutant is less effective when competing for nodulation with the wild-type bacteria.

Strain Rm1021 was marked with a neomycin resistance marker (Rm1021^{NmR}) (Table 2) and was used as co-inoculant with the strains Rm1021 and Rm1021 Δ *ribBA*. The nodule occupancy by Rm1021^{NmR} versus the tested strains (NmS) was compared with the initial bacterial titer in the co-inoculum (Fig. 3). Three independent experiments showed that the *ribBA* mutant had a decreased ability to compete with the parental strain for nodulation.

The *ribBA* mutant is impaired in alfalfa root colonization.

To confirm the role of RibBA in the interaction of *S. meliloti* with the host plant reported previously by Yang and associates

Table 2. Bacterial strains and plasmids

Strains	Genotype or characteristics	Source
<i>Sinorhizobium meliloti</i>		
Rm1021	Wild type	Galibert et al. 2001
Rm1021 Δ <i>ribA</i>	<i>ribA</i> deletion mutant of Rm1021	This work
Rm1021 Δ <i>ribBA</i>	<i>ribBA</i> deletion mutant of Rm1021	This work
Rm1021 Δ <i>ribD</i>	<i>ribD</i> deletion mutant of Rm1021	This work
<i>Escherichia coli</i>		
S17-1	<i>pro hsdR recA</i> [(RP4-2(Tc::Mu) (Km::Tn7)]	Simon et al. 1989
Plasmids		
pCPP30	IncP LacZ; Tc	Huang et al. 1992
pCPPribBA	pCPP30 (<i>S. meliloti ribBA</i> including a 500-bp promoter region)	This work
pK19mobsacB	pK19mob derivative <i>sacB</i>	Schafer et al. 1994
pK19mobsacB- Δ <i>ribA</i>	pK19mobsacB (500 bp flanking <i>ribA</i>)	This work
pK19mobsacB- Δ <i>ribBA</i>	pK19mobsacB (500 bp flanking <i>ribBA</i>)	This work
pK19mobsacB- Δ <i>ribD</i>	pK19mobsacB (500 bp flanking <i>ribD</i>)	This work
pJ1700G	pFAJ1700 (<i>XbaI-HindIII gusA</i>)	Yurgel et al. 2010
pJ1700-pRibBA	0.5 kb <i>ribBA</i> promoter	This work
p30LICRibBA	pET-30 Ek/LIC, RibBA expression	This work

(2002), alfalfa seedlings were inoculated with either the wild-type strain or the *ribBA* mutant, and the colonization was assessed by isolating the bacteria from the roots after 5 days of growth. In agreement with previously published report (Yang

et al. 2002), Rm1021 was much more effective in the host-plant root colonization than Rm1021 Δ *ribBA* (Fig. 4). At least sixfold fewer bacteria were isolated from the roots colonized by the *ribBA* mutant than from the roots colonized by

Fig. 3. Competitiveness of the *Sinorhizobium meliloti ribBA* mutant. Percentage of the strains in the inoculum and in the nodules (three independent experiments). The lower, black segment represents the proportion of the test strains Rm1021 or Rm1021 Δ *ribBA* in inoculum or the nodules. The upper, white segment represents the proportion of the reference strain Rm1021^{NmR}. The middle, gray bar represent the proportion of the nodules with double occupancy. The proportion of the nodules occupied by Rm1021 Δ *ribBA* was significantly less than the proportion of the strain in the corresponding inoculum ($P < 0.001$).

Fig. 4. Alfalfa root colonization by *Sinorhizobium meliloti ribBA* mutant. The plants were grown in nitrogen-free plant nutrient solution; (RF) = the plant nutrient solution was supplemented with 10 nM of riboflavin. Bacteria were isolated from alfalfa roots and plated on Min-mannitol-NH₄ plates. Number of colonies was scored after 5 days of growth. Data are average from at least three independent experiments. Letters a, b, c, and d = significantly different ($P < 0.05$).

Rm1021. The overexpression of *ribBA* improved the root colonization by the *ribBA* mutant.

The existence of mechanisms through which biofilm formation and motility mediate *S. meliloti* attachment or invasion was recently proposed (Janczarek et al. 2010; Rinaudi et al. 2006; Rinaudi and Gonzalez 2009). However, neither motility nor biofilm formation were affected in the Rm1021 Δ *ribBA* strain (Supplementary Table S1). On the other hand, supplementation of the plant growth medium with 10 nM riboflavin improved the ability of the *ribBA* mutant to colonize the host plant roots (Fig. 4), indicating a direct link between riboflavin secretion and root colonization in *S. meliloti*.

DISCUSSION

Riboflavin biosynthesis in bacteria is thought to be well understood, and all enzymes composing this pathway are considered identified. Like all other bacteria, *S. meliloti* has sequence homologs of the RibA and RibB enzymes catalyzing both initial steps of the convergent riboflavin biosynthetic pathway. Here, we report that the *S. meliloti* strain missing the protein with the RibA and RibB domains was still able to produce riboflavin. Additionally, the GTP cyclohydrolase II activity in the mutant was only slightly lower than in the parental strain. These data suggest that *S. meliloti* possesses an alternative enzyme with GTP cyclohydrolase II activity, and probably also an alternative enzyme with the dihydroxybutanone phosphate synthase activity. To our knowledge, this is the first example of an organism having two different types of enzyme with GTP cyclohydrolase II activity. On the other hand, we have shown that deleting *ribD* leads to riboflavin auxotrophy, suggesting that bifunctional 5-amino-6-(5-phosphoribosylamino) uracil reductase diaminohydroxy-phosphoribosyl aminopyrimidine deaminase (RibD) is the main enzyme capable of performing this reaction in *S. meliloti*.

The overall quantitative contribution of RibBA to riboflavin biosynthesis remains to be clarified. It is possible that RibBA is the major enzyme of the standard pathway, and that deleting the gene stimulates the expression or post-translational activation of the alternative enzymes. Alternatively, two specialized riboflavin biosynthetic pathway modules might have evolved in *S. meliloti*, with one functioning in riboflavin biosynthesis to supply the needs of essential internal bacterial metabolism and the other to supply flavins that are secreted to mediate interactions with the host during root colonization. The redundancy of riboflavin biosynthetic genes in flavin-secreting *Shewanella* spp. was proposed to represent a mechanism to satisfy the requirement for intracellular flavins while releasing them into environment (Brutinel and Gralnick 2012; Higa et al. 2010).

Flavin biosynthesis is an energy-consuming process—the biosynthesis of a single molecule of riboflavin requires 25 molecules of ATP (Bacher et al. 2000; Brutinel and Gralnick 2012; Higa et al. 2010). This implies that flavin secretion is important to *S. meliloti*. It was shown previously that riboflavin contributes to root colonization by *S. meliloti* and it was proposed that riboflavin produced by rhizobia increases bacterial growth indirectly through an effect on the host plant (Phillips et al. 1999; Yang et al. 2002). Our data show that bacteria-derived flavins play a role in efficient root colonization and nodulation competitiveness. The finding that the *ribBA* mutation did not affect bacterial growth, motility, and biofilm formation in vitro suggests that the decreased root colonization is a result of a less-effective cross talk between the bacteria and the host plant, caused by decreased secretion of flavins by the microsymbiont. In a single-strain inoculation, the *ribBA* mutant did not exhibit a decreased nodulation rate, suggesting that the decreased competitiveness of the mutant in nodulation

competition assays might be due to its impaired root colonization performance. When the wild-type strain was also present in the inoculum, providing considerable amounts of bacteria-derived flavins in the rhizosphere, the competitiveness of the *ribBA* mutant was decreased, suggesting that flavin secretion confers an advantage to the bacterium that produces it during the host plant infection.

It was previously postulated that lumichrome produced by *S. meliloti* enhances alfalfa root growth (Phillips et al. 1999; Yang et al. 2002). Our results indicate that free-living *S. meliloti* does not secrete lumichrome but that photolysis of the secreted riboflavin, FMN, and FAD is the main source of lumichrome detected in the *S. meliloti* growth medium. Therefore, these data suggest that plant enzymes, bacterial enzymes specifically activated during the *S. meliloti*–legume interaction, or both would produce any lumichrome derived from bacterial riboflavin during the *S. meliloti*–host plant interaction in the absence of light.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media.

The bacterial strains and plasmids used in the study are listed in Table 2. The *S. meliloti* strains were grown at 30°C on Luria-Bertani (LB) (Sambrook et al. 1989), MMNH₄, or yeast mannitol broth (YMB) medium (Somerville and Kahn 1983). The *E. coli* strains were grown at 37°C on LB medium (Sambrook et al. 1989). The antibiotics for *S. meliloti* were added at 200 µg/ml (streptomycin), 200 µg/ml (neomycin), or 10 µg/ml (tetracycline). For *E. coli*, the antibiotics were added at 10 µg/ml (tetracycline) or 40 µg/ml (kanamycin). Sigma-Aldrich agar was used for media preparation. All genetic manipulations with *S. meliloti* that involve *rib* regions were done on plates supplemented with 500 µM riboflavin. Strains not requiring riboflavin were routinely grown on media without riboflavin.

Genetic techniques.

DNA manipulations were carried out following standard procedures (Sambrook et al. 1989). The deletion mutants were constructed using an insertion/excision strategy (Yurgel and Kahn 2005), with some modifications. Sucrose selection of *rib* mutants was done on YMB plates supplemented with 5% sucrose and riboflavin. To construct the *S. meliloti* *ribA*, *ribBA*, and *ribD* deletion mutants, polymerase chain reaction (PCR) was used to amplify the two chromosomal regions (0.5 and 0.6 kb) flanking the regions to be deleted, using primers listed in Table 3 (Supplementary Fig. S6). The deletions were confirmed by PCR using primers located outside of the regions flanking those used to construct the deletions. The ability of the strains to grow without riboflavin was tested after verification of the gene excision.

To construct the neomycin-resistant strain Rm1021^{Nm^R}, a 438-bp segment of DNA (chromosomal position 560,651 to 561,089) was amplified in the intergenic region between SMc00251 and SMc00250 using primers PhoB_rep_F and PhoB_rep_R (Table 3). The resulting PCR fragment was purified using the Zymoclean gel DNA recovery kit (Zymo-Research, Irvine, CA, U.S.A.), digested with the *Eco*RI and *Bam*HI restriction enzymes, and subcloned into the *Eco*RI-*Bam*HI-digested pK19*mobsacB*, resulting in the plasmid pK19PhoB-rep. Using conjugation, the plasmid was integrated into the Rm1021 chromosome, marking it with a neomycin resistance gene contained within the plasmid.

RibBA expression in *S. meliloti*.

The *ribBA* open reading frame (ORF) was amplified by PCR using KOD polymerase (Novagen, Madison, WI, U.S.A.) and

primers *ribB*-F-1 and *ribA*-R-2 (Table 3). To generate an expression vector, the resulting PCR fragment was purified using the Zymoclean Gel DNA Recovery Kit (ZymoResearch), digested with the *Eco*RI restriction enzyme, and subcloned into the *Eco*RI-digested pCPP30, resulting in plasmid pCPPribBA, which contains the *ribBA* gene with a 500-bp native promoter region.

Construction of a translational fusion to the *ribBA* promoter was done as described previously (Yurgel et al. 2010). Promoter fragments of approximately 500 bp were amplified from the Rm1021 chromosomal DNA by PCR using the primers *ribBA*_P-F and *ribBA*_P-R (Table 3). The PCR fragment was digested with *Bam*HI and *Eco*RI restriction enzymes and subcloned into the *Bam*HI/*Eco*RI-digested pF1700G, resulting in the plasmids listed in Table 2.

Assay for the GTP cyclohydrolase II activity in bacterial extracts.

The strains were grown in 3 ml of MMNH₄⁺ medium at 30°C for 48 h. The bacteria were diluted 20-fold into fresh MMNH₄⁺ medium and grown at 30°C for 16 h. The bacteria were pelleted and resuspended in 1 ml of lysis buffer (50 mM Tris-HCl [pH 7.5], 10 mM MgCl₂, 1 mM Tris[hydroxypropyl]phosphine, and 1× BugBuster reagent [Novagen]). After a 15-min incubation at room temperature, cell lysates were cleared by centrifugation at 22,800 × *g* for 15 min at 4°C. The cleared lysates were then desalted using 2-ml Zeba Spin Desalting Columns equilibrated with desalting buffer (50 mM Tris-HCl [pH 7.5], 1 mM Tris[hydroxypropyl]phosphine, and 10% glycerol), and were immediately used for assaying the GTP cyclohydrolase II activity.

GTP cyclohydrolase II converts GTP to 2,5-diamino-6-ribosylamino-4(3H)-pyrimidinone 5'-phosphate. In the assay procedure we used, this product was reacted with diacetyl (2,3-butanedione) to generate 6,7-dimethylpterin, which was then detected by fluorescence-monitored high-performance liquid chromatography (HPLC) (Bacher et al. 1997). GTP cyclohydrolase II was assayed in a total volume of 50 µl in 100 mM Tris-HCl (pH 8.5) buffer containing 5 mM MgCl₂, 5 mM dithiothreitol (DTT), 1 mM GTP, and 25 µl of the cleared whole-cell lysate. After the incubation at 37°C for 30 min, the assays were terminated and the product derivatized by adding 2.5 µl of EDTA (1 M, pH 8.0) and 50 µl of a mix containing 1% (vol/vol) diacetyl and 15% (wt/vol) trichloroacetic acid, fol-

lowed by incubation at 70°C for 20 min. The blanks were performed under the same conditions as the assays but with enzyme added after the 37°C incubation. The mixtures were centrifuged at 3,000 × *g* for 10 min and the supernatant was analyzed by HPLC. The fluorescent products were separated on a SunFire C18 reverse-phase column (4.6 by 150 mm, 3.5 µm) using a Waters Alliance 2695 HPLC system and mobile phase containing 3% (vol/vol) methanol and 97% phosphoric acid at a flow rate of 1 ml/min. Excitation and emission wavelengths were 330 and 435 nm, respectively. Product formation was determined after subtraction of the blank, based on the comparison with the standards. The product accumulation was linear up to 40 min.

RibBA expression in *E. coli*.

The RibBA ORF was amplified by PCR using KOD polymerase (Novagen) and the primers LIC_ribBA_F and LIC_ribBA_R (Table 3). To generate the expression vector, the resulting PCR fragment was purified using the Zymoclean gel DNA recovery kit (ZymoResearch), treated with T4 DNA polymerase (Promega, Madison, WI, U.S.A.), then ligated into the pET-30 Ek/LIC vector (Novagen), according to the manufacturer's protocols. After transformation and recovery, the construct was verified by DNA sequencing. The expression vector was then introduced into the Rosetta BL21 strain of *E. coli* (Invitrogen, Carlsbad, CA, U.S.A.) to produce the recombinant RibBA protein. Bacteria carrying the expression vector were cultured at 37°C in LB medium containing kanamycin at 100 µg/ml until the absorbance at 600 nm reached 0.5; the cultures were then cooled to 15°C. After the addition of isopropyl-thiogalactopyranoside (100 µM final concentration), the cultures were further incubated at 155°C overnight. The induced bacteria were pelleted at 4,000 × *g* for 5 min at 45°C.

Purification of the recombinant RibBA.

The bacterial pellet was resuspended in 5 ml of lysis buffer containing phosphate-buffered saline (PBS) (20 mM phosphate and 0.5 M NaCl, pH 7.4), 0.1% Triton X-100, 5 mM imidazole, and 0.1% phenylmethylsulfonyl fluoride (PMSF). The cell suspension was then sonicated 5 × 15 s at 45°C (Fisher Sonic Dismembrator 300 with the intermediate size attachment; power setting of 60%; Fisher Scientific, Pittsburgh). Unbroken cells and the cellular debris were removed by centrifugation at 10,000 × *g* for 30 min at 4°C. Cleared cell lysates were incubated with

Table 3. Primers used in the study

Primer	Sequence
<i>ribB</i> -F-1	5'-CATGGAATTCGCTCGATCAGGATATCGCCGC-3'
<i>ribB</i> -R-1	5'-CATGGGATCCATATATTGCCTCCGCGAATATCT-3'
<i>ribA</i> _F-1	5'-CATGGAATTCATCATCGTGCGCCATACCTCCGGC-3'
<i>ribA</i> _R-1	5'-CATGGGATCCCTCGAAGGAATGGCCCTGCTCG-3'
<i>ribA</i> _F-2	5'-CATGGGATCCAATCCGGAAGAGCCCAAAATAAA-3'
<i>ribA</i> _R-2	5'-CATGGAATTCCTGCCGAACGGCCTCGGCCTCA-3'
<i>ribBA</i> _ver-F	5'-ATCTCGACGGCATCCGCAAA-3'
<i>ribBA</i> _ver-R	5'-CTGAAGTCGGTCTCTCGGTC-3'
<i>ribD</i> _F-1	5'-CATGGAATTCCTCCGCTTCACGACCTTCGAGC-3'
<i>ribD</i> _R-1	5'-CATGGGATCCACCGCTTGTCTCCGGGTGA-3'
<i>ribD</i> _F-2	5'-CATGGGATCCATGTTACAGGCATCATCACCGACA-3'
<i>ribD</i> _R-2	5'-CATGGAATTCGACGCCCATCCACCTTGTTC-3'
<i>ribD</i> _ver-F	5'-AGACAGTCAGGTGAAGGATT-3'
<i>ribD</i> _ver-R	5'-TTGACGAGATCGCCGACTT-3'
LIC_ribBA_F	5'-GACGACGACAAGATGTCTACGACCAGAAGCGCGT-3'
LIC_ribBA_R	5'-GAGGAGAAGCCCGTTACAGAATCTCGGTAGCGGCGAT-3'
pRibBA promoter	
<i>ribBA</i> _P-F	5'-TCATGAATTCGAATCTGATGTCCATCAACG-3'
<i>ribBA</i> _P-R	5'-TCATGGATCCCATATATATTGCCTCCGCGAAT-3'
pK19PhoB-rep	
PhoB_rep_F	5'-TCATGAATTCGAAGGACTGCTAGTGCCAGC-3'
PhoB_rep_R	5'-TCATGGATCCCGCATAGCGTCAAGCGTCAC-3'

0.5 ml of Ni-NTA His_Bind resin (Novagen) at 8°C on a rotary shaker set at 120 rpm for 1 h, followed by filtering the sample-resin mixture through a disposable polypropylene filter column (Fisher). Wash buffers contained PBS, 0.1% Triton X-100, 5 mM imidazole, and 100 mM (low-salt) or 1 M (high-salt) NaCl. Equilibration and elution buffers contained 25 mM HEPES, 70 mM KCl, 0.1 mM EDTA, 0.5 mM DTT, and 0.1% PMSF, without (equilibration) or with 300 mM (elution) imidazole. Eluate fractions were immediately desalted using PD-10 desalting columns (GE Healthcare, Piscataway, NJ, U.S.A.) equilibrated with buffer G (50 mM Tris-HCl [pH 8.0], 10 mM MgCl₂, 1 mM Tris(hydroxypropyl)phosphine, 10% glycerol, and 0.2% Tween 20). GTP cyclohydrolase II activity of the recombinant RibBA was assayed as described above for the whole cells.

Flavin quantification.

The strains were grown in 3 ml of MMNH₄⁺ medium at 30°C for 48 h. The bacteria were diluted 20-fold into fresh MMNH₄⁺ medium and grown at 30°C for 1 or 3 days in tubes covered with aluminum foil. The bacteria were then pelleted and the mass of the pellets was measured. The extracellular flavins were extracted from pellets using an adaptation of a previously published method (Sandoval et al. 2008). Cell pellets were resuspended in a buffer consisting of 100 mM ammonium formate, 100 mM formic acid, and 25% methanol. Suspensions were heated at 80°C for 10 min, then centrifuged at 20,000 × *g* for 20 min at 4°C. The flavin-containing supernatant was passed through a 0.45-μm filter before chromatographic separation of the flavins, as described below. To measure flavin secretion, the supernatant was passed through a 0.45-μm filter before separation by reverse-phase chromatography using a Waters Alliance 2695 HPLC system with a Waters SunFire C₁₈ column (4.6 by 150 mm, 3.5 μm) linked to a Waters 2475 fluorescence detector. Riboflavin was detected by fluorescence using an excitation wavelength of 470 nm and an emission wavelength of 530 nm, and the concentration determined by comparison to standards. The mobile phase contained 100 mM ammonium formate, 100 mM formic acid, and 25% methanol.

Gene expression assays.

The strains were grown in 3 ml of MMNH₄ medium at 30°C for 48 h. The bacteria were diluted 20-fold into fresh MMNH₄⁺ medium and grown at 30°C for 16 h. β-D-glucuronidase assays and estimation of β-D-glucuronidase activities (in units per cell for optical density at 600 nm [OD₆₀₀]) were carried out according to the protocol devised by Miller (Humann et al. 2008; Miller 1972). For the histochemical assays, the alfalfa nodules were cut by hand, fixed, and incubated with 5-bromo-4-chloro-3-indoyl X-Glu, as described by Jefferson and associates (1987). The staining time for nodule sections was 1 h. The sections were examined by light microscopy and photographed using an Olympus BH-2 light microscope with a Nikon Coolpix 990 digital camera.

Growth measurements.

To evaluate growth, the *S. meliloti* strains were first grown on YMB plates for 48 h. The cells were then resuspended in MMNH₄ to OD₆₀₀ = 0.5 and 2 μl of the cell suspension was added to 200 μl of the test medium. Growth at 30°C was monitored for 48 h by measuring the absorbance at 600 nm every 30 min using a SPECTRAMax 250 Microplate Spectrophotometer System (Molecular Devices, Menlo Park, CA, U.S.A.).

Alternatively, cells from 2- to 3-day-old plates were resuspended in Min-salt solution to OD₆₀₀ = 0.5 and the cell suspensions were diluted with Min-salt solution at 1 to 10, 1 to 100, 1 to 1,000, 1 to 10,000, and 1 to 100,000 times in a 96-well microplate. Aliquots of these suspensions were then trans-

ferred using a sterile bolt replicator onto plates containing solid medium. The colony size was scored after 2 to 5 days.

Motility assay.

Cells were prepared as described for the growth measurements. A motility assay was done as described previously (Yurgel et al. 2007). A 2-μl volume was placed onto 0.3% Bacto agar swarm plates containing MMNH₄, YMB, or LB medium and incubated at 30°C for 3 days. For each experiment, the diameter of the area occupied by the mutants was measured and normalized as a percentage of the diameter of the swarm formed by the Rm1021 parental strain. These values were used to calculate relative motility of the mutants. The experiment was repeated at least three times.

Biofilm formation.

Biofilm formation was analyzed using a microtiter plate assay, as described previously (Fujishige et al. 2006).

Plant tests.

Alfalfa (*Medicago sativa* 'Ladak') was used for all nodulation studies. The plant tests were performed as described previously (Yurgel and Kahn 2005). Seedlings (24 h old) growing in sterile Magenta boxes (BioWorld, Dublin, OH, U.S.A.) on sand containing the N-free nutrient solution were inoculated with 10⁷ CFU of bacteria per plant. Four to five weeks after the inoculation, the plants were harvested, the shoot dry mass was measured, and the root nodule formation examined.

To analyze nodulation kinetics of the *S. meliloti* strains, 30 magenta boxes with six 24-h-old seedlings in each box were inoculated with Rm1021 and Rm1021Δ*ribBA* strains (1.1 × 10⁸ ± 3.5 × 10⁷ and 1.1 × 10⁸ ± 2.6 × 10⁷ cells per box, respectively). Time of the appearance and the number of nodules were scored every 2 to 3 days from day 6 until day 28. Plants from three boxes (18 plants) for each inoculation were analyzed at every time-point. The experiment was repeated two times.

Assays for bacterial colonization of alfalfa roots were performed in sand as described previously (Yang et al. 2002). Three boxes with six sterile 24-h-old seedlings were inoculated with small numbers of cells, and the bacterial growth was assessed by isolating cells from roots after 5 days. The experiment was repeated at least three times.

Competitiveness test.

The strain Rm1021^{NmR}, which was marked with neomycin resistance, was used as co-inoculant with the neomycin-sensitive *ribBA* mutant. The tested and control strains were grown on plates for 2 to 3 days, and resuspended in plant growth medium to an OD₆₀₀ of approximately 0.4. To estimate the titer of the cell suspensions, serial dilutions of the cells were plated on minimal defined medium with and without neomycin. The plants were simultaneously inoculated with Rm1021Neo and the tested strain. As a control, some plants were inoculated with Rm1021Neo and Rm1021 strains. After 4 weeks post inoculation, the plants were harvested and at least 50 nodules from the plants with each co-inoculation treatment were picked, surface sterilized, and crushed. The bacteria from each nodule were plated onto rich medium and 10 colonies formed by bacteria from each nodule were tested for neomycin resistance. The ratio of NeoS and NeoR cells in the initial co-inoculation and the number of nodules formed by NeoS, NeoR, or both strains were compared with the estimate of bacterial competitiveness.

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