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Identification and Characterization of RibN, a Novel Family of Riboflavin Transporters from *Rhizobium leguminosarum* and Other Proteobacteria

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Rhizobia are symbiotic bacteria able to invade and colonize the roots of legume plants, inducing the formation of nodules, where bacteria reduce atmospheric nitrogen (N_2) to ammonia (NH_3). Riboflavin availability influences the capacity of rhizobia to survive in the rhizosphere and to colonize roots. In this study, we identified the *RL1692* gene of *Rhizobium leguminosarum* downstream of a flavin mononucleotide (FMN) riboswitch. *RL1692* encodes a putative transmembrane permease with two EamA domains. The presence of an FMN riboswitch regulating a transmembrane protein is usually observed in riboflavin transporters, suggesting that RL1692 may be involved in riboflavin uptake. The product of *RL1692*, which we named RibN, is conserved in members of the alpha-, beta-, and gammaproteobacteria and shares no significant identity with any riboflavin transporter previously identified. In this work, we show that RibN is localized in the membrane cellular fraction and its expression is downregulated by riboflavin. By heterologous expression in a *Brucella abortus* mutant auxotrophic for riboflavin, we demonstrate that RibN possesses flavin transport activity. Similarly, we also demonstrate that RibN orthologues from *Ochrobactrum anthropi* and *Vibrio cholerae* (which lacks the FMN riboswitch) are able to transport riboflavin. An *R. leguminosarum ribN* null mutant exhibited lower nodule occupancy levels in pea plants during symbiosis assays. Thus, we propose that RibN and its homologues belong to a novel family of riboflavin transporters. This work provides the first experimental description of riboflavin transporters in Gram-negative bacteria.

Rhizobium leguminosarum bv. viciae is a soil Gram-negative bacterium that infects and establishes a symbiotic relationship with its host, *Pisum sativum* (the pea plant). *R. leguminosarum* is a member of the rhizobial group, which is phylogenetically diverse and includes 12 genera and about 70 species of alpha- and betaproteobacteria. Rhizobia induce nitrogen-fixing nodules in the roots of legume plants. The order *Rhizobiales* is included in the class *Alphaproteobacteria*, where several rhizobia can be found along with the mammal zoonotic pathogens *Brucella* spp. (1, 2).

Riboflavin is the precursor of the cofactors flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), which are the typical cofactors of flavoproteins. Flavoproteins are essential for multiple cellular processes, including energy production, redox reactions, light emission, biosynthesis, and DNA repair (3, 4). Riboflavin availability can influence the onset of rhizobial symbiotic interactions. A *Rhizobium trifolii* strain auxotrophic for riboflavin requires the addition of riboflavin to the plant growth medium in order to attain effective symbiosis with red clover plants, and the effectiveness of the nodulation of this strain in other clover cultivars relates to the flavin content in nodules (5). Also, the addition of riboflavin to the rhizosphere can increase the colonization of alfalfa roots by *Sinorhizobium meliloti* (6). Riboflavin has also been implicated in the virulence of pathogenic bacteria. We have previously demonstrated that flavin metabolism is essential for intracellular survival and mouse colonization by *Brucella abortus* (7).

The majority of bacterial species are able to synthesize riboflavin *de novo* through a biochemical pathway that creates one riboflavin molecule from one molecule of GTP and two molecules of ribulose 5-phosphate. The enzymes involved in this pathway include GTP cyclohydrolase II, 3,4-dihydroxy-2-butanone 4-phosphate (3,4-DHBP) synthase, pyrimidine deaminase/reductase 6,7-dimethyl-8-ribityllumazine synthase (lumazine synthase), and riboflavin synthase (8). The genes coding for these enzymes can be clustered, or the enzymes are encoded as monocistronic elements scattered on the bacterial chromosome (9). The expression of some of these genes is often regulated by an FMN riboswitch (also known as an FMN element). The FMN riboswitch is an RNA sequence located within the 5′ untranslated region (5′ UTR) of the mRNA that usually downregulates transcription or translation by adopting alternative secondary structures after binding an FMN molecule (10, 11). Bacterial genomes may also encode riboflavin transport proteins in addition to or as a replacement for riboflavin biosynthetic genes. The flavin transporters of the ABC-like energy coupling factor (ECF) transport system from Gram-positive bacteria, which uses the RibU substrate specificity component (S component) from *Bacillus subtilis*, *Lactococcus lactis*, and *Listeria monocytogenes* and RibM from *Corynebacterium glutamicum* and *Streptomyces davawensis*, have been functionally

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characterized and have been shown to present different substrate specificities and binding affinities (3, 12–16). The identification of the S components and the RibM transporters was facilitated by the presence of an FMN riboswitch in their 5′ UTRs and the genetic linkage with riboflavin biosynthesis clusters. On the basis of the same criteria, the *rfnT* gene from *Agrobacterium tumefaciens* and the *impX* gene from *Fusobacterium nucleatum* have been predicted to encode riboflavin transporters (9). However, experimental evidence for these two transporter candidates is still lacking.

FMN riboswitches have exclusively been found upstream of prokaryotic riboflavin biosynthesis genes and flavin transporter proteins (9, 17). In the work presented here, we identified an FMN riboswitch upstream of the *R. leguminosarum* open reading frame (ORF) *RL1692* and also demonstrated that RL1692, named RibN, is a riboflavin transporter. In addition, we assessed the role of RibN in the colonization of pea plant roots by *R. leguminosarum*, contributing the first evidence of the importance of a flavin transporter in the life cycle of a bacterium.

MATERIALS AND METHODS

Bacterial strains, media, and culture conditions. *Rhizobium leguminosarum* bv. viciae 3841 strains were cultured at 28°C in TY or Y minimal medium containing 0.2% (wt/vol) mannitol as the carbon source (18) on a rotary shaker (250 rpm) or incubator for plates. *Escherichia coli* cultures were grown in Luria-Bertani (LB; Difco, BD) or modified M9 minimal medium (19) at 37°C. The antibiotic kanamycin (Km; 25 μg/ml), streptomycin (Str; 400 μg/ml), or chloramphenicol (Cm; 30 μg/ml) was added when needed. *Brucella abortus* 2308 strains were cultured in tryptic soy broth (TSB; Bacto, BD) or Gerhardt-Wilson (GW) minimal medium (20) at 37°C. The antibiotic nalidixic acid (Nal; 5 μg/ml) or Cm (20 μg/ml) was added when needed. All live *Brucella* strains were manipulated in a biosafety level 2 facility at the Fundación Instituto Leloir. Bacterial growth was monitored at an optical density at 600 nm (OD_{600}) using a Beckman Coulter DU-530 spectrophotometer. Plasmids were mobilized into *Rhizobium* or *Brucella* by biparental mating from donor *E. coli* S17-1 in conjugation experiments (21).

Mutant strains and plasmids. To construct the *R. leguminosarum* Δ*ribN* strain, a 702-bp fragment and a 663-bp fragment were amplified from 5′ and 3′ flanking sites of *RL1692* using oligonucleotides RL1692M1_F/RL1692M1_R and RL1692M2_F/RL1692M2_R, respectively. The two PCR products were amplified by overlapping PCR using oligonucleotides RL1692M1_F/RL1692M2_R. The resulting fragment was cloned into the pGEM-T Easy vector (Promega) to generate pGmutRL1692. Next, the deletion allele was excised from pGmutRL1692 by BamHI/EcoRI digestion and ligated into pK18mobSacB to obtain pK18RL1692, which was conjugated into *R. leguminosarum*. Km-resistant and sucrose-sensitive single recombinants were selected and cultured overnight in the absence of antibiotics. Double recombinants were selected in TY medium–10% sucrose plates. Finally, deletion of the *RL1692* ORF was confirmed by PCR using specific primers flanking the mutation site (RL1692flank_F/RL1692flank_R).

The *RL1692* gene and its 5′ UTR regulatory sequence were amplified by PCR using oligonucleotides RL1692_F/RL1692M2_R. The resulting fragment was cloned in pGEM-T Easy to generate pGTRL1692. The fragment was excised from pGTRL1692 by XbaI/BamHI digestion and ligated into the XbaI/BamHI sites of pBBR1MCS1 (22), to generate pRL1692. Similarly, to construct plasmid pRL1692Myc, the *RL1692* regulatory and coding sequence was amplified using primers RL1692_F/RL1692Myc_R. The resulting fragment was cloned into the XbaI/KpnI sites of pBBR1MCS1.

The *E. coli* Δ*ribB* strain was constructed by homologous recombination using the PCR fragment method as reported before (23). A fragment was amplified using primers ribBH1P1_F/ribH2P2_R and plasmid pKD3 (23) as the template. The resulting product was transformed into strain BW25141 bearing plasmid pKD46 (23) that had previously been grown at 30°C in medium with arabinose added. Colonies were selected in LB plates supplemented with Cm at 37°C and screened by PCR using primers ribBflank_F/ribBflank_R flanking the mutation site. To construct pRibB, a colony PCR using BW25141 and oligonucleotides ribBFw-ribBRv was performed. The resulting product was ligated into the pGEM-T Easy vector. To construct pRibNv, a PCR product was obtained using primers RibNv_F/RibNv_R and *Vibrio cholerae* 569B chromosomal DNA. This product was ligated into the pGEM-T Easy vector. Similarly, to obtain pRibNo, a PCR was performed using primers RibNo_F/RibNo_R and genomic DNA from *Ochrobactrum anthropi* ATCC 49188. The resulting fragment was ligated into pGEM-T Easy. All of the constructions were confirmed by DNA sequencing. All primers and their sequences are listed in Table 1.

TABLE 1 Oligonucleotides used in this study

Primer name	Sequence (5′ to 3′)
RL1692M1_F	AAGAATTCTGAACTCAAGAACAAGGGCGACATTC
RL1692M1_R	ATGATGAACCAATGCCGGCAAGGACCATCCAGAG
RL1692M2_F	CCGGCATTGGTTCATCATGCGAAACGAGATGCG
RL1692M2_R	TTGGATCCTGACGTCTTCGTCGCGCTCATTTTC
RL1692flank_F	AGAATGAACTCGTGTCCGC
RL1692flank_R	GCATCTGTGGGATCTTGC
RL1692_F	AATAGGATCGATTCTAGAACTAGTGCAACGTGC CGAAATTCACGC
RL1692Myc_R	CGGGAGCGCAAACCAGCCGAGCAGAAGCTGAT CAGCGAGGAGGACCTGTGAGGTACCATT
ribBH1P1_F	CATAATTTTAGTGAGGTTTTTTTACCATGAATCA GACGCTACTTTCTGTAGGCTGGAGCTGCTTCG
ribH2P2_R	GTTTCTTGATTAAGGCAGTAAATTAAGCAGCGG TTTTCAGCTGGCTCATATGAATATCCTCCTTAG
ribBflank_F	AAGGGTACCCGGGTCAATCATTGTAGTTTCC
ribBflank_R	AAGGAGCTCGAATTAACATCTTGCATAGCAGGG
RibNv_F	AAGGGGCCCAATCACACTGCCTTAAACTCCTG
RibNv_R	AAGGAGCTCGTTTTAGGCGATGTTTCTTTCACG
RibNo_F	AAGGAGCTCGAGAGTTTCCGATACGTCCATGC
RibNo_R	AAGGGTACCATAGGCAGGCATCCTCACCG
qRibN_F	ATCTGAAGCTGCCGTTGAAT
qRibN_R	AACAGCGAAGCAGACAGGAT
IF-1_F	CGAAAACGAACACGAGATCA
IF-1_R	GTAGGGCGTCATTTCCACAA

Radiolabeled riboflavin uptake. *B. abortus* cells were cultured overnight in TSB medium carrying the appropriate antibiotics. Media were inoculated with bacteria at an initial OD_{600} of 0.1, and the bacteria were incubated at 37°C until they reached late exponential phase. Then, cells were harvested at 4,000 rpm (3,220 × *g*), washed 2 times with phosphate-buffered saline (PBS) plus 1 mM glucose (transport buffer), and resuspended at a final OD_{600} of 2 in the same buffer. Uptake experiments were performed in 50-ml tubes under shaking (250 rpm) at 37°C. Cell suspensions were kept in the shakers for 15 min before the initiation of the uptake experiments. The assay started after addition of the riboflavin isotopic mixture containing [^{3}H]riboflavin (Moravek Biochemicals Inc.) and nonradioactive riboflavin (Sigma-Aldrich) at a specific activity of 78.6 MBq/mg and a final concentration of 2.5 μM. Aliquots of 0.5 ml were removed after 0, 7, 15, 30, 60, and 120 min and placed in 0.5 ml stop solution (ice-cold nonradioactive 500 μM riboflavin). Cells were centrifuged at 11,000 rpm (8,930 × *g*) for 4 min and washed three times with stop solution. Washed radioactive bacterial pellets were resuspended in PBS and analyzed by liquid scintillation counting.

Cell fractionation. *R. leguminosarum* was cultured for 72 h in 5 ml of TY medium and centrifuged at 12,000 rpm for 2 min. The bacterial pellet was resuspended in 500 μl PBS and mechanically disrupted in a cell homogenizer (Precellys 24). The homogenate was centrifuged at low speed

(3,000 rpm) for 15 min in a microcentrifuge. The supernatant was recovered and further centrifuged at 13,000 rpm for 30 min to obtain the membrane fraction. The membrane pellet was resuspended in 500 μl PBS.

Western blotting. Total extracts of bacteria grown under different riboflavin concentrations or membrane and cytoplasmic fractions were separated by SDS-PAGE and transferred to a nitrocellulose membrane. Next, the membranes were blocked by a solution of 5% nonfat milk in PBS for 1 h and incubated with monoclonal anti-Myc antibody (1:1,000) for 4 h at 4°C. Membranes were washed with PBS 3 times for 10 min and incubated with peroxidase-coupled goat antimouse antibody (1:5,000; Sigma) for 1 h at 4°C. After three washes with PBS for 10 min, signals were detected using a chemiluminescent ECL Plus Western blotting detection system (Amersham Biosciences) on a Storm image and detection system (Molecular Dynamics).

Sequence analysis. Riboflavin biosynthesis genes and surrounding sequences from *R. leguminosarum* 3841 and *B. abortus* 2308 were obtained from the Kyoto Encyclopedia of Genes and Genomes (KEGG; http://www.genome.jp/kegg/). Riboswitches were identified in the Rfam database (http://rfam.sanger.ac.uk/) and analyzed with RibEx software (24). RibN orthologs were identified using a BLAST search, and their sequences were retrieved from the National Center for Biotechnology Information (NCBI) or from KEGG. Multiple-sequence alignment was performed using the ClustalW2 program from the European Bioinformatics Institute (EBI; http://www.ebi.ac.uk/Tools/msa/clustalw2/), and a phylogenetic tree was constructed in the Seaview (version 4.3.0) program using PhyML software with the HIVb model and default settings.

Real-time quantitative RT-PCR. Reverse transcription (RT) was performed with a transcriptor first-strand SuperScript III cDNA kit (Invitrogen) using random decamer primers (Invitrogen) and RNasin RNase inhibitor (Promega). cDNA samples were used as the templates in real-time PCRs. Primer3 software (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) was used to design primers for PCR products that ranged from 90 to 110 bp (qRibN_F and qRibN_R). Real-time PCR with mixtures containing SYBR green was performed in 96-well plates using an Mx3005P Stratagene instrument, and the products were analyzed with MXPro and LinReg software. Relative quantification using a standard curve method was performed for each set of primers. Results for each target mRNA were normalized to the levels of expression of *R. leguminosarum* ORF RL0616 initiation factor 1 (IF-1) mRNA using specific primers (IF-1_F and IF-1_R). The primers and their sequences are listed in Table 1.

Symbiosis assays. Infections were performed using pea plants (*Pisum sativum* var. Frisson), as previously described (25). Bacteria were resuspended in Fahraeus (FP) medium to a final OD_{600} of 0.1. One milliliter of the bacterial suspension was placed into 0.5% (wt/vol) FP-agar medium in a 250-ml flask containing a 2-week-old pea plant. After inoculation, only the aerial part of the plant was exposed to the light. Plants were cultured at 21°C with a 16-h light/8-h dark cycle in an incubation chamber (I-30BLL; Percival Scientific, Inc.) equipped with cool white fluorescent light tubes. In each independent experiment, five plants were inoculated for each rhizobial strain. At 12, 21, and 28 days postinfection (p.i.), total, red, and white nodules were quantified. Twelve nodules from each plant were harvested and placed in wells of a 96-well plate containing 100 μl of 10% (vol/vol) glycerol. Nodules were mechanically disrupted, and the numbers of CFU per nodule were determined.

RESULTS

Genetic organization of riboflavin biosynthesis genes and FMN riboswitches in *R. leguminosarum*. In previous work, we demonstrated that the *B. abortus* 2308 riboflavin biosynthesis pathway harbors two paralogs for the lumazine synthase RibH. In particular, the *ribH2* gene contains an FMN riboswitch and encodes a functional lumazine synthase with a novel quaternary arrangement that is essential for *B. abortus* intracellular survival and virulence (7, 26–28). We sought to determine the arrangement of riboflavin biosynthesis genes and FMN riboswitches in the genome of *R. leguminosarum* strain 3841 (29). This search is depicted in Fig. 1, where *ribD* and *ribE* apparently form an independent bicistronic operon, while *ribH1* is clustered with *nusB*. The *ribH2* gene, which is the *ribH1* paralog, is encoded separately, similar to the situation in *B. abortus*. *R. leguminosarum* codes for a RibB homologue, in addition to a bifunctional RibBA enzyme. In some bacteria, the functions of 3,4-DHBP synthase and GTP cyclohydrolase II are encoded by two separate genes, *ribB* and *ribA*, respectively (9). Duplication of *rib* genes is not unusual, and riboflavin biosynthesis gene assortment and genetic organization vary among bacterial species (9). We also aimed to determine all FMN riboswitches within this genome. This analysis revealed that *ribB*, *ribH2*, and ORF *RL1692* of unknown function harbor FMN riboswitches in their upstream coding regions (Fig. 1).

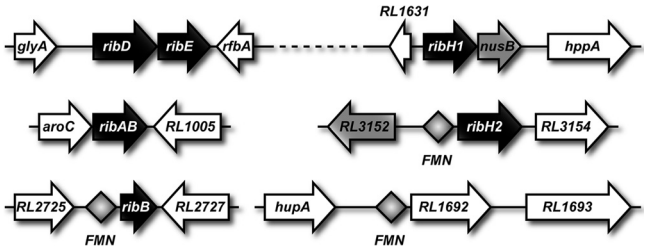

FIG 1 Schematic representation of riboflavin biosynthesis pathway genes and FMN riboswitches in the *R. leguminosarum* genome. Sequences from *R. leguminosarum* 3841 were retrieved from the KEGG database and analyzed. Black arrows, riboflavin biosynthesis-related genes; gray arrows, putative or previously characterized regulators; white arrows, unrelated or uncharacterized genes; gray diamonds, FMN riboswitches.

RL1692 encodes a putative transmembrane permease of 302 residues that we hypothesized functions as a riboflavin transporter. A BLAST search for proteins with significant identity with RL1692 retrieved sequences from species in 16 different families of that alpha-, beta-, and gammaproteobacteria, all of which are also uncharacterized proteins. A phylogenetic analysis using one representative RL1692 homologue from each family showed that the protein sequences from genes containing the FMN riboswitch clustered together (Fig. 2). All of the homologues that contained an FMN riboswitch belonged to species from the class *Alphaproteobacteria*. RL1692 was conserved and had high identity to proteins in other *R. leguminosarum* strains (data not shown), and the closest homologue to RL1692 in a different species was RHE_CH01591 from *Rhizobium etli* (Fig. 3). A noteworthy finding was that in most species, RL1692 orthologues lacked the FMN riboswitch (Fig. 2). This indicates that different regulatory mechanisms may have been selected across species. Alternatively, these homologues might transport substrates other than riboflavin.

***RL1692* encodes a flavin-regulated membrane protein.** A Pfam search using RL1692 and some orthologues showed that they contain EamA-like transporter family domains, making them putative transporter proteins (Fig. 3). The EamA domain family is contained in the membrane drug/metabolite transporter (DMT) clan (http://pfam.sanger.ac.uk/family/PF00892) (30). In addition, some EamA domain-containing proteins were previously characterized as nucleotide-sugar transporters (31). *In silico* prediction for transmembrane segments in the sequence of RL1692 retrieved eight putative transmembrane sections (Fig. 3). However, the number of predicted transmembrane domains var-

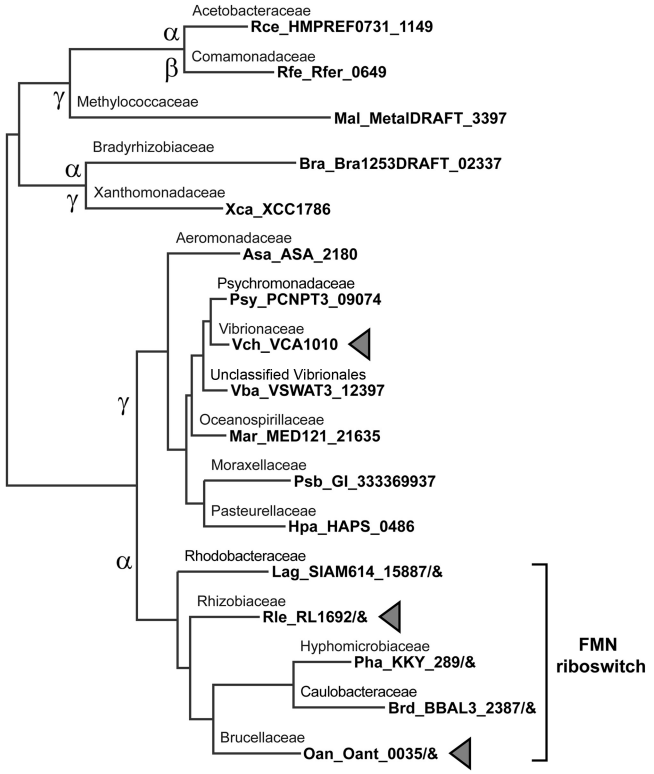

FIG 2 Phylogenetic tree of RL1692 homologues. Sequences from RL1692 and homologues were aligned using the ClustalW2 program, and the phylogenetic tree was constructed using PhyML software. The proteobacterial classes (alpha-, beta-, and gamma proteobacteria) and FMN riboswitch-containing genes are indicated. Rce, *Roseomonas cervicalis* ATCC 49957; Rfe, *Rhodoferax ferrireducens* T118; Mal, *Methylomicrobium album* BG8; Bra, *Bradyrhizobium* sp. strain WSM1253; Xca, *Xanthomonas campestris* pv. campestris strain ATCC 33913; Asa, *Aeromonas salmonicida* subsp. *salmonicida* A449; Psy, *Psychromonas* sp. strain CNPT3; Vch, *Vibrio cholerae* bv. El Tor; Vba, *Vibrionales bacterium* SWAT-3; Mar, *Marinomonas* sp. strain MED121; Psb, *Psychrobacter* sp. strain 1501; Hpa, *Haemophilus parasuis* SH0165; Lag, *Labrenzia aggregata* IAM 12614; Rle, *R. leguminosarum* bv. viciae 3841; Pha, *Pelagibacterium halotolerans* B2; Brd, *Brevundimonas* sp. strain BAL3; Oan, *Ochrobactrum anthropi* ATCC 49188. The homologues from *R. leguminosarum*, *O. anthropi*, and *V. cholerae* characterized in this study are indicated by gray arrowheads.

ied from seven to nine among the RL1692 orthologues (data not shown). Due to the presence of the FMN riboswitch in the RL1692 sequence and the prediction of transmembrane domains, it was likely that this gene encodes a transmembrane protein whose expression is regulated by FMN. To test if RL1692 is a membrane protein, we constructed a plasmid containing the complete RL1692 sequence, including its native regulatory region and a Myc tag fused at the C terminus (c-Myc) (Fig. 4A). This construct was transformed into the wild-type (WT) strain of *R. leguminosarum*, and then cells were fractionated into membrane and cytosolic fractions and subjected to Western blotting using an anti-c-Myc monoclonal antibody. The tagged RL1692 protein was detected exclusively in the membrane fraction (Fig. 4B). To analyze RL1692 expression, the strain was grown in the presence of different riboflavin concentrations and the RL1692-Myc protein was immunodetected in total extracts. The results showed that the expression of RL1692 is diminished at high riboflavin concentrations (Fig. 4C). Quantitative RT-PCR analysis of *RL1692* mRNA levels revealed no differences between bacteria cultured with 1,000 μM riboflavin and bacteria cultured in the absence of the metabolite (Fig. 4D). These results show that RL1692 is a membrane protein and that it is downregulated by riboflavin, probably through a translational attenuation mechanism.

***RL1692* encodes a riboflavin transporter.** The protein RL1692 and its orthologues share no significant similarity with previously characterized or putative riboflavin transporters. Thus, we speculated that these proteins constitute a new family of riboflavin transporters. In order to test the role of RL1692 in riboflavin transport, we introduced a plasmid harboring *RL1692* (pRL1692) into a *B. abortus* auxotrophic mutant strain and evaluated its riboflavin requirements. We have previously characterized the auxotrophic *B. abortus ribH1 ribH2* double mutant, which is unable to synthesize riboflavin (7). The genome of *B. abortus* does not harbor any obvious riboflavin transporter, and the *ribH1 ribH2* mutant requires up to 500 μM riboflavin in the culture medium to grow. A similar concentration (720 μM) is necessary to culture *Escherichia coli* riboflavin auxotrophs (32). It is likely that at such a high concentration of riboflavin crosses the membrane by simple diffusion rather than through a specialized transport system. As previously

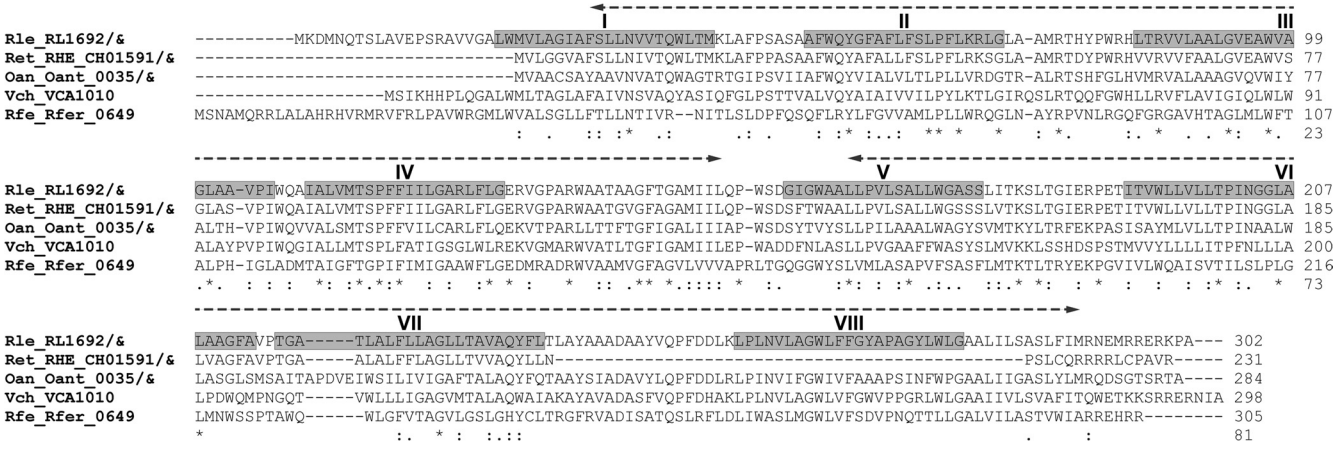

FIG 3 Sequence alignment of RL1692 orthologues. RL1692 and selected orthologues were aligned using the ClustalW2 program. Ampersands, presence of the FMN riboswitch in the corresponding gene; asterisks, identical amino acids; colons, conserved substitutions; periods, semiconserved substitutions. The putative transmembrane segments in RL1692 predicted by TMHMM software are highlighted in gray and designated by roman numerals. The two EamA domains of RL1692 are indicated by dashed double-headed arrows. Rle, *R. leguminosarum*; Ret, *R. etli*; Oan, *O. anthropi*; Vch, *V. cholerae*; Rfe, *R. ferrireducens*.

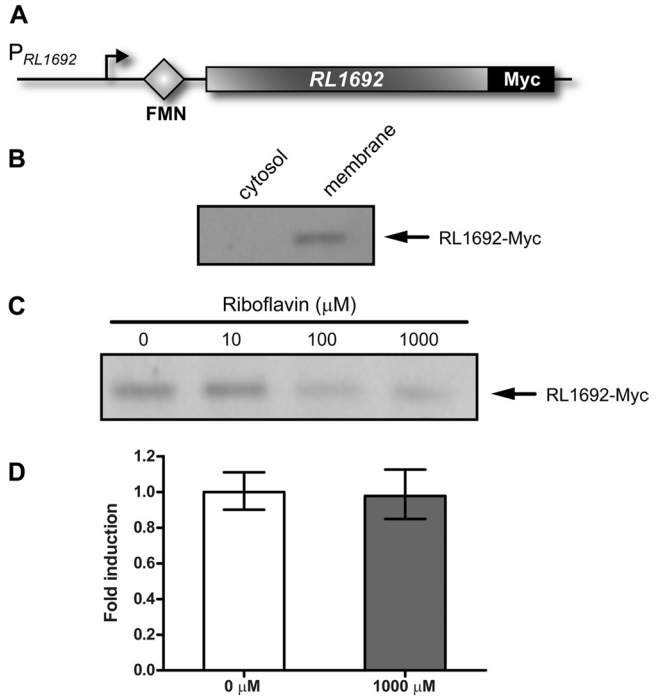

FIG 4 Subcellular localization and regulation of RL1692. (A) Schematic representation of the RL1692-Myc fusion from plasmid pRL1692Myc. RL1692 is expressed from its promoter (P_{RL1692}) and regulatory region, including the FMN riboswitch. (B) Western blot of cellular fractions obtained from *R. leguminosarum*/pRL1692Myc cultures. (C) Western blot of total cell lysates of *R. leguminosarum*/pRL1692Myc. Bacteria were cultured in TY medium at different riboflavin concentrations for 48 h. (D) The expression of RL1692 was assayed by RT-quantitative PCR in *R. leguminosarum*/pRL1692Myc cultured with 0 μM or 1,000 μM riboflavin using specific primers. The data shown are means ± SDs of triplicate samples from one representative experiment and are reported as the fold induction relative to the level of expression in 0 μM riboflavin. In panels B and C, the same amount of sample was loaded into the SDS-polyacrylamide gels and immunoblotted with an anti-Myc antibody.

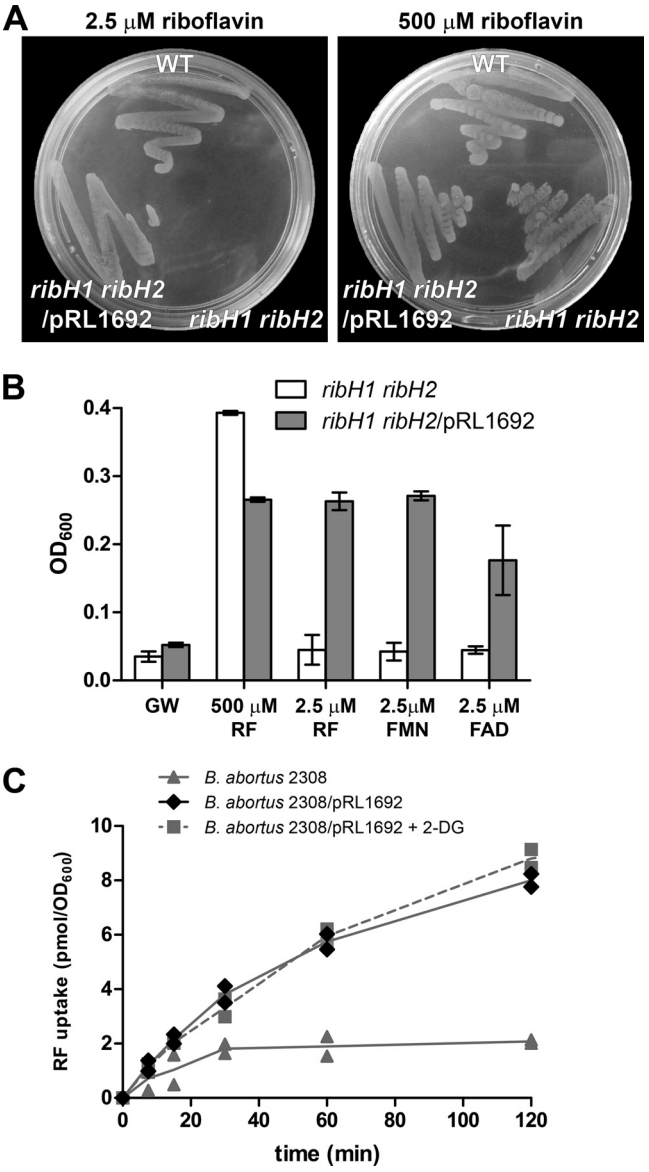

FIG 5 Characterization of RL1692 flavin transporter activity. (A) Tryptic soy agar plates to which a low concentration (2.5 μM) or a high concentration (500 μM) of riboflavin was added were inoculated with the indicated *B. abortus* 2308 strains and incubated for 48 h. (B) OD_{600} measurements from *B. abortus* strains cultured for 72 h in GW minimal medium with or without riboflavin, FMN, or FAD added. Values are expressed as means, and bars indicate the differences between duplicates. (C) [^{3}H]riboflavin (RF) uptake by *B. abortus* 2308 and *B. abortus* 2308/pRL1692. Cells suspended in transport buffer containing radiolabeled riboflavin were sampled at different times, washed, and assessed by liquid scintillation counting. Each data point represents a single replicate, and lines connect mean values.

reported, *B. abortus ribH1 ribH2* was not able to grow in plates with a low riboflavin concentration (2.5 μM) and the addition of 500 μM riboflavin completely restored growth (7). However, heterologous expression with pRL1692 restored growth at 2.5 μM riboflavin (Fig. 5A). The auxotrophic strain carrying pRL1692 was also able to grow in TSB rich medium even without the addition of riboflavin (data not shown), indicating the presence of flavin traces in the TSB. Similarly, growth of the *E. coli ribA* and *ribB* null mutants auxotrophic for riboflavin in rich medium but in the absence of additional riboflavin was restored by *S. davawensis* RibM overexpression (14). Figure 5B shows that the auxotroph *ribH1 ribH2* double mutant is unable to grow in liquid Gerhardt-Wilson (GW) defined minimal medium without riboflavin, irrespective of the presence of pRL1692. As expected, addition of 500 μM riboflavin to GW medium promoted the growth of the auxotroph independently of the expression of RL1692. In accordance with the results obtained in rich medium, only the double mutant bearing pRL1692 could grow at 2.5 μM riboflavin. Taken together, our observations indicate that RL1692 is a riboflavin transporter which we named RibN. In order to investigate whether RibN is able to import different flavins, we also cultured the strains in GW minimal medium to which 2.5 μM FMN or FAD was added. Both of these flavins were able to restore growth in the *ribH1 ribH2* mutant carrying pRL1692, indicating that both flavins can be imported. However, unlike riboflavin and FMN, FAD is not able to fully restore growth levels in this strain (Fig. 5B). In addition, radiolabeled riboflavin uptake experiments were performed with *B. abortus* 2308/pRL1692 to evaluate transport over time. In this experiment, the strain expressing RibN was able to incorporate riboflavin in a time-dependent manner, similar to the

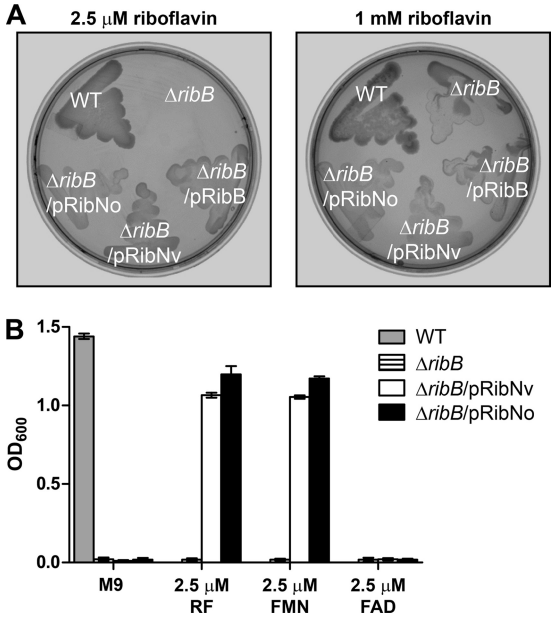

FIG 6 Heterologous expression of RibN homologues from *O. anthropi* and *V. cholerae*. (A) LB plates with 2.5 μM or 1,000 μM riboflavin were inoculated with the indicated *E. coli* strains and incubated for 18 h. (B) OD_{600} measurements from *E. coli* strains cultured for 20 h in modified M9 medium with or without the addition of 2.5 μM riboflavin (RF), FMN, or FAD. The WT strain was cultured in modified M9 medium without any flavin added. Values are expressed as means, and bars indicate the difference between duplicates.

findings for other reported riboflavin transporters (Fig. 5C). Addition of 2-deoxy-D-glucose (2-DG) did not affect the incorporation of radiolabeled riboflavin (Fig. 5C). The compound 2-DG leads to the depletion of ATP and also to the loss of membrane gradients. Together, these results show that RibN can transport flavins into the bacterial cell and suggest that the transporter is probably not coupled to proton uptake or any other coupling.

Functional characterization of other RibN homologues. We sought to test the ability of other RibN homologues to import riboflavin. For this purpose, we constructed plasmids carrying close and distant homologues of *R. leguminosarum ribN*: one from *Ochrobactrum anthropi* (pRibNo), a member of the alphaproteobacteria, and another from *Vibrio cholerae* (pRibNv), a member of the gammaproteobacteria. The RibN gene from *V. cholerae* comprises an interesting candidate because it represents the large group of *ribN* homologues that lack the FMN riboswitch (Fig. 2) and that are encoded independently of the riboflavin biosynthesis genes. Next, we evaluated the capacity of these two plasmids to restore the growth of an *E. coli* riboflavin auxotroph in low riboflavin. We constructed a Δ*ribB E. coli* strain that requires a high riboflavin concentration (1 mM) to sustain growth due to the impairment in riboflavin biosynthesis and its natural lack of transporters (Fig. 6B), similar to what has been reported previously (12, 16). As expected, the *ribB* copy provided in *trans* was able to restore growth in low riboflavin (Fig. 6A). Plasmid pRibNv or pRibNo was also able to restore the growth of this strain in low riboflavin, establishing that the RibN proteins from *O. anthropi* and *V. cholerae* function as riboflavin transporters. To test whether these proteins transport different flavins, the strains were grown under low concentrations of FMN or FAD. Interestingly, both transporters were able to restore growth in the presence of FMN but failed to do so with FAD (Fig. 6B). Altogether, these results indicate that RibN and its homologues comprise a family of functional riboflavin transporters.

RibN is involved in *R. leguminosarum* symbiosis. In order to fulfill their essential riboflavin requirement, some bacterial species have genes that exclusively encode the riboflavin biosynthesis pathway or riboflavin transporter proteins. The reason that some bacteria possess the ability to both synthesize and transport flavins is intriguing. To gain insight into the biological role of the RibN transporter, we constructed a *ribN* deletion mutant strain of *R. leguminosarum*. The Δ*ribN* strain has wild-type growth curves in rich TY or Y minimal medium, irrespective of the presence of exogenous riboflavin (data not shown). To evaluate some important *in vitro R. leguminosarum* phenotypes, we tested a Δ*ribN* strain for swimming motility on soft agar, biofilm formation on polystyrene culture plates, or production of acyl homoserine lactones (quorum-sensing inductors), analyzed by the induction of violacein production in *Chromobacterium violaceum*. No differences between the WT and the mutant were detected in any of these assays (data not shown).

Next, we evaluated the role of RibN in symbiosis. For that purpose we performed infections of pea plant roots with the WT and Δ*ribN* strains. Total, red, and white nodules were quantified at days 12, 21, and 28 p.i. Figure 7A presents red nodule numbers as a fraction of the total number of nodules per plant as an indicator of nodule maturation. Plants infected with the Δ*ribN* strain showed a modest delay in nodule maturation compared with those infected with the WT strain, while this feature was restored in plants infected with the Δ*ribN* strain complemented with pRL1692. Also, at day 21 p.i., intranodular rhizobia were quantified. Intranodular bacterial counts (number of CFU per nodule) were reduced over 22-fold in plants infected with the Δ*ribN* mutant compared with those infected with the WT strain (Fig. 7B). Complementation with plasmid pRL1692 partially restored the intranodular counts, confirming that the absence of RibN is responsible for the phenotype observed in the mutant strain. No other differences, such as the total number of nodules or weight, were detected between plants treated with *R. leguminosarum* Δ*ribN* and WT strains (data not shown). These observations indicate that the RibN transporter is required for normal nodule development during colonization by *R. leguminosarum*.

DISCUSSION

Riboswitch screening is a useful and reliable tool for the annotation of genes of unknown function (33). The identification of the two riboflavin transporter families characterized to date, RibU-ECF and RibM, was prompted by the presence of the FMN riboswitch sequence on the upstream regions of their genes (9, 12, 15, 34). In this work, by means of searching for FMN riboswitch target genes in *R. leguminosarum*, we identified RibN to be a putative riboflavin transporter. RibN represents the first flavin transporter experimentally characterized in Gram-negative bacteria. The FMN riboswitch-containing orthologues are distributed among the *Alphaproteobacteria*, while homologues lacking the FMN riboswitch are found in the *Betaproteobacteria* and the *Gammaproteobacteria*. The fact that these homologues are neither FMN regulated nor genetically associated with riboflavin biosynthesis genes raised the question of whether they are truly riboflavin transport-

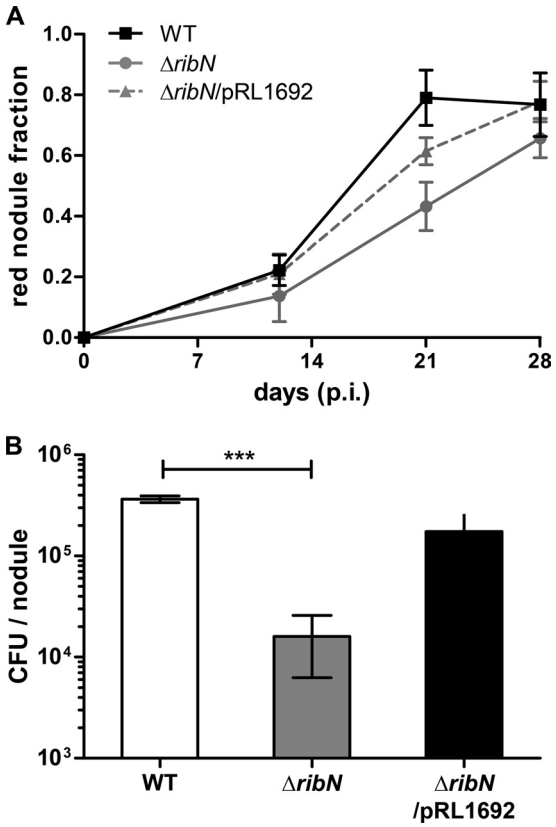

FIG 7 The RibN transporter is required by *R. leguminosarum* for effective nodule colonization in pea plants. (A) Pea plants were inoculated with the *R. leguminosarum* WT, the *ribN* mutant, or the *ribN* complemented strain. After different time points, red nodule numbers as a fraction of the total number of nodules per plant were determined. Values are expressed as the mean ± SEM ($n = 7$). (B) Intranodular bacterial counts were determined at day 21 p.i. Values are expressed as the mean ± SD ($n = 5$). Statistical analysis was carried out by performing a two-tailed unpaired Student's *t* test (***, $P < 0.001$).

ers. Functional characterization of the *V. cholerae* RibN protein confirms that homologues lacking the FMN riboswitch function as riboflavin transporters. Thus, it can be speculated that a number of bacterial riboflavin transporter families remain undiscovered by current screening methods. Recently, using structural and functional characterization of ligand-binding components, Deka et al. (35) identified the substrate binding component of the ABC transporter system RfuABCD from spirochetes that has the ability to bind riboflavin, which is highly suggestive of a riboflavin transport function for this system.

We have also demonstrated that riboflavin negatively regulates the expression of RibN. It is most likely that this regulation is mediated by the FMN riboswitch sensing intracellular FMN after riboflavin kinase synthesizes it from riboflavin. It is known that this element regulates riboflavin biosynthesis and transport genes by two alternative mechanisms: premature transcription termination and translational attenuation by sequestering the Shine-Dalgarno (SD) sequence (9–11). It has been reported that the production of RibU from *B. subtilis* is increased under riboflavin deficiency (12), and it is proposed that the FMN riboswitch regulates the translation of RibU and most of its orthologues by translational attenuation (9). However, riboflavin-mediated transcriptional repression has been experimentally observed for *ribU* in *L. lactis* (13) and *lmo1945*, a *ribU* orthologue, in *L. monocytogenes* (36). Our *in silico* analysis of *ribN* FMN riboswitches revealed an anti-SD motif only in *Oant_0035* from *O. anthropi* (data not shown). Conversely, our experimental evidence suggests the presence of a translational regulatory mechanism in *ribN* from *R. leguminosarum*.

Our results also show that FMN and FAD are able to restore the growth of a *B. abortus* auxotroph expressing RibN in minimal medium, although FAD seems to restore it less efficiently. This result is in agreement with the findings of previous studies for RibU in *B. subtilis*, where FAD was a less efficient competitor for transport than riboflavin and FMN in radioactive riboflavin uptake assays (12). Cumulative experimental data seem to support the idea that derivatives more distantly related to riboflavin, like FAD, acriflavine, and lumichrome, may be less structurally fit to passage through the transporter than close derivatives, such as FMN or roseoflavin (12–14).

An *R. leguminosarum ribN* mutant is able to grow up to WT levels in rich and minimal media with or without exogenous riboflavin. The Δ*ribN* strain may have sufficient riboflavin supplied by the endogenous biosynthesis pathway when cultured in the absence or presence of riboflavin, explaining why no differences in growth were observed. However, when we inoculated pea roots with the mutant strain, a clear handicap in nodule colonization was observed. This indicates that the ability of *R. leguminosarum* to import riboflavin plays a role in the symbiosis and strongly suggests that the abilities to synthesize and to import riboflavin are not redundant. Micronutrient transport has been related before to the capacity to establish rhizobial symbiotic interactions (37, 38). However, the mechanism by which the riboflavin transport function improves symbiotic efficiency is intriguing. It has been reported that iron deficiency induces riboflavin biosynthesis and secretion in the roots of some plants. This would make riboflavin available as a nutrient or signal molecule for bacteria in the rhizosphere (39–41). It is tempting to speculate that bacteria harboring a riboflavin transporter, in addition to a complete riboflavin biosynthesis pathway, are able to economize in synthesizing riboflavin when it is available from the environment. Also, the initial stages of the legume-rhizobium symbiosis involve molecular talk between the host plant and the specific symbiont (42, 43). Therefore, it is also possible that extracellular riboflavin is acting as a signaling molecule, triggering the expression of symbiosis-proficient bacterial genes. In *Pseudomonas aeruginosa*, riboflavin and lumichrome can stimulate the quorum-sensing receptor LasR, thus mimicking the *N*-3-oxo-dodecanoyl homoserine lactone signal (44). Using *C. violaceum* as a biosensor, we were not able to observe alterations in the production of quorum-sensing molecules in the *R. leguminosarum* Δ*ribN* mutant, but this does not rule out the possibility of impairment in signaling events not related to canonical quorum-sensing systems. Riboflavin transporters may play an important role for bacteria in plant colonization since RibN and RfnT homologues are conserved in several plant symbionts (e.g., *R. leguminosarum*, *R. etli*, *S. meliloti*, *Mesorhizobium loti*, and *Bradyrhizobium* species) and phytopathogens (e.g., *A. tumefaciens* and *X. campestris*). Interestingly, riboflavin transporters are absent in mammal pathogens closely related to rhizobia, such as members of the *Brucella* and *Bartonella* genera. A deeper analysis is required to understand the functions that riboflavin

transporters are performing in riboflavin autotrophs in general and in host-microbe interactions in particular.

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