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Post-transcriptional regulation of *NifA* expression by Hfq and RNase E complex in *Rhizobium leguminosarum* bv. *viciae*

Yinghua Zhang[1,2] and Guofan Hong[1*]

[1]State Key Laboratory of Molecular Biology, Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 200031, China
[2]Graduate School of the Chinese Academy of Sciences, Shanghai 200031, China
*Correspondence address. Tel: +86-21-54921223; Fax: +86-21-54921011; E-mail: gfhong@sibs.ac.cn

NifA is the general transcriptional activator of nitrogen fixation genes in diazotrophic bacteria. In *Rhizobium leguminosarum* bv. *viciae* strain 8401/pRL1JI, the *NifA* gene is part of a gene cluster (*fixABCXNifAB*). In this study, results showed that in *R. leguminosarum* bv. *viciae* 8401/pRL1JI, host factor required (Hfq), and RNase E were involved in the post-transcriptional regulation of *NifA* expression. It was found that Hfq-dependent RNase E cleavage of *NifA* mRNA was essential for NifA translation. The cleavage site is located at 32 nucleotides upstream of the *NifA* translational start codon. A possible explanation based on predicted RNA secondary structure of the *NifA* 5′-untranslated region was that the cleavage made ribosome-binding sites accessible for translation.

Keywords Hfq; RNase E; *NifA*; *Rhizobium leguminosarum* bv. *viciae*

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Introduction

NifA belongs to the bacterial enhancer-binding protein family of transcriptional regulators that activate gene expression in concert with RNA polymerase containing the specialized σ^{54} factor, which allows the polymerase core to recognize the −24/−12-type promoters [1–3]. In diazotrophic bacteria, NifA activates transcription of nitrogenase genes (*Nif* genes) and *fix* genes, particularly in the legume endosymbiotic bacteria. Although *NifA* genes are conserved in diazotrophic bacteria, their organization within *Nif* gene clusters differs [1]. In fast-growing rhizobia, such as *Sinorhizobium meliloti*, *Rhizobium leguminosarum* bv. *trifolii*, and *R. leguminosarum* bv. *viciae*, *NifA* is located in the symbiotic plasmid, between the *fixABC* operon and *NifB* gene.

There are different mechanisms for the regulation of *NifA* expression in root nodule bacteria (*Sinorhizobium meliloti* [4–7], *Bradyrhizobium japonicum* [8,9], and *Azorhizobium caulinodans* [10]). The regulation of *NifA* expression in *R. leguminosarum* bv. *viciae* has not been studied in detail. Nitrogenase is not expressed in free-living cells, probably because NifA is not functional under these conditions. The expression of the *NifA* gene is positively controlled by host factor required (*Hfq*) in *Azorhizobium caulinodans* [11,12] and *Rhodobacter capsulatus* [13]. However, whether and how *Hfq* regulates *NifA* gene expression in *R. leguminosarum* remains unknown.

The *Hfq* gene was initially identified as a gene encoding a bacterial Hfq for the replication of the bacteriophage Qβ RNA [14,15]. Inactivation of the *Hfq* gene in *Escherichia coli* causes a wide variety of phenotypes and alters the expression of many genes [16,17]. Hfq controls the translation [18,19] and decay [20] of some mRNAs in an sRNA-independent manner. Several antisense sRNAs, however, need Hfq to interact with target mRNA(s) that, in turn, modify mRNA translation and/or stability. Hfq also stabilizes the interacting sRNAs *in vivo* [21,22]. By facilitating the interaction between some sRNAs and their associated mRNA targets, the protein participates in the positive or negative regulation of translation initiation of these mRNAs [23,24]. The sRNA–mRNA interaction can result in the sequestration or exposure of the Shine–Dalgarno sequence from the mRNA targets or the initiation of mRNA degradation by RNase E [25] or RNase III [26]. In *E. coli*, 20% of the Hfq proteins co-purify with RNase E, but how the protein targets the mRNAs for degradation or stabilization is unknown [27].

Hfq can also induce mRNA stabilization with the help of an sRNA [28]. Several sRNAs that specifically bind the Hfq protein (e.g. DsrA, MicF, RyhB, SgrS, and RydC) control the translation of selected mRNAs in response to environmental stress [29].

RNase E is an essential endoribonuclease in bacteria. RNase E plays an important role in all aspects of RNA metabolism, including processing and/or decay of rRNAs, tRNAs, non-coding small RNAs, and mRNAs. Endoribonucleolytic cleavage by RNase E depends on 5′-terminal structures of RNAs [30,31] and occurs within single-stranded A- and/or U-rich segments [32–34].

In this study we found that in *R. leguminosarum* bv. *viciae* strain 8401 pRL1JI, *NifA* gene expression was regulated by Hfq and RNase E at the post-transcriptional level.

Materials and Methods

Bacteria and plasmids

Escherichia coli DH5α was used as a host for the cloning experiments, and *E. coli* ER2566 for overproduction of the recombinant proteins. Biparental conjugation was performed to mobilize broad-host-range plasmids from *E. coli* to *R. leguminosarum* as described by Simon *et al*. [35]. *Rhizobium leguminosarum* strain 8401 is a derivative of a biovar *phaseoli* isolate (strain 8002); it is streptomycin resistant and has been cured of the Sym plasmid pRP2JI. *Rhizobium leguminosarum* strain 8401/pRL1JI carries the biovar *viciae* Sym plasmid. The plasmids used in this study are described in **Table 1**.

Media

Rhizobium leguminosarum strains were routinely grown in TY medium (5 g tryptone, 3 g yeast extract, and 0.66 g $CaCl_2$ per liter) at 28°C. Starter cultures were prepared by inoculating a single colony from a TY plate stock into 3 ml of TY medium and incubating overnight at 28°C in a shaking incubator (300 rpm). A 0.3-ml portion of this culture was then subcultured into another 3 ml of TY medium and incubated to a suitable cell density (OD_{600} of 0.3–0.4). A 0.03-ml portion of this starter culture was then inoculated into 3 ml of nitrogen-rich medium [36] to give an initial optical density at 600 nm of 0.02. To investigate the nitrogen starvation effect, 3 ml of starter culture were centrifuged and 99% of the clarified medium was decanted; then the cell pellets were inoculated into 3 ml of nitrogen-free medium [36]. The nitrogen-free medium contained per liter: 5 g DL sodium lactate, 5 g disodium

Table 1 Plasmids used in this study

Name	Description	Source or reference
pTYb11	Amp^r, expression vector with chitin binding domain and intein	NEB
pTYb11-RHfq	pTYb11 derivative for expression of *Rhizobium* Hfq	This work
pRK415	Tc^r, broad-host-range expression vector, IncP	Lab stock
pRK415a	pRK415 derivative with *rrn*B terminator	This work
pKK223-3	Amp^r, expression vector	Pharmacia
pRK415a-FN	pRK415a derivative for the expression of *fixABCXNifAB* under Lac promoter and *rrn*B terminator	This work
pMP220	Tc^r IncP vector with promoterless *LacZ*	Lab stock
pMP220V	pMP220 derivative with *tac* promoter-LacZ fusion, carrying no RBS	This work
pMP220A	pMP220V derivative with *NifA* (−47)–(−1) region[a]	This work
pMP220B	pMP220V derivative with *NifA* (−32)–(−1) region[a]	This work
pGEM3zf(+)	Amp^r, vector for *in vitro* transcription	Pharmacia
pGEM-82	pGEM3zf(+) derivative with *NifA* (−47)–(+35) region under control of T7 promoter[a]	This work
pSUP202	Ap^r Cm^r Tc^r *oriT* from RP4	[35]
pSUP1011	Mob^+ Cm^r Km^r carrying Tn5	[37]
pSUP202a	pSUP202 derivative with Km^r cassette and *sacB* intact gene	This work
pSUP202a1	pSUP202a derivative with about 1.4 kb fragment downstream *Hfq* stop codon	This work
pSUP202a2	pSUP202a1 derivative with about 900 bp fragment upstream *Hfq* start codon	This work

[a]*NifA* start codon is +1.

succinate hexahydrate, 1.67 g K_2HPO_4, 0.87 g KH_2PO_4, 0.1 g $MgSO_4 \cdot H_2O$, 0.05 g NaCl, 40 mg $CaCl_2 \cdot 2H_2O$, 10 mg $FeCl_3 \cdot 6H_2O$, 5 mg $Na_2MoO_4 \cdot 2H_2O$, 2.5 mg $MnSO_4 \cdot H_2O$, 0.7 mg $ZnSO_4 \cdot 7H_2O$, 0.14 mg $CoSO_4 \cdot 7H_2O$, 0.12 mg $CuSO_4 \cdot 5H_2O$, 0.03 mg H_3BO_4, 0.02 mg biotin, 4 mg nicotinic acid, and 4 mg calcium pantothenate. The nitrogen-rich medium was the nitrogen-free medium supplemented with 1 g/L $(NH_4)_2SO_4$ and 1 g/L yeast extract. The nitrogen-free medium contained no nitrogen source at all and the poor growth of *R. leguminosarum* strains relied on the limited nitrogen source coming from starter cultures. *Escherichia coli* strains were grown in Luria–Bertani medium at 37°C.

Construction of modified suicide plasmid pSUP202a

A 1.5-kb kanamycin resistance cassette was amplified by PCR from plasmid pSUP1011 [37] using primers P19 (5′-CGCGAAGCTTGCGGCCGCCACGCTGCCGCAAGCACTC-3′) containing *Hin*dIII site and *Not*I site (underlined) and P20 (5′-CGCGGGATCCCCAAAGCGGCCATCGTGCC-3′) containing a *Bam*HI site (underlined). The fragment obtained was digested with *Bam*HI/*Hin*dIII and designated fragment K. A 1.8-kb fragment containing the complete *sacB* gene was amplified by PCR from *Bacillus subtilis* genomic DNA using primers P21 (5′-CGCGGGATCCTCTAGACGCCATTCTGTGCCGTTGCC-3′), which contains *Bam*HI site followed by *Xba*I site (underlined), and P22 (5′-CGCGGTCGACAATCCTTCAGTTAGAGATCC-3′), which contains an *Sal*I site. The fragment obtained was digested with *Bam*HI and *Sal*I and designated fragment S. Fragment K and fragment S were ligated with *Hin*dIII/*Sal*I digested pSUP202. The obtained plasmid was designated pSUP202a, which contained a kanamycin resistance gene (for positive screening with kanamycin) and the *sacB* gene (for negative screening with 10% sucrose [38]). The *sacB* gene encodes a levansucrase gene and levansucrase activity is lethal in the presence of sucrose for most gram-negative bacteria.

Construction of the *R. leguminosarum* bv. *viciae* Hfq mutant

The 2.5 kb *Hfq* region DNA was amplified by PCR from genomic DNA of *R. leguminosarum* bv. *viciae* strain 8401/pRL1JI using primers P27 (5′-GAGAATTGCCTATGTAAATGG-3′) and P30 (5′-CGGCCCGTTGCCGATGCGGC-3′). The fragment was sequenced by Shanghai GeneCore BioTechnologies (Shanghai, China). The sequence data have been submitted to the GenBank databases under accession No. FJ648549.

The 1.4 kb fragment downstream of the *Hfq* stop codon was obtained by PCR amplification from genomic DNA of *R. leguminosarum* bv. *viciae* strain 8401 using primers P23 (5′-CGCGGGATCCCAGGATCAGCCGTCATTTCG-3′), which contains a *Bam*HI site, and P24 (5′-CGCGTCTAGACGGTCGCTTCGGTTTCGGACAGG-3′) which contains an *Xba*I site. The 900 bp fragment upstream of the *Hfq* start codon was obtained by PCR amplification from genomic DNA of *R. leguminosarum* bv. *viciae* strain 8401 using primers P25 (5′-CGCGGCGGCCGCCCGATGCCAGCGTGCATATCGAGG-3′), which contains a *Not*I site, and P26 (5′-CGCGGCGGCCGCCGCGCCGCTTCTTTCTTTATTGCC-3′), which also contains a *Not*I site. The two fragments were then inserted into the *Bam*HI/*Xba*I and *Not*I sites of the modified suicide plasmid pSUP202a. The resulting plasmid pSUP202a2, in which the *Hfq* coding region was replaced by a kanamycin resistance cassette, was transferred into *R. leguminosarum* bv. *viciae* strain 8401/pRL1JI by biparental conjugation. After screening with kanamycin and 10% sucrose, one true marker exchange mutant (resulting from double crossover) was confirmed by PCR using primers P27 (5′-GAGAATTGCCTATGTAAATGG-3′)/P28 (5′-GAGTGCTTGCGGCAGCGTG-3′) and P29 (5′-GCATCCAGGAAACCAGCAGCG-3′)/P30 (5′-CCGGCCCGTTGCCGATGCGGC-3′).

DNA manipulations and sequence analysis

Rhizobium leguminosarum bv. *viciae* genomic DNA was extracted using a Promega genomic preparation kit (Madison, USA). DNA was sequenced by Shanghai GeneCore BioTechnologies. Nucleotide sequence data were analyzed with the DNA-star package. Homology searches were performed using the National Center for Biotechnology Information BLAST network server (http://www.ncbi.nlm.nih.gov/BLAST/).

Construction of Lac promoter-controlled *fixABCXNifAB* expression plasmid and strains

The 7.5 kb DNA fragment including *fixA*, *fixB*, *fixC*, *fixX*, *NifA*, and *NifB* genes was amplified by PCR from genomic DNA of *R. leguminosarum* bv. *viciae* strain 8401/pRL1JI using primers P35 (5′-TGTCGGCAACCCTACAAAACCCC-3′) and P6 (5′-TCAATTAGAGGGGCCTAAAGCCG-3′). The fragment was sequenced by Shanghai GeneCore BioTechnologies. The sequence data have been submitted to the GenBank database under accession No. FJ648550.

The *rrn*B terminator was amplified with primers P3 (5′-CGCGGGTACCCTGTTTTGGCGGATGAGAG-3′)

containing a *Kpn*I site/P4 (5′-CGCGGGTACCCAAAA-AGGCCATCCGTCAGG-3′) containing a *Kpn*I site from pKK223-3 plasmid DNA by PCR and inserted into the *Kpn*I site of pRK415 to generate the expression plasmid pRK415a. The 7 kb DNA fragment of the *fixABCXNifAB* gene region without its potential endogenous promoter upstream of *fixA* was amplified with primers P5 (5′-GGGTCCCTAAAGCCCGAATCCG-3′)/P6 (5′-TC-AATTAGAGGGGCCTAAAGCC-3′) from the 7.5 kb DNA by PCR and introduced into the *Hin*dIII site of pRK415a by blunt end ligation so that the 7 kb *fixABCXNifAB* DNA was under control of the Lac promoter (*Plac*) and the *rrn*B terminator. The resulting plasmid was designated pRK415a-FN and transferred into *R. leguminosarum* bv. *viciae* strain 8401/pRL1JI wild type or *Hfq* mutant strain (M21) by biparental conjugation.

Northern hybridization

Total cellular RNA was isolated and analyzed by northern hybridization. Briefly, cells were grown at 28°C in nitrogen-limited or nitrogen-rich medium to reach an optical density at 600 nm of 0.3–0.4. After being harvested and washed with ice-cold TE (10 mM Tris–HCl, pH 8.0, and 1 mM EDTA), the cells were suspended in 100 μl of TE and extracted directly by vigorously mixing them with an equal volume of equilibrated phenol/chloroform. After centrifugation (10,000 rpm), part of the upper layer was used for analysis. RNA samples were fractionated by formaldehyde-agarose gel electrophoresis and transferred onto positively charged nylon membranes by the capillary method. DNA probes were prepared by PCR amplification using primers P7 (5′-ATGATTAAACCAGAGGCGC-3′)/P8 (5′-TCACT-CCTTCTTCACATCGATACG-3′) for *NifA* and P9 (5′-CAACATGAGAGTTTGATCCTGG-3′)/P10 (5′-CGTC-TTACCAATTCCACAGC-3′) for 16S rRNA. The probes were labeled with ^{32}P by random primer labeling.

Purification of *Rhizobium* Hfq protein

Rhizobium Hfq was expressed and purified using the IMPACT^{TM}-CN protein purification system of New England Biolab (NEB; Beijing, China).

The coding region of the *Rhizobium Hfq* gene was amplified with primers P1 (5′-GGTGGTTGCTCTT-CCAACGCGGAACGTTCTCAGAATC-3′) containing a *Sap*I site/P2 (5′-CGCGCTGCAGTCAGGACGCTGCTT-CTTCGCTCTCG-3′) containing a *Pst*I site from *R. leguminosarum* bv. *viciae* strain 8401/pRL1JI genomic DNA by PCR and inserted into the *Sap*I /*Pst*I sites of the expression vector pTYb11 to generate the expression plasmid pTYb11-RHfq. The expression plasmid was introduced into *E. coli* strain ER2566 and protein purification was carried out according to the manufacturer's manual.

Protein samples were analyzed by SDS–PAGE. Protein concentrations were determined by the Bradford method.

***Rhizobium* RNase E complex purification**

RNase E complex was purified from *R. leguminosarum* bv. *viciae* 8401/pRLIJ1 wild type and *Hfq* mutant strain (M21) according to the method described previously [39,40]. Cells were cultured in TY medium. All purification steps were performed at 4°C. Buffers contained 2 μg/ml aprotinin, 0.8 μg/ml leupeptin, and 0.8 μg/ml pepstatin A. A suspension of 100 g *Rhizobium* cells in 100 ml of lysozyme-EDTA buffer containing 50 mM Tris–HCl, pH 7.5, 100 mM NaCl, 5% glycerol, 3 mM EDTA, 1 mM dithiothreitol (DTT), 1.5 mg/ml lysozyme, and 1 mM phenylmethylsulfonyl fluoride (PMSF) at room temperature was prepared. After 40 min on ice, 50 ml of room temperature DNase-Triton buffer containing 50 mM Tris–HCl, pH 7.5, 100 mM NaCl, 5% glycerol, 1 mM DTT, 3% Triton X-100, 30 mM magnesium acetate, 1 mM PMSF, and 20 μg/ml DNase I were added, followed by a 1-min low-speed blending. The lysate was kept on ice for 30 min and 37.5 ml of 5 M NH_4Cl were slowly added. The lysate was stirred for an additional 30 min and clarified for 1 h at 12,000 rpm. Proteins of this supernatant were precipitated with 40% ammonium sulfate, dissolved in 112.5 ml of buffer A containing 10 mM Tris–HCl, pH 7.5, 5% glycerol, 0.5% genapol X-080, 1 mM EDTA, 0.1 mM DTT, 0.1 mM PMSF, and 50 mM NaCl and loaded on a sulphopropyl (SP)-Sepharose cation-exchanger column (Pharmacia), equilibrated with buffer containing 50 mM NaCl. After washing with buffer A containing first 50 and then 300 mM NaCl, RNase E was eluted with 1 M NaCl and 0.5% genapol X-080 in buffer A. Fractions were analyzed with SDS–PAGE. Peak fractions from the SP-column were diluted 2 folds with buffer A and layered on a 10–30% (*w/v*) glycerol gradient containing buffer A with 300 mM NaCl. Centrifugation was performed at 4°C for 15 h at 37,000 rpm. Fractions were collected from the top of the gradient tube. The endoribonuleolytic activity of each fraction was tested using the *in vitro* transcribed 82 nt RNA fragment of the *NifA* gene (see below) as substrates. The fractions of high activity were collected and analyzed with SDS–PAGE.

Protein identification by MS spectrometry

Protein identification by MS spectrometry was conducted by RCPA (Research Centre for Proteome Analysis, Key

Lab of Proteomics, Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai, China). Briefly, the procedure involved excision of the band from the gel, in-gel trypsin digestion, MALDI-TOF MS (4800 Plus MALDI TOF/TOF Analyzer) of the tryptic peptides, and database searching of the peptide mass fingerprinting data.

RNase E cleavage experiments

The 82 nt RNA fragment that was located in the (−47)–(+35) region of the *NifA* gene (*NifA* translational start was +1), was *in vitro* transcribed with T7 RNA polymerase (TaKaRa, Dalian, China) according to the manufacturer's manual. The template for *in vitro* transcription was constructed as follows. The PCR products of primers P11 (5′-CGCGGAATTCTTCGTGCAGCAGGGTAAAAAGC-3′) containing an *Eco*RI site/P12 (5′-CGCGCTGCAGTAGAGTATATGGAGCCGCGCC-3′) containing a *Pst*I site were digested with *Eco*RI and *Pst*I, then cloned into the corresponding sites of plasmid pGEM3zf(+). The recombinant plasmid was designated pGEM-82 and linearized by *Pst*I to be used as a template. The *in vitro* transcribed RNA was labeled with ^{32}P by incorporating ^{32}P-CTP into it during the *in vitro* transcription reaction.

RNase E cleavage tests were carried as out described by Klein and Evguenieva-Hackenberg [40]. The reaction temperature was 28°C in this study.

Cleavage site determination

We determined the probable site of the 5′-untranslated region (UTR) of *NifA* mRNA cleavage by the RNase E complex. After the cleavage reaction, RNA fragments were size-fractionated, purified, and ligated to a 5′-DNA adapter (5′-TTTCTGCAGATGGCTAAGGGGCAATCTTTACAAG-3′) containing a *Pst*I site and a 3′-adapter (5′-GCAGATCGTCAGAATTCCAG-3′) containing an *Eco*RI site with T4 RNA ligase from NEB. The 3′-adapter was blocked by ddA with terminal transferase (NEB) at its 3′-terminus and phosphorylated by T4 polynucleotide kinase (NEB) at the 5′-terminus. The ligated RNA was reverse-transcribed into cDNA with the Access Quick reverse transcription-polymerase chain reaction (RT-PCR) system (Promega) with the 5′-adapter sequence and another primer complementary to the 3′-adapter. The RT-PCR product was amplified by PCR with the same primers and the DNA was cloned into the pGEM-T vector (Promega) for sequencing.

Total RNA was extracted from strain 8401/pRL1JI wild type cells carrying plasmid pRK415a-FN cultured in nitrogen-limited medium and the 3′-terminals of the predicted 4 kb mRNA were determined by 3′-RACE (rapid amplification of cDNA ends). RNA was polyadenylated using *E. coli* poly(A) polymerase, reverse transcribed using the primer 3′-RACE1: (5′-TCACGACTCACTATAGGATCCTTTTTTTTTTTTTN-3′), amplified with the specific primer P36 (5′-TTTGCGCCGTCGATAACACC-3′) and 3′-RACE2: (5′-TCACGACTCACTATAGGATCC-3′), and cloned into the TA cloning vector for sequencing.

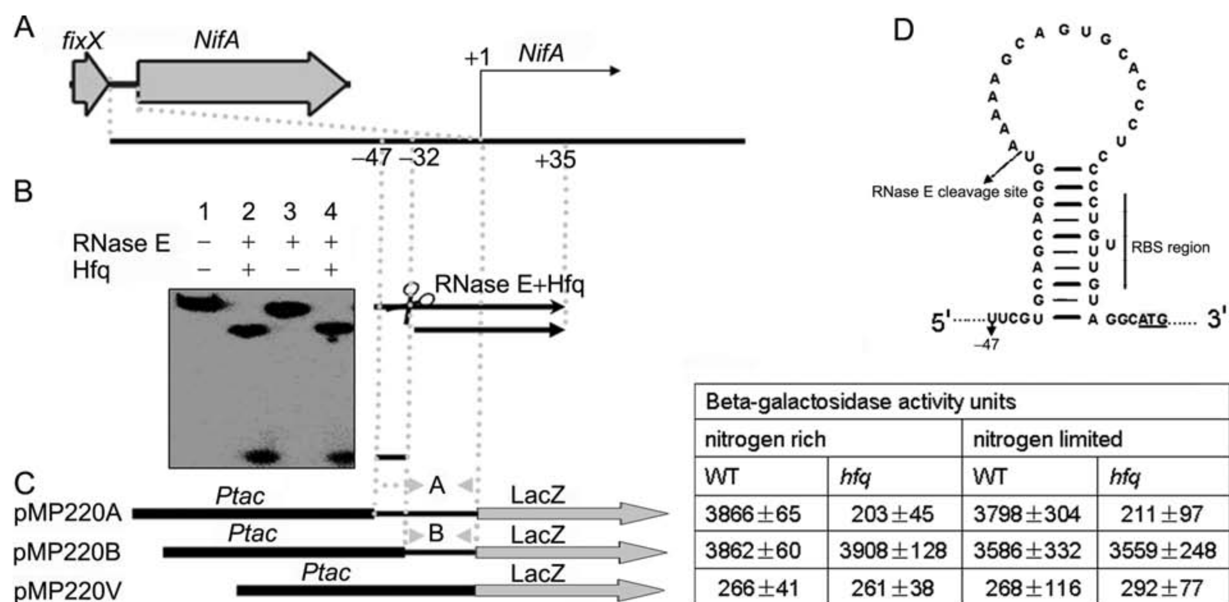
β-Galactosidase activity assays

The translational fusion *LacZ* reporter plasmids were constructed as follows. A Tac promoter (*Ptac*) without its ribosome-binding site (RBS) was amplified with primers P17 (5′-GCGAAGCTTGGCTGTGCAGGTCGTAAATC-3′) containing a *Hin*dIII site/P18 (5′-CGCGGGATCCGTGTGAAATTGTTATCCG-3′) containing a *Bam*HI site from plasmid pKK223-3 by PCR and inserted into the *Hin*dIII and *Bam*HI sites of the vector pMP220 to generate plasmid pMP220V. Fragment A was obtained by PCR amplification using primers P13 (5′-CGCGGGATCCGCCTACAAACAGGGGAGG-3′) containing a *Bam*HI site/P14 (5′-CGCGGGATCCTTCGTGCAGCAGGGTAAAAAGC-3′) containing a *Bam*HI site from pGEM-82 plasmid DNA. Fragment B was obtained by annealing two oligonucleotides P37 (5′-CGCGGGATCCAAAAAGCAGTGCACCCTCCCCTGTTTGTAGGC-3′) containing a *Bam*HI site and P38 (5′-CGCGGGATCCGCCTACAAACAGGGGAGGGTGCACTGCTTTTT-3′) containing a *Bam*HI site. Then fragment A and B were inserted into the *Bam*HI sites of plasmid pMP220V to generate translational fusion LacZ reporter plasmids pMP220A and pMP220B, respectively. Each reporter plasmid was transferred into *R. leguminosarum* bv. *viciae* strain 8401/pRL1JI wild type or *Hfq* mutant strain (M21) by biparental conjugation. β-Galactosidase activity assays were performed as described by Miller [41]. The locations of fragment A and B are indicated in **Fig. 1**.

Results

Construction of *R. leguminosarum* bv. *viciae* *Hfq* mutant

The genetic organization of the sequenced 2.5 kb *Hfq* region from the genome of *R. leguminosarum* bv. *viciae* 8401/pRL1JI is shown in **Fig. 2** upper panel. Upstream of the *Hfq* gene, there is a partial open reading frame (ORF) that was 971 bp long and encoded a *D*-alanine aminotransferase gene. Downstream of the *Hfq* gene



Beta-galactosidase activity units			
nitrogen rich		nitrogen limited	
WT	*hfq*	WT	*hfq*
3866±65	203±45	3798±304	211±97
3862±60	3908±128	3586±332	3559±248
266±41	261±38	268±116	292±77

Figure 1 The effects of RNase E and Hfq on *NifA* translation (A) Enlargement of the *fixX-NifA* intergenic region. The *NifA* translational start is numbered +1 and indicated by a line and horizontal arrow. (B) RNase E cleavage within the *NifA* 5′-UTR. ^{32}P-labeled 82 nt *in vitro* transcribed *NifA* 5′-UTR RNA, which was located at (−47)–(+35), was incubated with RNase E complex purified from wild type cells (lane 2), or RNase E complex purified from *Hfq* mutant cells (lane 3), or RNase E complex purified from *Hfq* mutant cells plus Hfq (lane 4) at 28°C. After incubation for 1 h, samples were phenol extracted and analyzed on a 10% sequencing gel. (C) Schematic representation of the *LacZ* reporter translational fusions (C) and their activities (in Miller units) in wild type and *Hfq* mutant *R. leguminosarum* bv. *viciae* strain 8401/pRL1JI. In plasmid pMP220A, the 47 bp DNA corresponding to *NifA* 5′-UTR (designated fragment A) was inserted between the *tac* promoter (*Ptac*) and promoterless *LacZ*. In plasmid pMP220B, the 32 bp DNA corresponding to *NifA* 5′-UTR (designated fragment B) was inserted between the *tac* promoter (*Ptac*) and promoterless *LacZ*. Translation from pMP220A and pMP220B transcripts utilizes the *NifA* RBS. Plasmid pMP220V was a negative control with no RBS. The locations of fragment A and fragment B are indicated by lines and numbers. Cells were cultured in nitrogen-limited or nitrogen-rich medium to 0.3–0.4 (OD_{600}). The numbers shown are averages obtained from at least three independent cultures. (D) Prediction of RNA secondary structure of the (−47)–(+3) region of *NifA* 5′-UTR by the MFOLD program (version 3.2) from Zuker [52]. The RNase E cleavage site, which is indicated by an arrow, is located between $U_{(-33)}$ and $A_{(-32)}$. The RBS is marked by a vertical line.

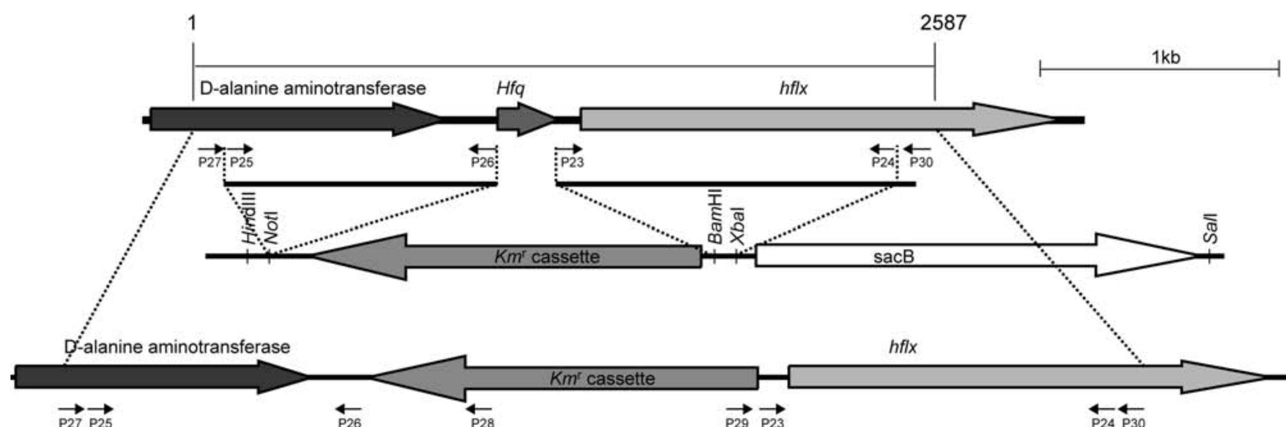


Figure 2 Physical map of the *R. leguminosarum* bv. *viciae* strain 8401/pRL1JI *Hfq* region Upper panel: *Hfq* gene organization. In addition to the *Hfq* gene, two partial ORFs are located on the cloned 2.5 kb fragment of genomic DNA corresponding to the *Hfq* region. *Hfq*, host factor required for Qβ replication; *hflx*, putative GTP-binding protein of unknown function. Primers used in the construction of *Hfq* mutant are indicated by horizontal arrows. For the construction of the *Hfq* mutant, PCR products of primers P25/P26 were introduced into the *Not*I site of modified suicide plasmid pSUP202a (middle panel) and PCR products of primers P23/P24 were introduced into the *BamH*I and *Xba*I sites. The *sacB* encodes a levansucrase gene and levansucrase activity is lethal in the presence of sucrose. Lower panel: *Hfq* mutant strain M21 is shown. Two pairs of primers P27/P28 and P29/30 were used to test the double crossover of recombination.

there is also a partial ORF that is 1373 bp long and encoded a GTP-binding protein hflx.

The *R. leguminosarum* bv. *viciae Hfq* mutant was constructed by replacing the *Hfq* coding region with a kanamycin resistance cassette as described in "Materials and Methods". The colony morphology of the *Hfq* mutant strain M21 was normal.

Analysis of the *NifA* DNA region

In *R. leguminosarum* bv. *viciae* strain 8401/pRL1JI, the *NifA* gene locus is located in plasmid pRL1JI [42]. The upper panel of **Fig. 3(A)** shows the genetic organization of the sequenced 7.5 kb *NifA* region. Genes and ORFs in this cluster were designated on the basis of their similarity to genes and ORFs previously described in *R. leguminosarum* bv. *viciae* 3841 (AM236084.1). The whole sequence of this 7.5 kb DNA shares 90% identity with *R. leguminosarum* bv. *viciae* strain 3841 and strain UPM791. The *fixA* upstream region contains a potential σ^{54}-binding sequence preceded by consensus NifA-binding sequences, suggesting the existence of NifA-dependent promoters (**Fig. 4**).

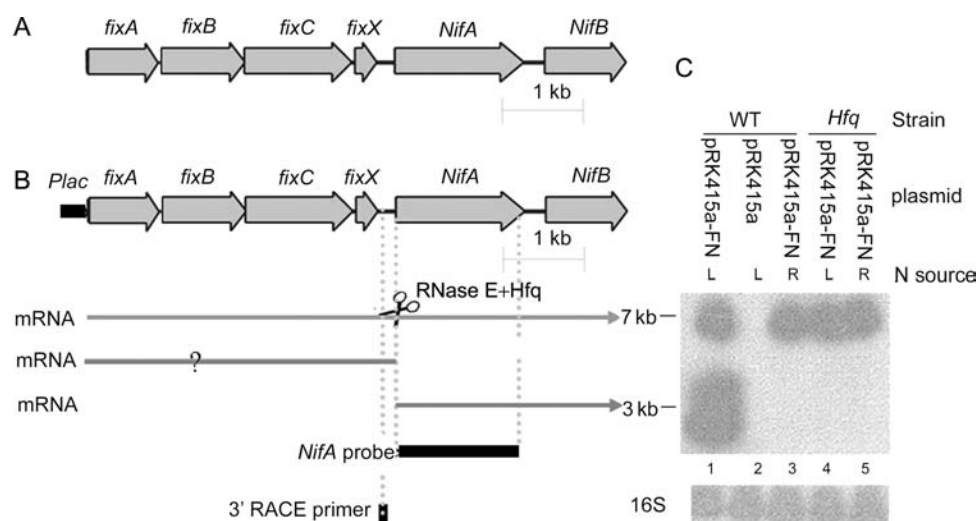


Figure 3 *R. leguminosarum* bv. *viciae* strain 8401/pRL1JI *NifA* mRNA expressed from broad-host-range plasmid pRK415a-FN In this plasmid, *fixABCXNifAB* was under control of an upstream *Lac* promoter, which took the place of the potential endogenous NifA-dependent promoter upstream of *fixA*. (A) Structure of the *NifA* DNA region from *R. leguminosarum* bv. *viciae* strain 8401/pRL1JI. The grey arrows correspond to ORFs identified in a 7.5-kb DNA fragment containing the *NifA* gene from *R. leguminosarum* and adjacent DNA (EMBL accession No. FJ648550). (B) Schematic depiction of mRNAs expressed from plasmid pRK415a-FN. Upper panel: *Lac* promoter (*Plac*) controlled *fixABCXNifAB* expression in plasmid pRK415a-FN. The grey arrows correspond to ORFs; the *Lac* promoter (*Plac*) is indicated by a black square. Lower panel: predicted mRNAs expressed from plasmid pRK415a-FN. The 7 kb *fixABCXNifAB* mRNA was cut into a 4 kb *fixABCX* part and a 3 kb *NifAB* part by RNase E and Hfq. The existence of 7 kb and 3 kb mRNAs were proved by northern hybridization with a *NifA* probe. The 4 kb mRNA was not checked, so a question mark is placed on it. The position of the *NifA* probe used for northern hybridizations is indicated by a black square. The position of the specific primer used for 3′-RACE is indicated by a black square. (C) Northern blot probed with a probe for *NifA* (upper panel) or a control probe for 16S RNA (lower panel). Wild type (lanes 1 and 3) and *Hfq* mutant (lanes 4 and 5) *R. leguminosarum* bv. *viciae* strain 8401/pRL1JI harboring pRK415a-FN were grown in either nitrogen-limited medium (L) (lanes 1 and 4) or nitrogen-rich medium (R) (lanes 3 and 5). Wild type cells harboring pRK415a were grown in nitrogen-limited medium (L) (lane 2). Plasmid pRK415a was the control empty vector.

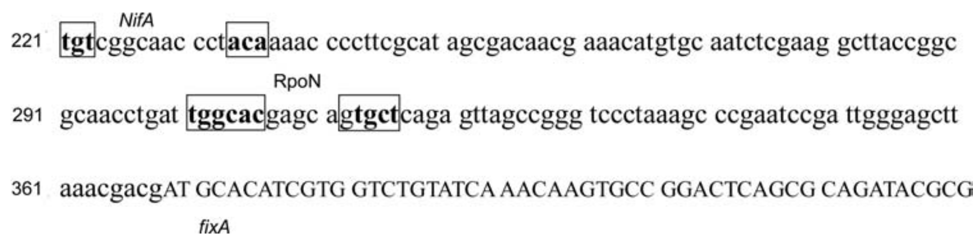


Figure 4 The nucleotide sequences of the region upstream of *fixA* and the 5′-terminal of *fixA* The numbers on the left indicate the positions of the first nucleotide according to the EMBL accession No. FJ648550 sequence. NifA- and σ^{54}-binding sites are enclosed in boxes.

Analysis of *NifA* mRNA expressed from plasmid pRK415a-FN

In *R. leguminosarum* bv. *viciae*, *NifA* is not expressed in free-living cells, even in a nitrogen-limited medium. We constructed plasmid pRK415a-FN as described in Materials and Methods. In this broad-host-range plasmid, *fixABCXNifAB* was expressed from the *Lac* promoter upstream of *fixA* [**Fig. 3(B)**, upper panel]. This plasmid was transferred into *R. leguminosarum* bv. *viciae* strain 8401/pRL1JI wild type and *Hfq* mutant strain (M21) to investigate *Hfq*'s effects. We detected *NifA* mRNA by northern hybridization using a *NifA* probe [**Fig. 3(B)**, lower panel]. Two forms of *NifA* mRNAs were detected on northern blot: one transcript of approximately predicted size is 7 kb, the other is smaller (3 kb) [**Fig. 3(C)**]. The smaller form was only detectable in wild type cells carrying pRK415a-FN grown in nitrogen-limited medium. In wild type cells carrying control empty vector pRK415a, no *NifA* mRNA was detectable. In *Hfq* mutant cells, the smaller form of *NifA* mRNA was not detectable, suggesting that *Hfq* may play a role in *NifA* expression. According to its length, the 3 kb smaller transcript was likely to be *NifAB*. This transcript was possibly generated by post-transcriptional cleavage of the 7 kb larger mRNA since there was no other active promoter upstream of *NifA* in free living cells except the *Lac* promoter upstream of *fixA*. 3′-RACE was conducted to determine the conjectured cleavage site within the *fixX-NifA* intergenic region. The specific primer used in 3′-RACE was located 100 bp upstream of the *NifA* translational start. The obtained 3′-end was located 32 nt upstream of the *NifA* translational start. This supported the idea that a cleavage occurred within the 7 kb larger mRNA and this cleavage was dependent on *Hfq* and the nitrogen source.

Hfq can be associated with RNase E complex in *R. leguminosarum* bv. *viciae*

There were seven major bands on the SDS–PAGE analysis of the purified RNase E complex of strain 8401/pRL1JI wild type cells (**Fig. 5**). The protein bands of about 180, 65, and 15 kDa were proved to be RNase E, RNA helicase, and Hfq, respectively, by MALDI-TOF-MS analysis. The weak band of 15 kDa of the RNase E complex purified from *Hfq* mutant cells was obviously generated by bromophenol blue, which is commonly used as an indicator in electrophoresis.

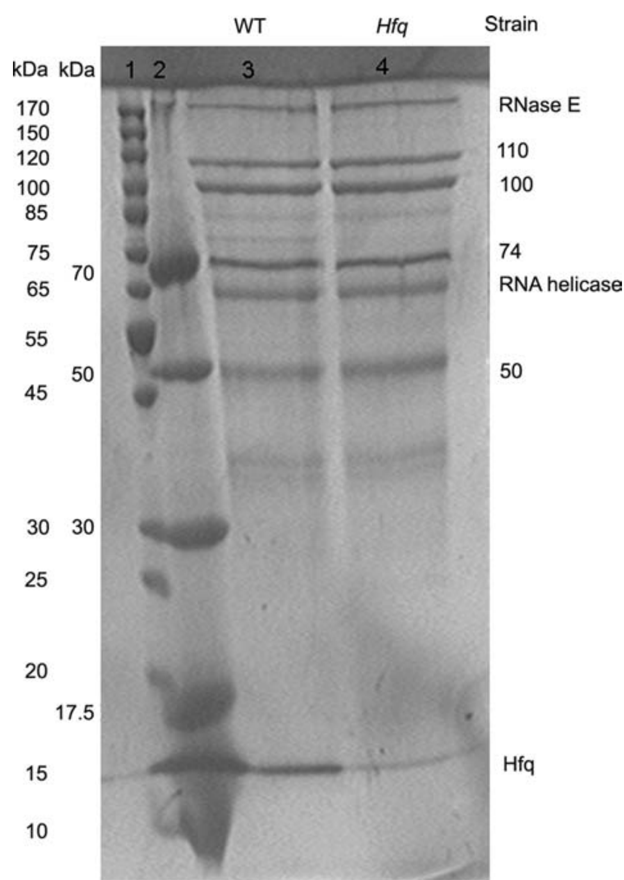


Figure 5 Hfq can be associated with RNase E complex measured by 10% Coomassie Brilliant Blue stained SDS–PAGE Lanes 1 and 2: protein molecular weight standards; lane 3: RNase E complex purified from *R. leguminosarum* bv. *viciae* strain 8401/pRL1JI wild type cells; lane 4: RNase E complex purified from *Hfq* mutant cells. The 180 kDa RNase E, 65 kDa RNA helicase, and 15 kDa Hfq were identified by MS analysis.

RNase E complex can cut the *in vitro* transcribed *NifA* 5′-mRNA fragment and this cleavage is dependent on Hfq

We *in vitro* transcribed an 82 nt RNA fragment that was located in the (−47)–(+35) region of *NifA* mRNA [**Fig. 1(B)**, right panel, *NifA* translational start is numbered +1] and conducted RNase E cleavage tests [**Fig. 1(B)**, left panel]. RNase E complex purified from wild type cells cut the 82 nt substrate RNA into two smaller fragments. On the contrary, the RNase E complex lost this cleavage activity in the absence of Hfq. It seemed that Hfq was essential for the cleavage activity of the RNase E complex. To determine the cut site, after the cleavage reaction the two RNA product fragments were recovered from gel and cloned (see Materials and Methods) according to the method of Yao

et al. [50]. The locations of the two fragments are indicated in **Fig. 1(B)**. Thus we concluded that the cut site was located at 32 nt upstream of the *NifA* translational start. This was consistent with the 3′-RACE result. Thus we came to the conclusion that the 3 kb *NifAB* part was cleaved from the larger 7 kb *fixABCXNifAB* mRNA by the RNase E complex in the presence of Hfq.

Hfq-dependent RNase E cleavage is essential for *NifA* translation, probably by making RBSs accessible

The translational fusions of *Ptac*-A-*lacZ* and *Ptac*-B-*lacZ* were constructed and assayed for β-galactosidase activity in *R. leguminosarum* bv. *viciae* strains at the same cell density (see Materials and Methods).The reporter gene structures and the locations of fragments A and B are indicated in **Fig. 1(C)**. β-Galactosidase activities were measured when cells were cultured in nitrogen-limited or nitrogen-rich medium. The translational activity of fragment A in wild type cells was 15 times higher than in *Hfq* mutant cells. However, the translational activity of fragment B did not depend on *Hfq*. The β-galactosidase activity of neither fragment A nor B was regulated by a nitrogen source. We predicted the secondary structure of *NifA* 5′-UTR [**Fig. 1(D)**] and found that the nucleotides of the RBS of the *NifA* gene were able to form a double-strand structure with adjacent nucleotides through base-pairing. This made the RBSs inaccessible to ribosomes, and so the translation of the *NifA* gene could not be initiated. The Hfq-dependent cleavage by RNase E at 32 nt upstream of the *NifA* translational start codon destroyed the double-strand structure and released the RBS; translation could then be initiated. This hypothesis could explain why fragment B had high translational activity in both wild type cells and *Hfq* mutant cells while fragment A only had high activity in wild type cells. Thus we came to a conclusion that Hfq-dependent RNase E cleavage was essential for *NifA* translation.

Discussion

The *NifA* expression in symbiosis has not been studied in strain 8401/pRL1JI. In *R. leguminosarum* bv. *viciae* UPM791, *NifA* is expressed in symbiosis with peas from a σ54-dependent NifA-autoregulated promoter located upstream of the *orf71 orf79 fixW orf5 fixABCX NifA NifB* operon, although basal levels of symbiotic NifA expression were obtained from a second promoter located upstream of the *fixX-NifA* intergenic region [51]. In strain 8401/pRL1JI, NifA- and σ^{54}-binding sites were identified upstream of *fixA*. It is reasonable to propose that 8401/pRL1JI *NifA* is expressed from this potential promoter upstream of *fixA*. We used the *Lac* promoter-controlled *fixABCXNifAB* expression system as the study target of *NifA* expression on the basis of this hypothesis.

The regulatory function of Hfq in gene expression is somehow mediated by RNase E activity, at least in some cases [27,43–46]. In general, RNase E forms a multi-protein complex with an exoribonuclease PNPase, an RNA helicase, enolase, and several other minor components [39,40,47–49]. In *E. coli*, the RNase E complex contains RNase E, PNPase, an RNA helicase, and enolase. Hfq has been reported to be co-purified with RNase E in *E. coli* [27]. However, enolase, Hfq, and PNPase were not found in the high molecular weight complex containing RNase E in *R. capsulatus* [39] and *R. leguminosarum* [40] but rather two RNA helicases (65 and 74 kDa) and a 50 kDa Rho factor. It is not clear whether Hfq is one of the components of RNase E complex in *R. capsulatus* since the proteins that migrated faster than 32.5 kDa on SDS–PAGE were not shown in [39]. We purified the RNase E complex of *R. leguminosarum* bv. *viciae* strain 8401/pRL1JI cells and analyzed the components of RNase E complex by SDS–PAGE followed by Coomassie Brilliant Blue staining. We observed seven major protein bands which migrated as 180, 110,100, 74, 65, 50, and 15 kDa on SDS–PAGE, respectively (**Fig. 5**). By MS analysis, the 180, 65, and 15 kDa bands were proved to be generated by RNase E, an RNA helicase, and Hfq, respectively. The 110 kDa band and the 100 kDa band might be generated by the degradation products of full length RNase E. The 74 kDa band might be generated by another RNA helicase. The 50 kDa band might be generated by Rho factor. *Rhizobium leguminosarum* Hfq is 80 amino acids long, but it migrated with a molecular mass of about 15 kDa, presumably following the behavior of the *E. coli* Hfq, which migrates as 14 kDa on SDS–PAGE in spite of its 11 kDa molecular weight.

Although the *in vitro* transcribed 82 nt RNA of *NifA* 5′-UTR could be cleaved by RNase E complex in the presence of Hfq *in vitro*, the *in vivo* cleavage was regulated by the nitrogen source [**Fig. 3(C)**]. Unfortunately, we cannot give a clear explanation for this difference now. In *Azorhizobium caulinodans*, *Hfq* gene expression is not regulated by nitrogen [11]. In strain 8401/pRL1JI, we purified an RNase E complex containing Hfq protein when cells were cultured in TY medium, which was nitrogen-rich. Consequently, Hfq might not be

responsible for nitrogen regulation. Since RNase E cleavage is dependent on RNA structure, we guessed that other factor(s) and/or nucleotides upstream of fragment A might be responsible for nitrogen regulation. In *E. coli*, Hfq and RNase E often act together with sRNA to regulate gene expression. However, no sRNA has yet been reported in Rhizobia.

The mechanism revealed in this study is possibly common in *R. leguminosarum* because the *NifA* gene organization is the same and the sequence of the *fix-NifA* intergenic region shares 100% identity among strains UPM791, 8401/pRL1JI, and 3841. However, this mechanism is not common among other species of Rhizobia. Since the *NifA* gene organization of *Sinorhizobium meliloti* is the same as that of *R. leguminosarum*, it is most likely that this mechanism is suitable for *Sinorhizobium meliloti*. However, the RBS region of the *NifA* gene in *Sinorhizobium meliloti* is unable to form a double-strand structure like that in *R. leguminosarum*.

It has been reported that in *E. coli*, Hfq protects RNA against RNase E cleavage *in vitro* [20,55]. This is opposite to the conclusion of this paper. However, Brigid and Matthew have reported that the primary transcripts of MicX, a *Vibrio cholerae* sRNA, are processed in an RNase E- and Hfq-dependent fashion to a shorter, still active and much more stable form [53]. What cause the opposite role of Hfq in RNase E-dependent cleavage remains unclear. Given the role proposed above for RNase E in the processing of the 7 kb *NifA* transcripts, coupled with the absence of reports of Hfq-mediated RNA cleavage, it seems unlikely that Hfq is directly responsible for cleavage of this RNA. Instead, we hypothesize that Hfq, which has been shown to alter the structure of other RNA species, may promote folding of *NifA* 5′-UTR into a structure susceptible to RNase E cleavage. An alternative explanation for the absence of processed 3 kb *NifA* transcripts in an *Hfq* mutant is that processed transcripts may be extremely unstable in this genetic background. Analyses of sRNAs have revealed that Hfq can play a dramatic role in transcript stabilization [54] and it seems likely that Hfq stabilizes the 3 kb *NifA* transcripts as well. However, as we never observed the 3 kb *NifA* mRNA in the absence of Hfq and we could not observe the same 3′ extremity by 3′-RACE experiment in an *Hfq* mutant, there is currently no evidence to suggest that it is formed. Consequently, if Hfq is not required for RNase E-mediated cleavage of the 7 kb *NifA* transcripts, as proposed above, then it is not clear why some low levels of the 3 kb *NifA* transcripts are not detected.

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