Chapter 20

Identification of Small RNA–Protein Partners in Plant Symbiotic Bacteria

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Abstract

The identification of the protein partners of bacterial small noncoding RNAs (sRNAs) is essential to understand the mechanistic principles and functions of riboregulation in prokaryotic cells. Here, we describe an optimized affinity chromatography protocol that enables purification of in vivo formed sRNA–protein complexes in *Sinorhizobium meliloti*, a genetically tractable nitrogen-fixing plant symbiotic bacterium. The procedure requires the tagging of the desired sRNA with the MS2 aptamer, which is affinity-captured by the MS2-MBP protein conjugated to an amylose resin. As proof of principle, we show recovery of the RNA chaperone Hfq associated to the strictly Hfq-dependent AbcR2 *trans*-sRNA. This method can be applied for the investigation of sRNA–protein interactions on a broad range of genetically tractable α-proteobacteria.

Key words Rhizobia, *trans*-sRNA, MS2, Maltose-binding protein

1 Introduction

Posttranscriptional regulation of gene expression by small noncoding RNAs (sRNAs) is ubiquitous in bacteria. The vast majority of sRNAs rely on antisense interactions with one or multiple mRNAs to control translation and/or stability of the targeted transcripts. However, sequence complementarity between sRNAs and *trans*-encoded target mRNAs is typically limited to short and discontinuous nucleotide stretches, and therefore these interactions require the assistance of proteins. Previous work on classical model enterobacteria identified Hfq and, more recently, ProQ as RNA chaperones acting as global sRNA stabilizers and matchmakers in riboregulation [1, 2]. However, these proteins are not widely distributed across bacterial kingdom. Even in bacteria encoding a functional Hfq homolog, this protein has a limited impact or is fully dispensable in riboregulation, anticipating that other yet undiscovered proteins may fulfill similar chaperone roles in sRNA

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regulatory networks. It is also well known that the enterobacterial Hfq establishes a higher-order protein complex with the RNA degradosome consisting of the major single-strand RNase E endoribonuclease, the 3′–5′ exoribonuclease polynucleotide phosphorylase (PNPase), RNA helicase B, and enolase [3]. The degradosome promotes target mRNA decay upon base-pairing interaction with its cognate partner Hfq-dependent sRNAs. On the other hand, other classes of bacterial sRNAs act by target mimicry rather than through base-pairing interactions to counteract the activity of specific proteins functionally related to the flow of genetic information. Well-characterized examples of this class of riboregulators are the 6S and CsrB sRNA families, which antagonize the activity of the σ^{70} RNA polymerase holoenzyme or the translational repressors of the CsrA/RmsA family, respectively [4, 5].

Understanding the function and activity mechanisms of bacterial sRNAs therefore requires the identification of their protein partners. In this regard, research on phylogenetically distant bacteria exhibiting complex lifestyles is expected to add new paradigms to what is known about protein-assisted riboregulation in classical model enterobacteria. We have optimized an affinity chromatography protocol to capture proteins interacting with sRNAs identified in the nitrogen-fixing symbiotic α-proteobacterium *Sinorhizobium meliloti*. The procedure relies on 5′-tagging of bait sRNAs with the RNA aptamer recognized by the MS2 coat protein, which is fused to a maltose-binding protein (MBP) to allow its immobilization on an affinity matrix. As a proof of principle, we show reliable recovery of Hfq with its known *S. meliloti* AbcR2 sRNA partner.

2 Materials

Standard equipment in molecular biology (e.g., incubators, gel electrophoresis devices, refrigerated centrifuges) is required for the following protocols. To avoid RNA degradation, special attention should be kept on glassware and equipment cleanness. Working solutions were prepared in ultrafiltered sterile water. Commercial RNase-free water and plasticware were used for in vitro assays. Below, the specific material needed to carry out the methods described in Subheading 3 is listed.

2.1 Culture and Harvest of Bacteria

1. Rhizobial strains: wild-type and derivatives expressing a FLAG-tagged Hfq (e.g., Sm*hfq*^{FLAG} in Sm2B3001) [6] or carrying a deletion of the corresponding sRNA *locus* (e.g., Sm2B3001Δ*abcR2*) [7].
2. Media: LB medium was routinely used to grow *E. coli* at 37 °C and complex tryptone yeast (TY) [8] or defined minimal medium (MM) [9] for growing rhizobia at 30 °C.

3. Antibiotics: streptomycin (600 mg/mL), tetracycline (10 mg/mL), or kanamycin (50 mg/mL for *E. coli* and 180 mg/mL for *Sinorhizobium* strains).
4. Cultures (usually 200 mL cultures in 1 L flasks) were incubated at 30 °C and 180 rpm.
5. Although most of the bacterial sRNAs are upregulated in early stationary phase [10], optimal expression conditions for the sRNA of interest must be assessed prior the study.
6. Cells are harvested by centrifugation (10 min at 5500 × *g* and 4 °C). Cell pellets are washed once with 0.1% sarcosyl in Tris-EDTA pH: 8.0 (TE Buffer) to facilitate subsequent cell disruption and once again with the working buffer (e.g., buffer A for affinity purification) prior to storage at −80 °C.

2.2 sRNA Aptamer Tagging

1. Vector pSRK-C, which is an engineered pSRKKm [11], lacking the LacIQ operator is used as backbone for aptamer-tagged sRNA constructions (confers Km resistance).
2. DNA oligonucleotides (100 pmol/mL) for direct annealing of aptamer and terminator sequences and for PCR amplification of the sRNA (see details for primer design in Subheading 3.1).
3. Conventional PCR: Phusion High-Fidelity DNA Polymerase (Finnzymes), 5× Phusion HF Buffer, 10 mM dNTPs.
4. DNA electrophoresis: 6× DNA loading dye, 10× TAE buffer (0.4 M Tris, 17.4 M acetic acid, 0.02 M EDTA pH: 8.2), and agarose.
5. PCR and plasmid DNA purification kits.
6. Cloning: Restriction enzymes, T4 DNA ligase (5000 U/mL), and 10× T4 DNA ligation buffer.
7. Rubidium chloride competent *E. coli* DH5α (for cloning) and S17.1 cells (for conjugation by biparental mating).

2.3 Northern Hybridization

Materials listed below are required to purify RNA, including sRNAs, without the use of columns, although commercial kits are available (*see* **Note 1**).

1. RNA isolation and DNA digestion: lysis solution (1.4% SDS, 4 mM EDTA, 50 μg proteinase K), 5 M sodium chloride (NaCl), ethanol (EtOH), RNase-free DNase I.
2. RNA extraction and precipitation: phenol (pH: 4.5):chloroform:isoamyl alcohol solution (25:24:1, v/v), EtOH and sodium acetate 3 M.
3. Acrylamide gel: 10× TBE (0.89 M Tris, 0.89 M boric acid, 0.02 M EDTA pH: 8.0); 40% acrylamide/bisacrylamide solution, 7 M urea, 10% ammonium persulfate (APS, prepare fresh or store aliquots at −20 °C), and tetramethylethylenediamine (TEMED).

4. Electrophoresis: 2× RNA loading buffer (97.5% formamide, 10 mM EDTA pH 7.5, 0.3% xylene cyanol, 0.3% bromophenol blue); RNA molecular weight marker.
5. Membrane blotting: 3 mm Whatman paper, positively charged nylon membrane, semidry electroblot transfer apparatus.
6. Hybridization: Buffer (0.5 M sodium phosphate buffer pH 7.2, 7% SDS, 10 mM EDTA), hybridization tubes, and oven.
7. Probe labeling: T4 phosphonucleotidekinase (PNK) provided with 10× reaction buffer, 10 mCi/mL ɣ-[^{32}P] ATP, 20-25mer oligonucleotide probes (50 pmol/μL; complementary to the sRNA under study, the aptamer, and to the 5S rRNA), and Sephadex G-25 spin column.
8. Membrane washes: 20× SSC (3 M NaCl, 300 mM trisodium citrate pH: 7.0) and 1% SDS.
9. Detection: phosphorimager cassette, screen, scanner, and image analysis software.

2.4 Affinity Purification of Aptamer-Tagged sRNAs

1. Purification: Buffer A (20 mM Tris–HCl pH: 8.0, 150 mM KCl, 1 mM $MgCl_2$, 1 mM DTT), sonicator, MS2-MBP, amylose resin (NEB), disposable chromatography columns, and maltose.
2. RNA extraction and precipitation (*see* **item 2** in Subheading 2.3).
3. Protein precipitation: acetone.

2.5 RT-PCR Analysis

1. Reverse transcription: random hexamers, SuperScript™ II RT, 5× First-Strand Buffer (Invitrogen), 0.1 M DTT, and RNaseOUT™ (Invitrogen).
2. PCR: specific primers to amplify the sRNAs under study and conventional reagents (*see* **item 3** in Subheading 2.2).

2.6 Protein Electrophoresis and Western Blotting

1. Acrylamide gel: Tris–HCl buffer 1.5/1 M pH: 8.8/6.8 resolving/stacking gel; 40% acrylamide/bisacrylamide solution, 10% APS, 10% SDS, TEMED, and butanol.
2. Electrophoresis: 10× SDS running buffer (250 mM Tris base, 1.92 M glycine and 1% SDS in 1 L); protein loading buffer containing β-mercaptoethanol; protein size marker.
3. Gel staining: silver stain kit.
4. Membrane blotting: 3 mm Whatman paper, polyvinylidene difluoride membrane (P 0.45 PVDF, Amersham), methanol, transfer buffer (25 mM Tris pH: 8.3, 192 mM glycine, and 20% methanol).
5. Immunoassay: TBST20 buffer (20 mM Tris–HCl pH: 8.0, 0.18 M NaCl, 0.1% Tween 20), blocking reagent (Amersham),

monoclonal anti-FLAG antibody (1:5000 in TBST20), and anti-mouse antibody conjugated to horseradish peroxidase (1:100,000).

6. Detection: blotting detection reagent (ECL, Amersham) and imaging system.

3 Methods

The experimental strategy to engineer, analyze, and purify aptamer-tagged sRNAs and in vivo bound proteins is overviewed in Fig. 1a. The *S. meliloti* Hfq-dependent *trans*-sRNA AbcR2 [6, 12], which is widely conserved in α-proteobacteria, has been used to successfully implement and optimize the following protocol. Figures 1 and 2 illustrate the detailed protocol using AbcR2 as a model sRNA.

3.1 Plasmid-Based MS2-Aptamer Tagging of sRNAs

sRNAs can be theoretically tagged with a wide variety of aptamer tags (e.g., MS2 tandem repeats, boxB, elF4A, or streptomycin) at any position between its transcription start site (TSS) and the beginning of the terminator [13]. However, experiments in *E. coli* have revealed that MS2 tagging at the sRNA 5′-end yields the most homogenous transcripts and efficient target regulation [13] (*see* **Note 2**). This section describes the 5′-MS2-aptamer tagging of the full-length AbcR2 sRNA (Fig. 1b) under the control of the P_{Lac} promoter in the engineered plasmid pSRK-C, which has been successfully used for constitutive overexpression of sRNAs in rhizobia.

1. The MS2 aptamer is generated by annealing of complementary DNA oligonucleotides that leave *Bam*HI and *Xba*I compatible overhangs. 1 μL of FMS2 and RMS2 oligonucleotides (Table 1) are mixed in a final volume of 10 μL, heated for 5 min at 90 °C, and let cool down to enable primer annealing.
2. The obtained 48 bp fragment is directly ligated into pSRK-C digested with *Bam*HI and *Xba*I enzymes, generating the pSRK-C derivative harboring the MS2 aptamer sequence.
3. *E. coli* DH5α competent cells are used for transformation with ligation reaction and plated in kanamycin-containing LB plates (*see* **Note 3**).
4. Successful integration of the insert is verified by colony PCR using flanking reverse primer PCR1 (CGGGCCTCTTCG CTATT) and forward PCR2 (TTAGCTCACTCATTAGG).
5. Plasmid DNA from colonies showing the correct size is isolated and sequenced with PCR2.

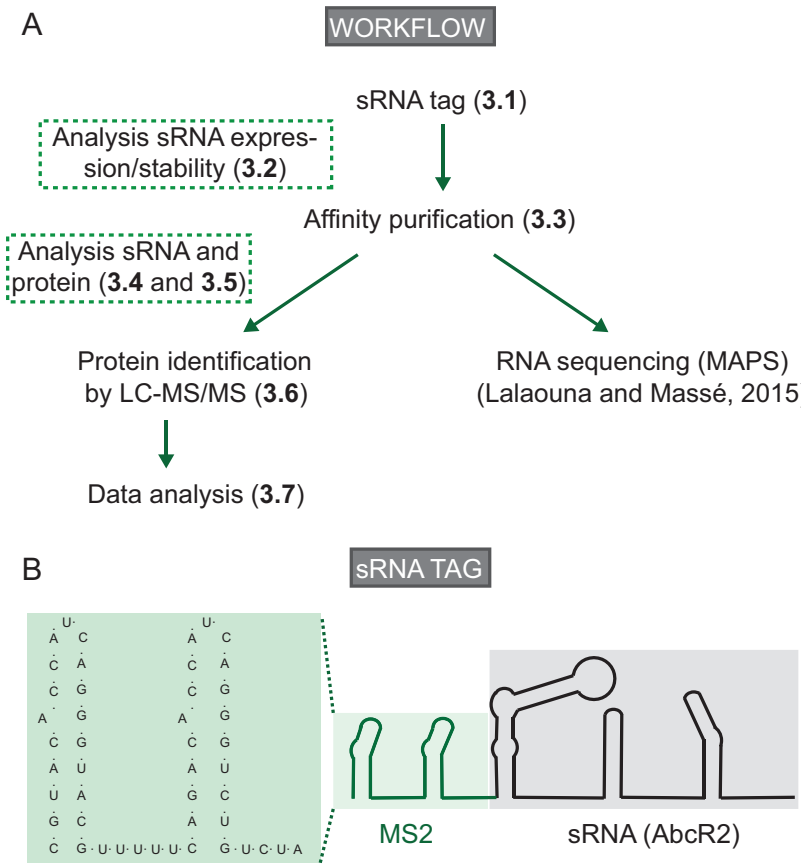

Fig. 1 (**a**) Schematic view of the workflow. (**b**) Schematic representation of the secondary structure of the sRNA AbcR2 tagged with the MS2 epitope. Left panel shows the sequence of the MS2 tandem tag

6. To subsequently generate vectors expressing the aptamer-sRNA fusion (e.g., MS2-AbcR2), primer pairs incorporating *Xba*I and *Hind*III sites to the 5′- and 3′-ends of the sRNA of interest, respectively, are designed.
7. PCR amplification is performed using genomic DNA as template and obtained products are digested with the aforementioned enzymes.
8. In parallel, to construct a control vector with the MS2 aptamer sequence followed by a transcription terminator (e.g., T1), the fragment is generated as in **step 1** by oligonucleotide hybridization using primers T1X/H and T1cX/H listed in Table 1, which leave *Xba*I and *Hind*III compatible overhangs.
9. Purified fragments obtained in **steps 7** and **8** are ligated into *Xba*I-*Hind*III digested pSRK-C containing the MS2 sequence.
10. **Steps 3**–**5** are repeated.
11. Correct plasmids are purified, sequenced, and subsequently transformed into *E. coli* S17.1 cells for biparental mating with the corresponding rhizobial strain (*see* **Note 4**).

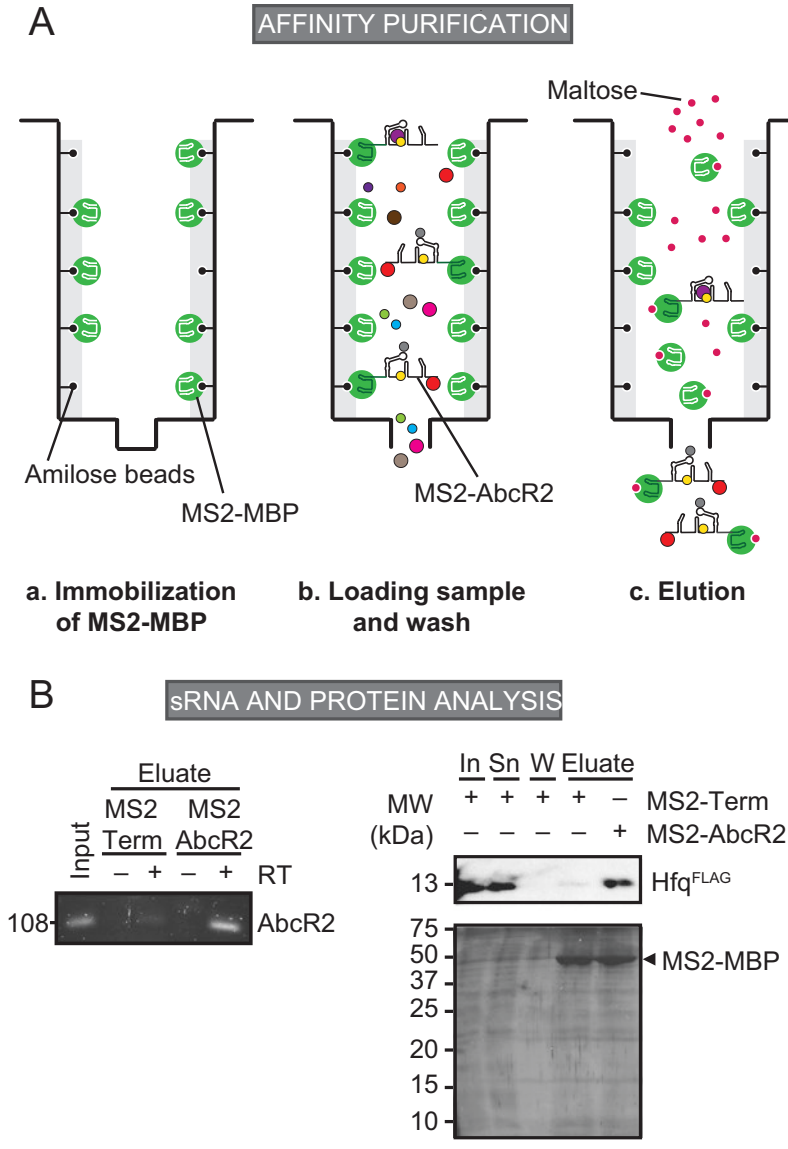

Fig. 2 (**a**) Schematic view of the affinity purification procedure. MS2-MBP protein is immobilized in an amylose column. Cell lysate containing the tagged MS2-AbcR2, previously pre-incubated with MS2-MBP is applied to the amylose column. After several column washes, addition of a maltose enables the elution of both the MS2-AbcR2- and the AbcR2-binding proteins. (**b**) sRNA and protein analysis. Agarose gel (left panel) showing products from RT-PCR reactions for detection of AbcR2 within the eluates from MS2-Term and MS2-AbcR2. Input: total RNA. RT: reverse transcription. Immunoblot and silver-stained polyacrylamide gel (right panel) to monitor specific proteins (Hfq^{FLAG}) and protein pattern across the procedure when using cells expressing MS2-AbcR2 or MS2-Term as control. Input, total lysate; Sn, supernatant after incubation with amylose column; W, wash fraction. MS2-MBP in the silver-stained PAA gel is indicated with an arrow

Table 1
DNA oligonucleotides encoding the MS2 and T1 that leave *Bam*HI/*Xba*I or *Xba*I/*Hind*III compatible overhangs for construction of pSRK-MS2-(Term)

Oligo	Sequence
FMS2	GATCCGTACACCATCAGGGTACGTTTTTCAGACACCATCAGGGTCTGT
RMS2	CTAGACAGACCCTGATGGTGTCTGAAAAACGTACCCTGATGGTGTACG
T1X/H	CTAGATGAAAAAACGACAAAGCAGCACTGATTACAGTGCTGCTTTT TTTATCCCTGTA
T1cX/H	AGCTTACAGGGATAAAAAAAGCAGCACTGTAATCAGTGCTGCTTTG TCGTTTTTTCAT

3.2 Analysis of Aptamer-Tagged sRNAs by Northern Hybridization

Before affinity purification, the impact of tagging on secondary structure, expression, stability, and, if possible, functionality of the sRNA should be assessed (i.e., the Hfq-binding, regulatory potential or susceptibility to RNase degradation). Northern blot hybridization was used to assess the stability and the transcript size of aptamer-tagged sRNAs, which should not be more than 48 nt longer than the wild-type version (size of the tandem MS2 aptamer and the restriction site). As control vector, a construct in which the MS2 aptamer is followed by a transcription terminator (MS2-Term) can be included. An oligonucleotide probe corresponding to the aptamer region is generally used to ensure specificity, but membranes can also be probed with oligonucleotides designed to detect the sRNA under study.

1. Bacterial pellets (*see* details in Subheading 2.1) are split into 1.5 mL microtubes containing cells equivalent to OD_{600} ~3 (e.g., 5 mL culture of OD_{600} 0.6), gently resuspended in 300 μL of lysis solution and incubated for 10 min at 65 °C with regular mixing.
2. Lysates are chilled on ice and 125 μL 5 M NaCl is added to each 1.5 mL microtube.
3. After 10 min on ice, samples are centrifuged (15 min, 16,000 × *g*, 4 °C).
4. The aqueous (upper) phase is transferred to a new 1.5 mL microtube with 1.35 mL of cold 100% EtOH.
5. Tubes are mixed by inversion and stored at −80 °C at least 1 h prior to centrifugation (30 min, 16,000 × *g*, 4 °C).
6. EtOH is completely removed and pellets are resuspended in 42.5 μL of water and pooled together for DNAse I treatment according to the manufacturer's instructions.
7. After incubation, 1× vol of cold phenol:chloroform:isoamyl alcohol is added. Samples are mixed by vortex and the organic

and inorganic phases are separated by centrifugation (15 min, 16,000 × *g*, 4 °C).

8. The aqueous (upper) phase is transferred to a new microtube containing 20 µL 3 M NaAc (pH: 5.2) and 600 µL EtOH and mixed by inversion. **Step 5** is repeated.
9. Supernatant is removed and RNA pellet is washed with 700 µL cold 70% EtOH, avoiding pipetting or vortex.
10. Precipitated RNA is pelleted by centrifugation (30 min, 16,000 × *g*, 4 °C) and supernatant is carefully removed.
11. Samples are air-dried at room temperature with open lids for 10 min.
12. RNA pellets are resuspended in 25 µL of RNase-free water, and RNA concentration is determined by measuring optical density at 260 nm with a Nanodrop device (~1 µg/µL).
13. A 6% polyacrylamide 7 M urea solution is prepared (*see* **Note 5**).
14. Per mL of gel solution, 10 µL of 10% APS and 1 µL of TEMED are added.
15. Gel mixture is immediately poured in between two glass plates, separated by 1-mm spacers, and a comb is inserted (5 mm-depth into the glass plates is sufficient and allows better resolution) avoiding air bubbles (*see* **Note 6**).
16. Electrophoresis device is casted and the required volume of 1× TBE is added.
17. Prior to samples loading, a pre-electrophoresis step is performed (current set at ~30 mA) to warm up the gel.
18. To adjust sample volumes, equal amounts of total RNA (5–15 µg) are dried in a vacuum concentrator and 10–20 µL 1× RNA loading buffer are added.
19. Samples are denatured by 5 min-boiling at 95 °C and cooled on ice.
20. After stopping pre-electrophoresis, gel wells are flushed with 1× TBE with a syringe to remove the urea and unpolymerized acrylamide.
21. Samples are spin down and loaded into the wells (loading volume should not exceed 25 µL). As a size reference, a RNA molecular weight marker (MWM) is loaded.
22. Electrophoresis is set at ~30 mA until bromophenol blue reaches the bottom part of the gel.
23. After the electrophoresis, the lane containing the MWM is excised and stained with ethidium bromide (EtBr) or GelRed.
24. RNA is transferred onto a nylon membrane according to the manufacturer's instructions (*see* **Note 7**) which is subsequently

exposed to UV light at 254 nm for 5 min to enable a covalent link between the RNA and the membrane.

25. The nylon membrane can be then stored or directly placed in a glass hybridization tube, RNA facing inside, with 10–20 mL hybridization buffer and incubated at 42 °C with rotation for 30–120 min in a hybridization oven.
26. Oligonucleotide labeling reaction is set as follows (*see* **Note 8**): 5 μL of RNase-free H_2O, 1 μL of 50 pmol/mL oligonucleotide, 1 μL of 10× reaction buffer, 1 μL of T4-PNK, and 2 μL of γ-[^{32}P]ATP. Labeling reactions are incubated for 1 h at 37 °C.
27. RNAse-free H_2O is added to the reaction (final volume 25 μL) and the mixture is applied to the center of a Sephadex G-25 spin column in which the storage buffer has been already removed by centrifugation.
28. The column is transferred to a new 1.5 mL microtube and centrifuged (2 min, 3500 × *g*) to purify the oligonucleotide probe by eliminating the unincorporated γ-[^{32}P]ATP.
29. Eluted purified RNA probe (25 μL) is heated at 95 °C for 5 min and added into the hybridization bottle (*see* **Note 9**).
30. After overnight incubation at 42 °C, the hybridization solution is discarded and the membrane is washed twice for 5 min with 2× SSC-0.1% SDS solution and twice for 15 min with 1× SSC-0.1% SDS solution. All the washes are performed at the hybridization temperature.
31. Membrane is dried with Whatman paper, wrapped in plastic, and exposed onto a phosphorimager screen overnight.
32. After scanning, the same membrane is stripped by boiling at 95 °C in 0.1% SDS twice for 15 min with shaking. The protocol is repeated from **step 23** using a 5S rRNA probe (TACTCTCCCGCGTCTTAAGACGAA) as loading control.
33. For quantitative comparison of samples, an image analysis software is used.

The functionality of the tagged sRNA, i.e., the ability to regulate its targets or to trigger a specific phenotype upon overexpression, should also be checked in comparison with the untagged sRNA. Detailed methods for in vivo verification of sRNA–mRNA interactions in *S. meliloti* have been already described [14]. If tagging impairs sRNA function, other alternative approaches should be undertaken, e.g., MS2 incorporation into different positions within the sRNA, as detailed previously [13], or the use of biotinylated probes.

3.3 Affinity Purification of Aptamer-Tagged sRNAs

The previously established affinity chromatography protocol for *E. coli* [13, 15] was adapted for *S. meliloti*, assuming that FLAG-tagged Hfq should be detected in the eluates from bacteria harboring MS2-tagged AbcR2 sRNA. The bait protein MS2-MBP was

firstly purified by FPLC over amylose and heparin columns (*see* **Note 10**). A schematic view of the experimental approach to purify cognate proteins bound to MS2-tagged AbcR2 in vivo is represented in Fig. 2a. Briefly, *S. meliloti* expressing MS2-AbcR2 were harvested and disrupted. The whole-cell cleared lysate was then incubated with the MS2-MBP protein that binds the MS2-AbcR2 and applied to an amylose column, which interacts non-covalently with the MBP moiety. After removing unspecific-bound by several column washes, addition of a maltose-buffer disrupts the interactions between the MBP and the amylose from the column, facilitating the elution of both the MS2-AbcR2 and the AbcR2-binding proteins. The detailed protocol is described below. All the steps were performed at 4 °C or on ice.

1. Cells equivalent to 240 OD_{600} (e.g., 100 mL of a culture with OD_{600} ~2.4) were harvested as described in Subheading 2.1 and stored at −80 °C.
2. Bacterial cells were thawed on ice, resuspended in 8 mL buffer A (*see* **Note 11**), and split into 2 mL RNAse-free tubes (6–10 tubes are used to facilitate sonication). An analytical sample is collected at this point for subsequent monitoring of sRNA and protein within this fraction by RT-PCR and Western blot, respectively (*see* Subheadings 3.4 and 3.5).
3. Cells are broken using a sonicator with a microprobe by three rounds of 10 s bursts at 32 W. Lysates are chilled on ice between sonication rounds (*see* **Note 12**).
4. Cell lysates are cleared by centrifugation (15 min, 16,000 × *g*, 4 °C) to remove cell debris.
5. During centrifugation, affinity column is prepared. Columns are washed three times with 800 μL buffer A prior to resin application. On the other hand, amylose beads are briefly centrifuged to remove the storage solution from the affinity medium in a 1.5 mL microtube (100 μL per sample), washed with 800 μL buffer A and resuspended again in 800 μL buffer A to be loaded into the column together with 200 pmol of MS2-MBP (*see* **Note 13**).
6. After centrifugation of lysates, soluble cell fractions are transferred to a new microtube. An analytical sample (input-In) is kept as reference for further analysis. MS2-MBP (200 pmol) is added and the mixture is incubated for 5 min with soft soaking (*see* **Note 14**).
7. Cell lysates containing half of the bait protein mixture were then applied into the amylose column to interact with the MBP moiety (*see* **Note 15**).
8. An aliquot of the flow-through fraction (supernatant–Sn) is collected for reference. Buffer A (600 μL) is added three times to remove unspecific-bound to the column.

9. An aliquot from the wash fraction (wash-W) is collected prior to loading 600 μL of buffer A containing 12 mM maltose to enable the elution of MS2-AbcR2- and AbcR2-binding proteins (*see* **Note 16**).
10. An aliquot of the eluate fraction (eluate-E) is stored separately. For protein and RNA dissociation, 1× vol. phenol:chloroform:isoamylalcohol [25:24:1 (v/v)] is added and mixed by vortex for 20 s.
11. The mixture is centrifuged (30 min, 16,000 × *g*, 4 °C) enabling separation of water and organic phases.
12. RNA precipitation: the aqueous upper phase is transferred to a new microtube. 3× vol. EtOH and 20 μL 3 M NaAc pH: 5.2 are added.
13. Protein precipitation: 3× vol. acetone are added to the organic lower phase.
14. Samples from **steps 12** and **13** are mixed by inversion and stored overnight at −20 °C.
15. RNA and proteins are precipitated by centrifugation (30 min, 16,000 × *g*, 4 °C).
16. RNA and protein pellets are washed with 500 μL of cold 70% EtOH and 500 μL of acetone, respectively.
17. Samples from **step 16** are mixed by tube inversion and centrifuged (10 min, 16,000 × *g*, 4 °C).
18. EtOH or acetone is carefully removed in two steps and pellets are air-dried at room temperature.
19. RNA is resuspended in 10 μL of RNase-free water and stored at −80 °C (*see* **Note 17**), whereas the protein pellet is dissolved in 50 μL 1× protein loading buffer and stored at −20 °C.

3.4 RT-PCR Analysis of (AbcR2) sRNA Co-purification

To address whether the MS2 tag allows enrichment of MS2-sRNA during the purification process compared to control, fractions eluted upon affinity chromatography can be analyzed by RNA reverse transcription followed by PCR to monitor the presence of the sRNA of interest. Figure 2b shows the RT-PCR product for AbcR2 obtained when using the control (MS2-Term) and MS2-AbcR2 eluates, confirming that aptamer-tagged AbcR2 is successfully recovered from the affinity chromatography assay. This assay is performed as follows:

1. After treatment with DNAse I (*see* **Note 16**), eluted RNA samples are diluted 1:10 and 1 μL (2.4 OD_{600} equivalents) is PCR amplified with sRNA specific primers and conventional reagents to rule out the presence of genomic DNA.
2. The remaining 9 μL are subjected to first-strand cDNA synthesis using SuperScript™ II reverse transcriptase. The reaction is set mixing the RNA with 1 μL random hexamers

(100 ng/μL), 1 μL dNTP mix (10 mM each), and water (up to 12.5 μL).

3. The mixture is heated to 65 °C for 5 min and quickly chilled on ice.
4. The following reagents are subsequently added: 4 μL 5× first-strand buffer, 2 μL 0.1 M DTT, and 1 μL RNaseOUT™.
5. The contents are gently mixed and incubated at 42 °C for 2 min (*see* **Note 18**).
6. Finally, 0.5 μL (100 units) of RT are added to half of the reactions, mixed by pipetting, and incubated at 42 °C for 50 min.
7. The reaction is inactivated by heating at 70 °C for 15 min.
8. The first-strand reaction (1 μL) is now used as template for PCR with the same sRNA-amplifying primers and conventional reagents.
9. PCR products are electrophoresed in 2–2.5% agarose gel in TBE to allow for visualization, and PCR fragments of correct size are sequenced.

The remaining RNA sample from the soluble fraction can be subjected to RNA-seq (*see* **Note 17**) to decipher the sRNA "targetome," i.e., the array of mRNAs that are targeted by a sRNA under specific conditions. This approach, known as MAPS (MS2-affinity purification with RNA sequencing) has been already described elsewhere [16].

3.5 Western Blotting Analysis of Co-purified (Hfq) Proteins

To further evaluate the output of the affinity chromatography, protein aliquots collected across the experimental procedure are resolved by SDS-PAGE and visualized by silver staining and/or Western blot as described below (Fig. 2c). Gel profiling allows direct comparison between eluate fractions. Distinct protein patterns across control and experimental samples can be observed anticipating different binding proteomes that must be subsequently analyzed by LC-MS/MS. Of note, MS2-MBP is clearly enriched in all eluate fractions indicating that the affinity purification is working with the same efficiency for control and tagged-sRNA samples.

AbcR2 binds tightly to Hfq both in vivo and in vitro and this strong association has allowed enrichment of AbcR2 by co-immunoprecipitation with Hfq from *S. meliloti* lysates [6]. Given that the affinity chromatography approach recovered stable MS2-AbcR2 (Fig. 2b), Hfq is expected to be present within the MS2-AbcR2 eluate. Figure 2c shows enrichment of Hfq in the MS2-AbcR2 when compared to MS2-term, confirming a successful affinity purification procedure. As it has been already reported in *E. coli* [13], Hfq can weakly associate with MS2 alone, but the significant higher recovery with MS2-AbcR2 indicates that Hfq was purified specifically through the interaction with AbcR2.

1. Aliquots of collected proteins to be resolved by SDS-PAGE are thawed.
2. Two 15% polyacrylamide gels are prepared.
3. Immediately after addition of 10 μL of 10% APS, 10 μL of 10% SDS, and 0.4 μL of TEMED per mL of gel solution, the mixture is poured in between the glass plates up to ~2 cm of the top.
4. Separation gels are overlaid with butanol.
5. After polymerization of the resolving gel, the butanol is removed with filter or Whatman paper.
6. A 5% polyacrylamide stacking gel is prepared with the same proportion of APS, SDS, and TEMED per mL as for the separating gel (*see* **step 3**), mixed to homogeneity, and poured on top of the resolving gel.
7. Gel combs are immediately placed over the gel avoiding bubbles formation.
8. Two sets of protein samples are prepared: one for subsequent gel staining with silver containing aliquots equivalent to 0.05 OD of the lysate, flow-through and wash fractions and other set to 0.2 OD for Western blot. Half of the elution fractions can be loaded into each gel (120 OD).
9. Samples are resuspended in 2× protein loading buffer and, together with half of the elution fractions (120 OD), denatured by heating at 95 °C for 5 min.
10. Electrophoresis device is casted and the necessary 1× SDS-running buffer is added.
11. After gel polymerization, combs are removed and wells are carefully flushed with running buffer prior to sample and protein marker loading.
12. Electrophoresis is set at ~30 mA until desired protein separation, monitored by the pre-stained markers (1.30–2 h).
13. One gel is subsequently stained with silver for protein visualization.
14. For immunoblot analysis, prior to protein transfer, the PVDF membrane must be shortly activated in methanol, washed with ddH_2O, and finally immersed in 1× transfer buffer, together with the gel and the Whatman paper (*see* **Note 7**).
15. Proteins are blotted onto the PVDF typically for 50 min at 0.8 mA/cm^2. At this point, the membrane can be stored at 4 °C (*see* **Note 19**) or directly subjected to immunoassay at room temperature with shaking.
16. Membrane is incubated 1 h in TBST20 with 1.5% blocking reagent.

17. After a short rinse with TBST20, membrane is incubated for 1 h with a monoclonal anti-FLAG antibody (1:5000 in TBST20) to detect FLAG-tagged Hfq.
18. Membrane is then rinsed and washed in TBST20 six times for 5 min each.
19. After removal of the primary antibody, membrane is incubated with an anti-mouse antibody conjugated to horseradish peroxidase (1:100,000 in TBST20).
20. Membrane is then rinsed and washed again in TBST20 six times for 5 min each.
21. Proteins are detected by incubation with blotting detection reagent (5 min, room temperature in the dark) and visualized with an image documentation system.

3.6 Protein Identification by Mass Spectrometry

The protein complexity of the eluted fractions can be examined by mass spectrometry with the aim of identifying novel protein partners for specific sRNAs. This analysis will help to gain insights into the unknown cellular functions of the sRNA of interest. The combination of this technique together with the RNA-seq can shed some light on the role of sRNAs in *S. meliloti*. The protocol detailed below can be adapted depending on the proteomics facility available (e.g., mass spectrometer can vary and therefore the settings described below).

1. Protein samples equivalent to 120 OD_{600} were run 10 min in a 4% SDS-PAGE.
2. Each gel lane corresponding to the different samples was cut into 10 slices.
3. Gel slices are subjected to in-gel tryptic manual digestion.
4. The resulting peptides are fractionated using an Easy n-LC II chromatography system (Proxeon) in line with an Amazon Speed ETD mass spectrometer (Bruker Daltonics).
5. CaptiveSpray Ion Source (Bruker Daltonics) set at 1300 V is used to ionize the peptides.
6. Amazon Speed ETD Ion Trap mass spectrometer controlled by TrapControl software v7.2 (Bruker-Daltonics) and operated in AutoMS(2) acquisition mode is used to acquire tandem mass spectra.
7. Ion Trap set to analyze the survey scans in the mass range m/z 400–1400 in Enhanced Resolution MS mode and the top ten multiply charged ions in each duty cycle selected for MS/MS in UltraScan MS/MS mode.
8. DataAnalysis software v4.3 (Bruker-Daltonics) and search against the UniProtTrembl database using Mascot 2.4 (Matrix Science) integrated together with ProteinScape v4.0 (Bruker-Daltonics) was used to process the raw data files.

9. Peptide precursor mass tolerance was set at 0.5 Da, and MS/MS tolerance was set at 0.5 Da. Search criteria included carbamidomethylation of cysteine (+57.02 Da) as a fixed modification and oxidation of methionine (+15.99 Da) as a variable modification.
10. A maximum of 2 missed cleavages for tryptic digestion was set to perform the search.
11. The reverse database search option was enabled and all peptide data was filtered to satisfy false discovery rate (FDR) of ≤2%.

Detected proteins associated in vivo with the sRNAs identified using the above-described protocol may be unspecific or form part of complexes. Therefore, a set of protein candidates should be validated, e.g., by immunoprecipitation followed by sRNA co-purification detection as described before [6]. Furthermore, functionality of the sRNA–protein complex should be tested, ideally comparing wild-type and derivative strains carrying a deletion in the protein coding gene. Anyway, a suitable assay depending on the expected protein function should be designed (Northern blot to check sRNA stability in the absence of the protein, sRNA degradation assays, or double plasmid assay to assess protein involvement in target regulation).

3.7 Data Analysis

Once obtained the list of proteins identified by mass spectrometry, it is essential to demarcate the range of nonspecific binding proteins. The nonspecific proteins identified by affinity purification experiments can vary considerably and depend on the experimental conditions and also the construction used to assess the contamination, i.e., different proteins will associate with the MS2 moiety itself or with the type of matrix used for the purification. Thus, all proteins associated with negative controls, either containing only the MS2 aptamer or the sRNAs should be discarded from the analysis. To ensure that proteins cataloged as putative contaminants are indeed indirect partners, it is important to analyze the correlation between samples, calculating the Pearson correlation coefficient. When different proteins are identified across samples and corresponding controls, it is expected to have a bad linear correlation between samples ($r < 0.4$). However, when samples present a similar protein pattern or when biological replicates are performed, a higher correlation is expected ($r > 0.75$). This criterion can be used to remove potential contaminants although is commonly accepted to remove only those proteins not significantly enriched in the samples when compared to the corresponding control after a label-free quantification analysis. The major limitation of this quantification is the number of biological and technical replicates required. Therefore, when limited samples are available for the analysis and quantification cannot be performed, proteins identified in control samples should be excluded to avoid false positives.

Stringent thresholds can then be applied to redefine the protein list. One commonly applied threshold is the exclusion of those proteins identified by less than two peptides. However, the length of the protein must be considered when applying this criterion. Shorter proteins, such as Hfq, will produce fewer unique peptides, and thus might be artificially excluded using the two peptide cutoff.

Gene Ontology (GO) enrichment analysis is performed to explore protein properties. Several online tools are available for this purpose; some examples are DAVID [17] or comparative GO [18]. The main difference between these two tools is the statistical method applied to calculate the enrichment level. DAVID calculates the p-value of the enrichment by the Fischer exact test and allows for p-value correction. Conversely, comparative GO calculates the p-value by a hypergeometric test, resulting in a more relaxed analysis. Regardless the online tool chosen for the enrichment analysis, it is essential that the number of genes annotated for the corresponding strain and species, in this case *S. meliloti* 1021 would be complete, otherwise the results obtained will be biased. In other words, the selected tool must have the majority of the proteins, if not all, annotated for the organism of interest to be used as the reference proteome. Moreover, DAVID allows for exploring other protein properties such as protein domains or cellular pathways where these proteins are involved.

4 Notes

1. The majority of the commercial kits for RNA isolation are based on columns that do not retain RNA molecules smaller than ~200 nt and, therefore, to ensure that isolation of the small RNA fraction, RNA purification should be performed with specifically designed kits like the miRNeasy Mini Kit from QIAGEN [19].
2. The existence of alternative 5′ processed ends in sRNAs (e.g., EcpR1 sRNA) [19], which can be anticipated in RNAseq data and confirmed by Northern blot analysis, should be taken into account to implement this strategy. Cloning of the functional stable sRNA version starting at the processed end could be the best strategy to handle with these molecules, avoiding loss of the aptamer sequence by ribonucleolytic activity on the full-length version.
3. Blue/white selection of transformants with IPTG and X-gal is not possible in pSRK-C derivatives.
4. Preferably, a markerless deletion mutant of the sRNA under study should be used as recipient strain.

5. The mix should be heated to completely dissolve urea, filtered, and stored at 4 °C if not used immediately.
6. Polymerization takes approximately 1 h, but at this point the cast gel can be stored overnight at 4 °C.
7. For electrophoretic transfer during Northern and Western blotting, membrane and Whatman papers are cut slightly larger than the size of the acrylamide gel. A dry Whatman paper is placed onto the gel to facilitate gel removal and prevent introduction of air bubbles. The gel and the membrane are then surrounded by 3 Whatman papers, previously soaked in the corresponding transfer buffer and placed on the electroblot transfer device following the right order, considering that RNA and proteins migrate to the positively charged pole.
8. If detection using radioactivity is not possible, digoxigenin-labeled RNA (riboprobes) or DNA probes can also be used. Even though that detection may not be as sensitive as when using radioactive-labeled oligonucleotides, the fact that riboprobes are homolog to the (full) sRNA coding sequence and internally labeled, make these methods reliable for sRNA detection by Northern hybridization. Membranes hybridized with either nonradioactive labeled riboprobes (synthesized with the Maxiscript kit from Ambion) or DNA probes obtained by PCR can be subjected to chemiluminescence detection (DIG Luminescent Detection Kit, Roche) as described before [19, 20].
9. To avoid signal background, do not apply the probe directly on the membrane but on the hybridization buffer.
10. Recombinant MS2-MBP is composed by the MS2 coat protein N-terminally fused to maltose-binding protein. The fusion protein purification is described in [21]. Protein purity is checked by SDS-PAGE and Commassie blue gel staining and concentration is determined with Bradford dye-binding method (Bio-Rad).
11. Establishment and maintenance of RNA–protein complexes considerably depends on salt concentration and Hfq has been reported to co-purify better with InvR-MS2 RNA increasing concentration of KCl in Buffer A [13, 22]. We also compared MS2-AbcR2 eluates obtained with different KCl concentrations (150 mM, 500 mM and 1 M). However, we did not observe better binding of FLAG-tagged Hfq or other proteins with KCl concentrations higher than 150 mM, but an excess of salt precipitates in the eluates that hampered subsequent steps.
12. Alternatively, rhizobial cells can also be disrupted in a French press.
13. Considering the starting amount of cells and the conditions used here, a total of 400–500 pmol was the optimal amount of

MS2-MBP to recover FLAG-tagged Hfq with MS2-AbcR2 and, therefore, is recommended in this protocol. Lower amounts (100–200 pmol) of MS2-MBP did not recover Hfq so efficiently and increasing MS2-MBP concentration (1000 pmol) did result in more purified Hfq yield, but seemed to lead to unspecific protein binding.

14. Brief pre-incubation of MS2-MBP protein with the lysate prior to loading into the column had a positive effect on Hfq recovery with MS2-AbcR2, disregarding the incubation time. Considering that some MS2-MBP may not bind efficiently to the tagged-sRNA during incubation with the lysate or once into the column, the effect of additive application in two steps (200 pmol MS2-MBP each) was also tested. This experimental set-up showed the best Hfq recovery rates when MS2-AbcR2 was used as a bait and thus it is recommended.
15. If the column flow rate is high, the lysate can be applied twice to the resin, increasing the chance for complex binding.
16. DNAse I digestion of total or part of the eluates can be performed at this step to enable subsequent RT-PCR analysis of this fractions without further phenol-chloroform treatment.
17. If the samples are going to be either stored for long periods or shipped, RNA sample pellets can be stored dry.
18. A conventional PCR thermocycler can be set to follow the RT steps to avoid using multiple incubators.
19. If the PVDF membrane gets dry during storage, it must be shortly activated in methanol again prior to proceeding with immunoassay.

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