Arch Microbiol
DOI 10.1007/s00203-011-0701-1

ORIGINAL PAPER

Evidences of autoregulation of *hfq* expression in *Sinorhizobium meliloti* strain 2011

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Received: 4 February 2011 / Revised: 16 March 2011 / Accepted: 18 March 2011

Abstract Riboregulation comprises gene expression regulatory mechanisms that rely upon the activity of small non-coding RNAs (sRNAs) and in most cases RNA binding proteins. In γ-proteobacteria, the Sm-like protein Hfq is a key player in riboregulatory processes, because it promotes sRNA–mRNA interactions and influences mRNA polyadenylation or translation. In the α-proteobacterium *Sinorhizobium meliloti*, the large number of detected small RNA transcripts and the pleiotropic effects of *hfq* mutations lead to the hypothesis that riboregulatory mechanisms are important in this soil microorganism to adjust gene expression both in free-living conditions and as a nitrogen-fixing endosymbiont within legume root nodules. In this study, homology modeling of *S. meliloti* Hfq protein and cross-complementation experiments of *S. meliloti* and *Escherichia coli* mutants indicates that hfq_{Sm} encodes an RNA chaperone that can be functionally exchanged by its homolog from *E. coli*. A transcriptional and translational analysis of *S. meliloti hfq* expression by means of *lacZ* reporter fusions strongly suggests that the *S. meliloti* Hfq protein autocontrols its expression at the translational level, a phenomenon that was evident in the natural host *S. meliloti* as well as in the heterologous host *E. coli*.

Communicated by Jorge Membrillo-Hernandez.

Electronic supplementary material The online version of this article (doi:10.1007/s00203-011-0701-1) contains supplementary material, which is available to authorized users.

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Keywords *Sinorhizobium meliloti* · *Hfq* · Autoregulation · Riboregulation

Introduction

Over the past decade, there has been an increasing research activity to uncover the role of prokaryotic small, non-protein coding, regulatory RNAs (sRNAs) (Waters and Storz 2009; Sharma and Vogel 2009). In response to a variety of external stimuli, sRNAs act as post-transcriptional modulators of gene expression, affecting the translation rate and/or stability of target mRNAs (Waters and Storz 2009; Valverde and Haas 2008; Frohlich and Vogel 2009). The majority of the characterized sRNAs act by antisense pairing with their target mRNAs, usually at or around the ribosome access region (Frohlich and Vogel 2009). Most sRNA–mRNA interactions characterized so far in Gram-negatives are facilitated by the chaperone protein Hfq (Aiba 2007; Valentin-Hansen et al. 2004). Hfq was originally discovered as a protein required for the replication of the bacteriophage Qβ in *Escherichia coli* (Kajitani and Ishihama 1991). Structural, biochemical, and genetic data show that Hfq is a homohexamer structurally related to the RNA-binding proteins of the Sm family (Brennan and Link 2007), which binds to RNA sequence-structure motifs and facilitates RNA reshaping and RNA–RNA annealing (Brennan and Link 2007). The documented pleiotropic phenotypes of *hfq* deletion mutants in a variety of pathogenic bacterial species, including altered growth, motility, stress resistance, biofilm formation and virulence (Chao and Vogel 2010), indicate that Hfq is involved in the regulation of multiple cellular responses because it participates in antisense interactions of many mRNA–sRNA pairs.

Published online: 12 April 2011

Recent studies reported on the identification and detection of sRNAs in α-proteobacterial species such as *Caulobacter crescentus*, *Rhodobacter sphaeroides*, *Rhizobium etli* and *Sinorhizobium meliloti* (Ulve et al. 2007; Berghoff et al. 2009; Landt et al. 2008; Del Val et al. 2007; Vercruysse et al. 2010; Schluter et al. 2010; Valverde et al. 2008), although the biology of the identified sRNAs remains unknown. *S. meliloti* is a soil bacterium existing either in free-living forms or in symbiosis within root nodules of legumes belonging to the *Medicago–Melilotus–Trigonella* tribe, where they differentiate into nitrogen-fixing bacteroides (Gage 2004; Jones et al. 2007). This makes *S. meliloti* an interesting model to study the role of sRNAs under both environmental conditions. A series of parallel papers recently described the pleiotropic effects of *hfq* mutations at the physiological, transcriptional, and proteomic levels in the sequenced strain *S. meliloti* 1021 (Torres-Quesada et al. 2010; Gao et al. 2010; Barra-Bily et al. 2010a, b), pointing to a major role of Hfq in controlling free-living and symbiotic phenotypes. In order to better understand the role of Hfq as a participant in sRNA-dependent regulatory processes in *S. meliloti* strain 2011, we performed homology modeling of *S. meliloti* Hfq protein and carried out functional exchange of *hfq* homolog genes between *S. meliloti* and *E. coli*. We next studied *hfq* expression at the transcriptional and translational levels and provide clear evidences of autoregulation of *hfq* expression.

Materials and methods

Strains, media, and growth conditions

Strains, plasmids, and primers used in this study are listed in Table 1. *E. coli* was grown at 37°C in nutrient yeast broth (NYB; in g l^{-1}: nutrient broth, 20; yeast extract, 5), and *S. meliloti* was cultured at 28°C in tryptone-yeast extract (TY; in g l^{-1}: tryptone, 5; yeast extract, 3, $CaCl_2$, 0.7) or RDM (Vincent 1970). The following antibiotics were used: for *E. coli*, 100 μg ml^{-1} ampicillin, 25 μg ml^{-1} kanamycin, 10 μg ml^{-1} gentamicin, 25 μg ml^{-1} tetracycline; for *S. meliloti*, 400 μg ml^{-1} streptomycin, 100 μg ml^{-1} neomycin, 40 μg ml^{-1} gentamicin, and 5 μg ml^{-1} tetracycline. Growth was monitored by measuring OD in cultures shaken at 120 rpm.

Modeling of *S. meliloti* Hfq structure

Sequence alignments were generated with ClustalW (Larkin et al. 2007). The JPRED2 algorithm was used for secondary structure prediction (Cuff et al. 1998). A model of *S. meliloti* Hfq tertiary structure was generated by a homology-based procedure using *E. coli* Hfq (PDB 1hk9) as a template. Models were obtained with the program MODELLER (Sali et al. 1995), run in the TITO server (Labesse and Mornon 1998), and structures were validated by calculating energetic (PROSAII) or geometric parameters (RAMPAGE—Ramachandran Plot assessment) (Wiederstein and Sippl 2007; Lovell et al. 2003). Surface potential was calculated with APBS (Adaptative Poisson–Boltzmann Solver) (Baker et al. 2001) and visualized with PyMol (http://www.pymol.org/).

Qβ replication assay

Overnight cultures of *E. coli* strains MC4100 (wild type) and AMIII (*hfq*$^-$) harboring pESE102 (hfq_{Ec}), pUChfq_{Sm} (hfq_{Sm}) or pUC19 plasmids were grown in NYB containing the corresponding antibiotics and 3 mM IPTG, and the Qβ titer (PFU/ml) was determined in the lysates as reported (Sonnleitner et al. 2002).

Swimming motility assay

Strains were grown in TY to exponential phase (OD_{600} = 0.5–0.7) and standardized to the same OD_{600} before spotting 2 μl onto Bromfield agar plates (Sourjik and Schmitt 1996). After 3 days at 28°C, the swimming diameter was measured to the nearest millimeter. The data shown correspond to the swimming diameter minus the diameter of the spotted inoculum.

EPS extraction and quantification

The EPS fraction was extracted from log-phase cultures grown in TY medium. CTAB precipitation and total carbohydrate content were determined by anthrone colorimetry as described (Wells et al. 2007). Cell protein content was estimated with the Bradford method (Bradford 1976), using BSA as standard.

DNA manipulations

DNA preparations were obtained and cloning steps were carried out according to standard protocols (Sambrook et al. 1989). Small-scale plasmid preparations were obtained with the one-tube CTAB method (Del Sal et al. 1988) and high-quality plasmid preparations with the Jet-Quick miniprep spin kit (Genomed GmbH). All cloned PCR products were verified by sequencing from both ends by Macrogen Inc. (Korea). To construct a Δ*hfq* in-frame deletion strain (*S. meliloti* 20PS01), the strain 2011 wild-type *hfq* allele was replaced by double recombination with an *hfq* copy bearing an in-frame deletion of 69 nucleotides that removes amino acid residues 37–59 within the Sm1

Table 1 Bacterial strains, plasmids, and oligonucleotides used in this study

Strain, plasmid, or oligonucleotide	Genotype, features, or sequence	Reference or source
E. coli		
AMIII	MC4100 *hfq-1*::Ω, Km^R	Muffler et al. (1996)
DH5α	F^- *endA1 hsdR17 supE44 thi-1 recA1 gyrA96 relA1* Δ(*lacZYA-argF*)U169 *deoR* (Φ80d*lacZ*ΔM15)	Sambrook et al. (1989)
MC4100	F^- [*araD*139] Δ(*argF-lac*)169 λ^- e14- *flhD*5301 Δ(*fruK-yeiR*)725 (*fruA*25) *relA1 rpsL*150(*strR*) *rbsR*22 Δ(*fimB-fimE*)632(::IS1) *deoC*1	Casadaban (1976)
S17-1 *λpir*	F^- *pro thi hsdR recA*; chromosome::RP4-2 Tc::Mu Km::Tn*7* Tp^R, Sp^R *λpir*	Simon et al. (1983)
S. meliloti		
2011	Wild type, Sm^R	Meade and Signer (1977)
20PS01	2011 with an in-frame $hfq\Delta^{37-59}$ deletion, Sm^R	This work
20PS07	2011 with a control Phfq_{Sm}-*Zcal* chromosomal transcriptional fusion, Sm^R, Nm^R, Gm^R	This work
20PS08	2011 with a Phfq_{Sm}-*lacZ* chromosomal transcriptional fusion, Sm^R, Nm^R, Gm^R	This work
20PS15	2011 with an hfq_{Sm}'-'*lacZ* chromosomal translational fusion, Sm^R, Nm^R	This work
20PS16	20PS01 with an hfq_{Sm}'-'*lacZ* chromosomal translational fusion, Sm^R, Nm^R	This work
Plasmids		
pAB2001	Contains a promoterless *lacZ-accC1* cassette for construction of transcriptional *lacZ* fusions, Ap^R, Gm^R	Voss et al. (2009)
pAB5002	Contains a '*lacZ-accC1* cassette for construction of translational *lacZ* fusions, Ap^R, Gm^R	Ugalde et al. (1998)
pBluescript	Cloning vector, ColE1 replicon, MCS KS+, Ap^R	Stratagene (Agilent)
pESE102	pUC19 with hfq_{Ec} under P*lac* promoter	Sonnleitner et al. (2002)
pFAJ1708	Derivative of RK-2, contains *npt*II promoter from pUC18-2, Ap^R, Tc^R	Dombrecht et al. (2002)
pK18mob2	Mobilizable and suicide vector in *S. meliloti* for insertional mutagenesis, Km^R	Tauch et al. (1998)
pK18mobSacB	pK18mob derivative with *sacB*, Km^R, $sucrose^S$	Schafer et al. (1994)
phfq_{Ec}	pFAJ1708 with hfq_{Ec} under P*npt*II promoter, Ap^R, Tc^R	This work
phfq_{Sm}	pFAJ1708 with hfq_{Sm} under its own promoter, Ap^R, Tc^R	This work
pPS07	pK18mob2 derivative with a Phfq_{Sm}-*Zcal* transcriptional fusion, Km^R, Gm^R	This work
pPS08	pK18mob2 derivative with a Phfq_{Sm}-*lacZ* transcriptional fusion, Km^R Gm^R	This work
pPS15	pAB5002 with an hfq_{Sm}'-'*lacZ* translational fusion, Ap^R	This work
pUC19	Cloning vector, ColE1 cloning vector, Ap^R	New England Biolabs
pUChfq_{Sm}	pUC19 with hfq_{Sm} under P*lac* promoter, Ap^R	This work
Oligonucleotides[a] (5′ → 3′)		
BH1E (*Eco*RI)	CGTGAATTCGGCGATCTTCGTCGACGTGG, forward, anneals between −455 and −436	This work
BH1H (*Hind*III)	CGATAAGCTTACACCCGTCAGCTTGACGCC, reverse, anneals between +88 and +107 (denoted as 4 in RT–PCR shown in Fig. 2a)	This work
BH2H (*Hind*III)	GCTAAAGCTTCGCCATCTCGACGATCATGC, forward, anneals between +176 and +196 (denoted as 3 in RT–PCR shown in Fig. 2a)	This work
BH2B (*Bam*HI)	CGGGATCCTTGAGATGTGCGAGATCGACC, reverse, anneals between +708 and +728 (denoted as 6 in RT–PCR shown in Fig. 2a)	This work
HflXbam (*Bam*HI)	AGTTAGGATCCGCCAGACCAAGTCAGTGGG reverse, anneals between +1,785 and +1,803	This work
NrfAfwd (*Eco*RI)	ATCCGAGAATTCCGCCGGCAAATAAGAAAGAAGC, forward, anneals between −28 and −7 (denoted as 2 in RT–PCR shown in Fig. 2a)	This work
NrfArev (*Bam*HI)	TGAATAGGATCCTGTTCCTCACATCCCGTCAGG, reverse, anneals between +239 and +259 (denoted as 5 in RT–PCR shown in Fig. 2a)	This work
Y01047F (*Eco*RI)	ATCCAGAATTCACGAGGTCTGCGAGGTGC, forward, anneals between −867 and −850 (denoted as 1 in RT–PCR shown in Fig. 2a)	This work

[a] Specified restriction sites are underlined. The annealing positions are relative to the first nucleotide of the hfq_{Sm} start codon

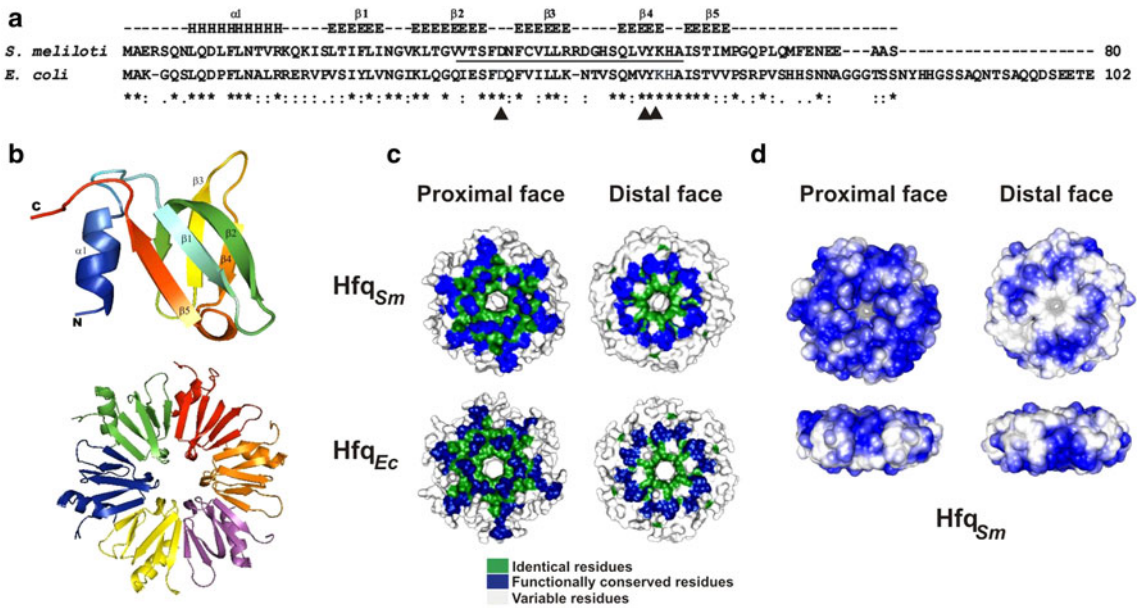

Fig. 1 Structural modeling of the *S. meliloti* Hfq protein. **a** Sequence homology between *S. meliloti* (Hfq$_{Sm}$) and *E. coli* (Hfq$_{Ec}$) proteins. *Arrowheads* point to critical conserved residues important for Hfq function in *E. coli*. The residues deleted in the Hfq$_{Sm}$ mutant studied in this work are *underlined*. The predicted α-helical (α_i) and β-stranded (β_i) regions for Hfq$_{Sm}$ are shown above the sequence. **b** Predicted ribbon model of the Hfq$_{Sm}$ monomer and the putative hexamer using the *E. coli* structure (PDB 1hk9) as a template. The model was validated by RAMPAGE (91.4% of the residues are in favorable regions of the Ramachandran plot) and by a PROSAII z score = −10.78, which fall within the range typically found for native proteins of similar size (Wiederstein and Sippl 2007; Lovell et al. 2003). **c** Location of identical and functionally conserved amino acid residues in *S. meliloti* and *E. coli* hexamers. **d** Both faces of the Hfq$_{Sm}$ ring model show positively charged residues (*highlighted as dark regions* on the hexamer surface), with a higher charge density on the proximal face

domain and avoids polarity on *hflX* (Fig. 1a). The Δ*hfq* mutation was confirmed by PCR and Southern blot. For complementation of the Δ*hfq* mutant, a 1,126 bp-PCR fragment containing the *hfq* gene and its own promoter was cloned into pFAJ1708 in opposite direction to the vector *npt*II promoter to generate p*hfq*$_{Sm}$, and this construct was mobilized into strain 20PS01 by conjugation. For complementation of *E. coli hfq* mutants, a 287-bp PCR fragment bearing the *hfq* RBS was cloned into pUC19 under the control of the vector *lac* promoter. For heterologous complementation of the *S. meliloti hfq* mutant, an *Eco*RI-*Bam*HI fragment from pESE102 was subcloned into pFAJ1708 to give p*hfq*$_{Ec}$, in which the *E. coli* K12 *hfq* gene and its own RBS lie under the control of the *npt*II promoter.

Construction of *lacZ* reporter fusions and β-galactosidase assay

To construct a chromosomal *hfq* transcriptional fusion, a 560-bp PCR fragment containing the putative *hfq* promoter was cloned into pK18mob2 upstream the promoterless *lacZ* from pAB2001, to generate pPS08. To construct an *hfq* translational fusion, the same amplicon was subcloned into the suicide vector pAB5002, thus generating an in-frame fusion between the first 25 residues of *S. meliloti* Hfq and the eighth β-galactosidase codon (pPS15). pPS08 and pPS15 were mobilized into *S. meliloti* by conjugation. β-Galactosidase activity was measured in cells from liquid cultures that were permeabilized with 5% (v/v) toluene (Miller 1972).

RNA manipulations

The total RNA was extracted with Trizol (Invitrogen) from stationary phase cultures of *S. meliloti* grown in TY, following manufacturer's instructions. RNA quality was assessed by denaturing agarose electrophoresis, and RNA concentration was estimated by UV spectrophotometry (Sambrook et al. 1989). Samples were analyzed by Northern blot as reported (Valverde et al. 2008). In vivo sRNA stability was analyzed after addition of 200 μg ml^{-1} rifampicin to the cultures followed by RNA extraction at indicated times and further analysis by Northern blot. For RT–PCR analysis, 5 μg of total RNA was used as template for each 20-μl reverse transcription reaction containing 2 U of M-MuLV reverse transcriptase and 20 pmol of the appropriate DNA primer (Table 1), following the protocol supplied by the manufacturer (Fermentas); 5 μl of each RT reaction served as template for a PCR reaction with the corresponding DNA primers indicated for each experiment.

Results and discussion

Structural modeling of the *S. meliloti* Hfq protein

The *Sinorhizobium meliloti hfq* gene (hereafter hfq_{Sm}) encodes a polypeptide of 80 residues that is 47% identical to *E. coli* Hfq (Fig. 1a). The core sequence of the prokaryotic Hfq proteins (first 60–65 residues), encompassing the Sm1 and Sm2 motifs typical of Sm-RNA binding proteins, is highly conserved, and the variability among Hfq homologs occurs at the C-terminal region (Sun et al. 2002). Key residues required for Hfq activity in *E. coli*, such as D40, Y55, and K56 (Mikulecky et al. 2004; Ziolkowska et al. 2006), are conserved in Hfq_{Sm} (Fig. 1a). The strong primary and secondary structure similarity between *S. meliloti* and *E. coli* Hfq core sequence (Fig. 1a) supported modeling of the Hfq_{Sm} structure (Fig. 1b). The donut-shaped Hfq_{Sm} hexamer contains differentially charged surfaces, and most of the identical and conserved residues lay in the furrows present in the proximal and distal sides (Fig. 1c), where interactions with A/U-rich sequences and with poly-A tracts take place, respectively (Link et al. 2009; Schumacher et al. 2002). The calculated surface distribution charges on both sides of the predicted Hfq_{Sm} hexamer match the positive and asymmetric charge distribution of the crystallized *E. coli* Hfq protein (Fig. 1c) (Brennan and Link 2007). It differs, however, from the surface charge properties of *S. aureus* Hfq, which seems to be dispensable for RNA–RNA interactions (Jousselin et al. 2009).

Functional replacement of *hfq* homologs in *S. meliloti* strain 2011 and *E. coli*

The result of two-dimensional and three-dimensional Hfq_{Sm} structure predictions (Fig. 1) strongly suggests that hfq_{Sm} encodes a protein having similar functions as the *E. coli* RNA chaperone, which was originally identified in *E. coli* as a host factor for replication of the phage Qβ (Kajitani and Ishihama 1991). As expected, the *E. coli hfq* mutant AMIII is a restrictive host for Qβ propagation (Table 2) and normal Qβ titers were obtained for AMIII complemented with the hfq_{Ec} allele (Table 2). Expression of the *S. meliloti hfq* homolog partially restored Qβ-replication (Table 2). This suggests that Hfq_{Sm}, although bearing a shorter C-terminal extension than Hfq_{Ec} (Fig. 1a), it can partially replace *E. coli* Hfq in its RNA chaperoning role during the Qβ phage cycle.

To test the ability of the *E. coli hfq* gene (hfq_{Ec}) to functionally replace strain 2011 hfq_{Sm}, we constructed an isogenic in-frame *hfq* deletion mutant that avoids polarity on *hflX* (Fig. 2). In agreement with recent reports (Gao et al. 2010; Barra-Bily et al. 2010a, b; Torres-Quesada et al. 2010), the hfq_{Sm} mutant strain 20PS01 displayed a lower growth rate, a longer lag phase, and a lower final yield in both complex and defined media, reduced swimming motility, reduced resistance to H_2O_2, reduced survival rate when exposed to UV radiation, and reduced symbiotic performance (Table 3, Table S1 and Figs. S1–S3). In addition, the hfq_{Sm} mutant developed mucoid colonies in TY plates that fluoresced brighter than the wild type in the presence of the exopolysaccharide (EPS) I-specific dye calcofluor (Fig. S3) and overproduced CTAB-precipitable EPSs on a cellular protein basis in liquid culture (Table 3). In terms of its role on sRNA metabolism, the *hfq* mutation in strain 2011 had a strong negative impact on the steady state concentration and stability of the previously identified small RNA transcripts Sm8, Sm12, Sm26, Sm84, and Sm145 (Valverde et al. 2008) (Fig. S3), as reported for the SmrC16 sRNA (Voss et al. 2009).

Table 2 Hfq_{Sm} facilitates Qβ replication in *E. coli*

E. coli strain	Genotype	Qβ titer (PFU/ml)
MC4100	Wild type	11.06 ± 0.05^{a}
AMIII	Δhfq	5.03 ± 0.15^{b}
AMIII/pESE102	Δhfq, hfq_{Ec}	11.04 ± 0.16^{a}
AMIII/pUChfq_{Sm}	Δhfq, hfq_{Sm}	8.08 ± 0.18^{c}
AMIII/pUC19	Δhfq	5.32 ± 0.10^{b}

Each titer represents the average log value of three different cultures ±SD. Averages were statistically compared using one-way ANOVA followed by Tukey's multiple comparison test against the parental strain. Different superscript letters indicate that the averages are significantly different with $P < 0.001$. The experiment was repeated thrice, with essentially the same results

Complementation of the mutant strain 20PS01 with hfq_{Sm} resulted in full restoration of growth in TY medium (Table 3; Fig. 3a). In the minimal and defined RDM medium, the hfq_{Sm} copy failed to fully restore wild-type growth (Table 3; Fig. 3b). We attribute this effect to the higher hfq_{Sm} expression caused by the vector used, as we observed that the growth of wild-type strain 2011 was also negatively affected by phfq_{Sm} in RDM medium (Fig. S4). Complementation with the 2011 *hfq* allele did also partially revert the motility and EPS mutant phenotypes, confirming that the hfq_{Sm} gene is involved in their control (Table 3 and Fig. S3).

hfq_{Ec} fully restored wild-type growth in TY medium but partially in RDM medium, resulting almost indistinguishable from the mutant complemented with the hfq_{Sm} copy in both media (Table 3; Fig. 3). hfq_{Ec} also complemented the hfq_{Sm} mutant for EPS production, to the same extent as hfq_{Sm} (Table 3; Fig. S3). However, the defective motility in semisolid medium could not be restored to near-wild-type level by the heterologous hfq_{Ec} (Table 3; Fig. S3). These results on functional

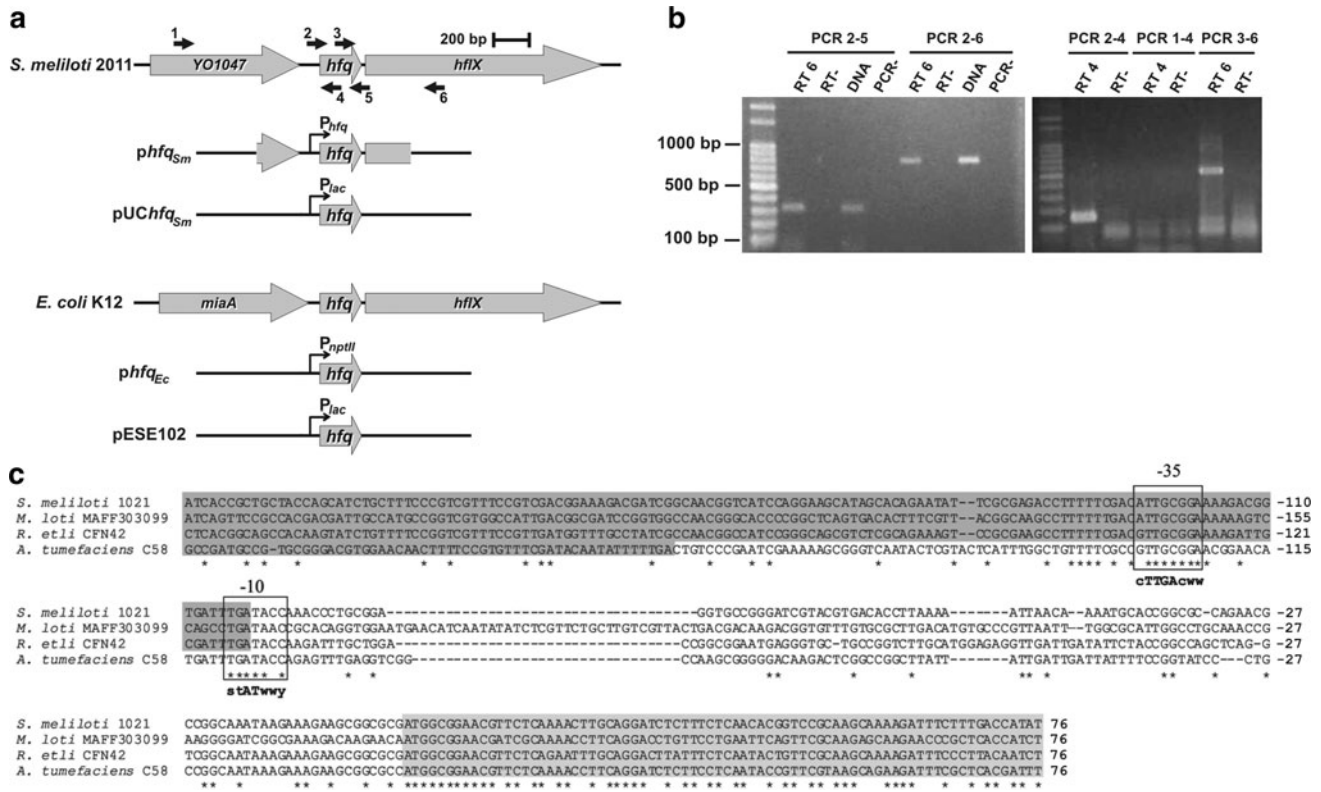

Fig. 2 Expression of hfq_{Sm} in free-living *S. meliloti* strain 2011. **a** *hfq* locus in *S. meliloti* strain 2011 and *E. coli* K12. The approximate location of the forward (1–3) and reverse (4–6) primers used for the RT–PCR analysis is indicated by small arrows on strain 2011 locus (see Table 1 for exact annealing positions). The fragments used for complementation vectors are schematically depicted under the corresponding genetic loci of *S. meliloti* and *E. coli*. **b** RT–PCR analysis of the hfq_{Sm} locus. PCR was performed on RNA after reverse transcription with primer 4 (RT4) or primer 6 (RT6), on RNA without reverse transcription as control (-RT), on genomic DNA (DNA), or without DNA as control (PCR-). The primers used for RT and PCR are indicated. **c** DNA alignment of *hfq* upstream regions from the α-proteobacteria *S. meliloti* 2011, *Mesorhizobium loti* MAFF303099, *Agrobacterium tumefaciens* C58, and *Rhizobium etli* CFN42. The nucleotides shaded in *light gray* correspond to the *hfq* CDS and those shaded in *dark gray* correspond to the upstream CDS Y01047 (that encodes a D-alanine aminotransferase), conserved in all strains but *A. tumefaciens*. *Asterisks* denote identical bases. The nucleotide positions are relative to the first base of the start codon. Note the overall lack of conservation upstream the *hfq* CDS, except for a DNA stretch located at −131 to −97 that coincides with the sequence of a predicted σ^{70}-like promoter (http://www.fruitfly.org/seq_tools/promoter.html) (−35 box TTGCGG and −10 box TGATAC, score 0.94 out of 1.0) and also with the sequence consensus of the proposed rhizobial −35 and −10 boxes (MacLellan et al. 2006) indicated below the conserved putative −10 and −35 regions

replacement of *S. meliloti* and *E. coli hfq* genes add to the previously reported observations of heterologous complementation of *hfq* mutants between Gram-negative bacteria, Gram-positive bacteria, and even an archaeon (Nielsen et al. 2010; Sittka et al. 2009). The general RNA chaperoning role of Hfq homologs may not be totally interchangeable as in the case of the motility assay (Table 3), as there might be some kind of RNA-Hfq specificity determinants.

hfq transcriptional and translational expression pattern in *S. meliloti* strain 2011

The sequence of the *S. meliloti* 2011 *hfq* locus is 100% identical to that of strain 1021 along a fragment of 1,182 bp (GenBank accession GU725393). The *hfq-hflX* tandem (Fig. 2a) is highly conserved in α- and γ-proteobacteria. By RT–PCR (Fig. 2b), we showed that hfq_{Sm} is co-transcribed with *hflX*, which encodes a putative GTP-binding protein of yet unknown function. *hfq-hflX* co-transcription has been demonstrated only in *E. coli* (Tsui et al. 1996) and in *Francisella tularensis* (Meibom et al. 2009). The RT–PCR product obtained with primer pair 2–4 (Fig. 2b) indicates that the hfq_{Sm} promoter lies at least 30 bp upstream the start codon. In silico analysis of the DNA sequence upstream the hfq_{Sm} CDS revealed the presence of a putative rhizobial transcriptional start site (MacLellan et al. 2006), conserved in other α-proteobacteria, with the -10 box centered at 100 bp upstream the start codon (Fig. 2c).

A chromosomal Phfq_{Sm}-*lacZ* transcriptional fusion drove *lacZ* expression from a 560-bp fragment containing the hfq_{Sm} promoter and any possible upstream regulatory

Table 3 Functional complementation of the hfq_{Sm} mutant by the *E. coli hfq* homolog

S. meliloti strain (genotype)	Growth rate (h^{-1})[a]		Swimming halo[b] (cm)	EPS production[c] (mg glucose mg protein^{-1})
	TY	RDM		
2011 (wt)	0.257 ± 0.003	0.091 ± 0.003	1.07 ± 0.12	0.08 ± 0.02
20PS01 (Δ*hfq*)	0.165 ± 0.022*	0.070 ± 0.004*	0.42 ± 0.03*	0.17 ± 0.01*
20PS01 (Δ*hfq*)/phfq_{Sm}	0.255 ± 0.021	0.077 ± 0.012	0.82 ± 0.08	0.08 ± 0.01
20PS01 (Δ*hfq*)/phfq_{Ec}	0.223 ± 0.011	0.081 ± 0.007	0.38 ± 0.03*	0.08 ± 0.01
20PS01 (Δ*hfq*)/pFAJ1708	0.153 ± 0.006*	0.061 ± 0.005*	0.28 ± 0.08*	0.17 ± 0.01*

[a] Growth rate was estimated from growth curves shown in Fig. 3. Each value is the average from three different cultures ±SD. The experiment was repeated thrice, with essentially the same results

[b] The swimming halos were measured at 72 h after inoculation of semisolid Bromfield agar plates. The data represent the average from four replicates ±SD. The experiment was repeated thrice, with essentially the same results

[c] The data shown represent the average CTAB-precipitable EPS content from three TY replicate cultures ±SD. The experiment was repeated twice, with essentially the same results. For every phenotype, the average values were statistically compared using one-way ANOVA followed by Dunnet multiple comparison test against the wild-type strain

* The average is significantly different from the parental strain with $P < 0.001$

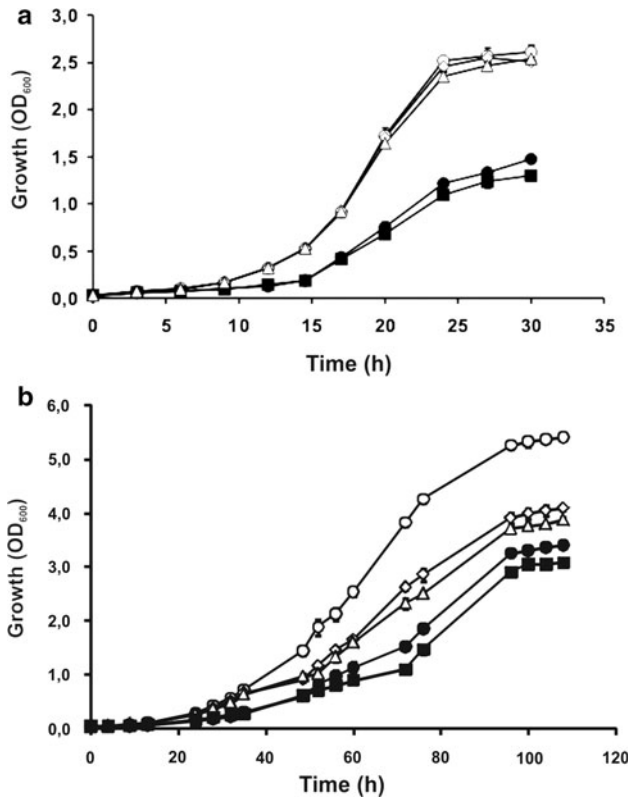

Fig. 3 Functional complementation of *S. meliloti* Δ*hfq* mutant growth by the *E. coli hfq* allele. Growth curves in tryptone-yeast broth TY (**a**) or rhizobial defined medium RDM (**b**); *open circle* wild-type strain 2011, *filled circle* Δ*hfq* mutant strain 20PS01, *open triangle* 20PS01-bearing complementing plasmid phfq_{Sm}, *open diamond* 20PS01-bearing complementing plasmid phfq_{Ec}, *filled square* 20PS01 bearing control plasmid pFAJ1078. Each curve represents the average from three different cultures ±SD. The experiment was repeated twice, with essentially the same results

region, in both complex TY and defined RDM media (Table 4; Fig. 4). We observed a threefold increment in *lacZ* expression when TY cultures entered into stationary phase (Table 4; Fig. 4a), confirming previous observations in strain 1021 (Barra-Bily et al. 2010a). This growth-phase-dependent induction was not detected in defined RDM medium (Table 4; Fig. 4b). These results suggest that hfq_{Sm} expression is not regulated by any cell density-dependent mechanism. All along the growth curve in RDM, the Phfq_{Sm}-*lacZ* activity was relatively constant and showed slightly higher values than those of stationary phase in rich TY medium (Table 4; Fig. 4b). Thus, in pure cultures, hfq_{Sm} transcription is positively influenced by the growth phase in rich medium and/or by the nutritional conditions of the growth medium. The hfq_{Sm} promoter was also active *in planta* during all stages of the symbiotic association (Fig. S5).

Translational regulation of hfq_{Sm} was studied by means of a chromosomal hfq_{Sm}'-'*lacZ* fusion, which was active in both free-living (Table 4) and symbiotic conditions (Fig. S5). Unlike the transcriptional reporter, the hfq_{Sm}'-'*lacZ* construct was expressed constitutively in both TY and RDM media (Table 4; Fig. 4c, d). This suggests the operation of a post-transcriptional control on hfq_{Sm} expression, which was evident in complex TY medium.

Hfq_{Sm} is involved in negative regulation of its own expression

The activity of the chromosomal hfq_{Sm}'-'*lacZ* translational fusion in TY medium was clearly higher in the hfq_{Sm} mutant strain 20PS01 (Table 4; Fig. 4c). Moreover, the hfq_{Sm} mutant displayed a growth-phase-dependent hfq_{Sm}'-'*lacZ*

Table 4 Transcriptional and translational regulation of hfq_{Sm} expression in *S. meliloti* strain 2011

Chromosomal reporter fusion[a]	*S. meliloti* strain (genotype)	β-Galactosidase activity (Miller units)[b]			
		TY medium		RDM medium	
		Exponential	Stationary	Exponential	Stationary
–	2011 (wt)	8.7 ± 2.6a	3.9 ± 1.9a	5.4 ± 1.9a	6.3 ± 0.9a
Phfq_{Sm}-*Zcal*	20PS07 (wt)	9.1 ± 7.3a	2.4 ± 0.7a	13.7 ± 2.1a	10.2 ± 1.4a
Phfq_{Sm}-*lacZ*	20PS08 (wt)	346.8 ± 8.3b	1,245.0 ± 56.4b	1,522.2 ± 103.1b	1,527.1 ± 191.5b
hfq_{Sm}'-'*lacZ*	20PS15 (wt)	1,171.5 ± 32.3a	1,134.6 ± 67.2a	1,287.9 ± 165.1a	1,300.0 ± 62.6a
	20PS16 (Δ*hfq*)	5,926.2 ± 585.6b	10,427.0 ± 423.4b	4,675.1 ± 286.6b	4,661.3 ± 267.0b
	20PS17 (Δ*hfq*/phfq_{Sm})	2,996.4 ± 60.9c	2,298.9 ± 188.6c	2,788.4 ± 126.3c	2,629.2 ± 417.2c
	20PS18 (Δ*hfq*/phfq_{Ec})	2,794.1 ± 241.5c	3,141.4 ± 159.3c	2,915.7 ± 346.5c	2,762.6 ± 316.8c
	20PS19 (Δ*hfq*/pFAJ1708)	5,766.1 ± 281.6b	11,366.3 ± 282.1b	4,928.1 ± 300.4b	4,979.1 ± 86.3b

[a] In the transcriptional Phfq_{Sm}-*lacZ* fusion, *lacZ* expression is controlled by the *hfq* promoter. In the Phfq_{Sm}-*Zcal* construct, the promoterless *lacZ* gene has been cloned in the opposite orientation with respect to the *hfq* promoter. The hfq_{Sm}'-'*lacZ* construct bears an in-frame *hfq*'-'*lacZ* fusion controlled by the *hfq* promoter

[b] The data shown for exponential phase correspond to OD_{600} ranging 0.5–0.8. For stationary phase data, OD_{600} ranged 1.5–2.5 for TY medium and 2.5–4.5 for RDM medium. Each value is the average from three different cultures ±SD. The data for each fusion and condition (medium, growth phase) were statistically compared with one-way ANOVA followed by the Tukey's multiple comparison test against parental strains (2011 for Phfq_{Sm}-*lacZ* and 20PS15 (wt) for hfq_{Sm}'-'*lacZ*). Different superscript letters indicate that the averages are significantly different with $P < 0.001$. The experiment was repeated thrice, with essentially the same results

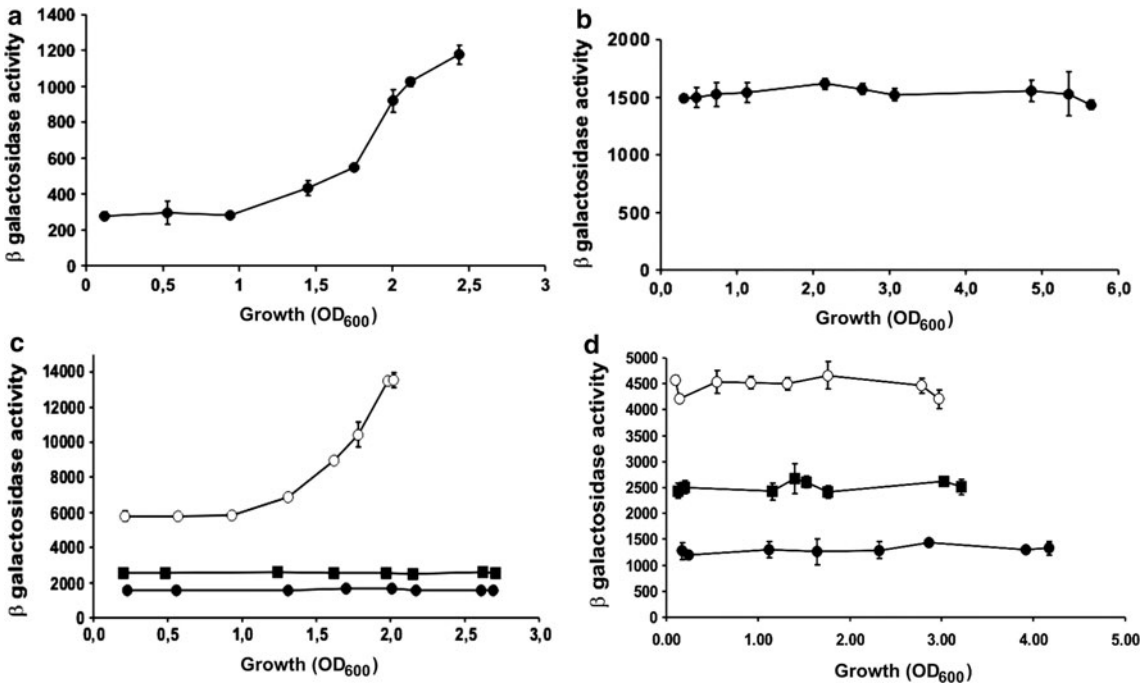

Fig. 4 Transcriptional control of *hfq* expression and translational autoregulation in *S. meliloti* strain 2011. β-Galactosidase activity of a chromosomally integrated transcriptional Phfq_{Sm}-*lacZ* fusion was determined in strain 20PS08 (wild type), growing in TY medium (**a**) or in RDM medium (**b**). Similarly, the β-galactosidase activity of a chromosomally integrated translational hfq_{Sm}'-'*lacZ* fusion was determined in strains 20PS15 (*filled circle* wild type), 20PS16 (*open circle hfq* mutant), and 20PS16/phfq_{Sm} (*filled square* hfq_{Sm} complemented mutant), growing in TY medium (**c**) or in RDM medium (**d**). Each value is the average from three different cultures ±SD. The experiment was repeated thrice, with essentially the same results

expression (Fig. 4c), showing that in the absence of the Hfq_{Sm} protein, translation of the hfq_{Sm} mRNA is deregulated and that the reporter mRNA level responds to the activity of the stationary-phase-activated Phfq_{Sm} promoter (Table 3; Fig. 4a). The negative autocontrol of *hfq* translation was also evident in the defined RDM medium (Table 4; Fig. 4d).

Notably, the negative regulation on hfq_{Sm} mRNA translation could be restored by complementation with a wild-type hfq_{Sm} allele (Table 4, Fig. 4c, d). Thus, the Hfq_{Sm} protein is either directly or indirectly involved in controlling its own expression. Interestingly, the *E. coli hfq* allele also conferred autocontrol of hfq_{Sm} mRNA translation (Table 4), giving further evidence of overlapping functions between these Hfq homologs (Table 3 and Fig. S3), in spite of the different C-terminal regions (Fig. 1a).

Hfq_{Sm} autoregulates its own expression in the heterologous host *E. coli*

If Hfq_{Sm} protein controls translation of its own mRNA (Table 4; Fig. 4), the phenomenon should be reproducible in a heterologous system. To test this, we mobilized the required *S. meliloti* genetic factors to the hfq^- *E. coli* strain AMIII (Muffler et al. 1996). The hfq_{Sm}'-'*lacZ* fusion was functional in the hfq^- background (Fig. 5), but when the hfq_{Sm} gene was provided *in trans*, the β-galactosidase activity was significantly reduced (Fig. 5), reflecting the gain of translational autocontrol. Again, the *E. coli hfq* allele was also able to introduce translational autocontrol *on* hfq_{Sm}'-'*lacZ* expression, when expressed from either a plasmid in the hfq^- background or the chromosome of the wild-type hfq^+ *E. coli* (Fig. 5). These results in the heterologous *E. coli* host show that the sole presence of Hfq_{Sm} or Hfq_{Ec} suffices to execute a negative control on *S. meliloti hfq* mRNA translation.

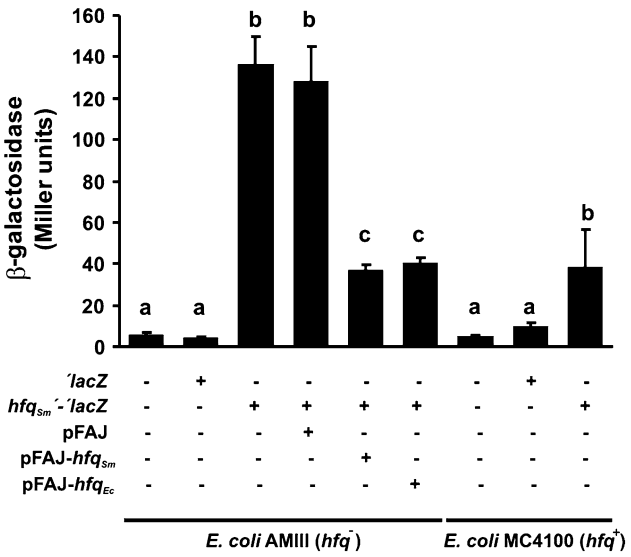

Fig. 5 Translational autocontrol of hfq_{Sm} expression also operates in the heterologous host *E. coli*. The data correspond to cultures sampled in stationary phase at OD_{600} ranging 2.5–3.5. Each value is the average from three different cultures ±SD. The data for each *E. coli* strain (AMIII and MC4100) were statistically compared with one-way ANOVA followed by the Tukey's multiple comparison test against parental strains. *Letters* on *top* of columns indicate that the averages are significantly different with $P < 0.01$. The experiment was repeated twice, with essentially the same results

In conclusion, homology modeling of *S. meliloti* Hfq protein and cross-complementation experiments of hfq_{Sm} associated phenotypes indicates that hfq_{Sm} encodes an RNA chaperone that can be functionally replaced by its homolog from *E. coli* (Figs. 1, 3; Tables 2, 3; Fig. S3). With the aid of *lacZ* reporter fusions analyzed both in the natural host *S. meliloti* and in the heterologous host *E. coli* (Table 4; Figs. 4, 5), we obtained strong evidence of translational negative autoregulation of *S. meliloti hfq* expression. Whether this is a generalized feature of Hfq proteins, as it has also been reported for *E. coli* Hfq (Vecerek et al. 2005), it remains to be determined, together with the molecular mechanism responsible for the autoregulatory loop.

Acknowledgments This work was supported by grants PICT 2004-25396, PICT 2007-700, PME 2006-1730 (Agencia Nacional de Promoción Científica y Tecnológica, Argentina), PIP 2004-5812 (CONICET, Argentina) and PUNQ 0395/07 (Universidad Nacional de Quilmes, Argentina). We thank Dr. A. Lagares for providing *S. meliloti* strain 2011 and vectors, Dr. Elisabeth Sonnleitner for the generous gift of a Qβ lysate and plasmid pESE102, Dr. Branislav Večerek for providing *E. coli* strains MC4100 and AMIII, and Rizobacter Argentina S.A. for providing alfalfa seeds. PS holds a Ph.D. fellowship from CONICET. CV is member of CONICET.

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