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A modified protocol for MS2-affinity purification coupled with RNA sequencing in Gram-positive bacteria

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Dr. Jaydev Upponi
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David Lalaouna
CRCN CNRS

Dear Dr. Jaydev Upponi,

We respectfully submit a revised version of our manuscript “A modified protocol for MS2-affinity purification coupled with RNA sequencing in Gram-positive bacteria” for which the reviews were sent back to us on July 13rd, 2020.

We have addressed every comment raised by you and the reviewers. We believe the revised manuscript has improved tremendously from these comments. We hope the revised version of our manuscript is responding to the standards of JoVE.

If you have any questions, please do not hesitate to contact me directly.

Yours sincerely,

David Lalaouna

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TITLE

MS2-Affinity Purification Coupled with RNA Sequencing in Gram-Positive Bacteria

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KEYWORDS

MAPS; Regulatory RNA; RNA sequencing; Targetome; Regulatory network; Co-purification; MS2 aptamer; Gram-positive bacteria; *Staphylococcus aureus*

SUMMARY

MAPS technology has been developed to scrutinize the targetome of a specific regulatory RNA in vivo. The sRNA of interest is tagged with a MS2 aptamer enabling the co-purification of its RNA partners and their identification by RNA sequencing. This modified protocol is particularly suited for Gram-positive bacteria.

ABSTRACT

Although small regulatory RNAs (sRNAs) are widespread among the bacterial domain of life, the functions of many of them remain poorly characterized notably due to the difficulty of identifying their mRNA targets. Here, we described a modified protocol of the MS2-Affinity Purification coupled with RNA Sequencing (MAPS) technology, aiming to reveal all RNA partners of a specific sRNA in vivo. Broadly, the MS2 aptamer is fused to the 5' extremity of the sRNA of interest. This construct is then expressed in vivo, allowing the MS2-sRNA to interact with its cellular partners. After bacterial harvesting, cells are mechanically lysed. The crude extract is loaded into an amylose-based chromatography column previously coated with the MS2 protein fused to the maltose binding protein. This enables the specific capture of MS2-sRNA and interacting RNAs. After elution, co-purified RNAs are identified by high-throughput RNA sequencing and subsequent bioinformatic analysis. The following protocol has been implemented in the Gram-positive human pathogen *Staphylococcus aureus* and is, in principle, transposable to any Gram-positive bacteria. To sum up, MAPS technology constitutes an efficient

method to deeply explore the regulatory network of a particular sRNA, offering a snapshot of its whole targetome. However, it is important to keep in mind that putative targets identified by MAPS still need to be validated by complementary experimental approaches.

INTRODUCTION

Hundreds, perhaps even thousands of small regulatory RNAs (sRNAs) have been identified in most bacterial genomes, but the functions of the vast majority of them remain uncharacterized. Overall, sRNAs are short non-coding molecules, playing major roles in bacterial physiology and adaptation to fluctuating environments[1-3]. Indeed, these macromolecules are at the center of numerous intricate regulatory networks, impacting metabolic pathways, stress responses but also virulence and antibiotic resistance. Logically, their synthesis is triggered by specific environment stimuli (e.g., nutrient starvation, oxidative or membrane stresses). Most sRNAs regulate multiple target mRNAs at the post-transcriptional level through short and non-contiguous base pairing. They usually prevent translation initiation by competing with ribosomes for translation initiation regions[4]. The formation of sRNA:mRNA duplexes also often results in the active degradation of the target mRNA by recruitment of specific RNases.

The characterization of an sRNA targetome (i.e., the whole set of its target RNAs) allows the identification of the metabolic pathways in which it intervenes and the potential signal it answers to. Consequently, the functions of a specific sRNA can generally be inferred from its targetome. For this purpose, several in silico prediction tools have been developed such as IntaRNA and CopraRNA[5-7]. They notably rely on sequence complementarity, pairing energy and accessibility of the potential interaction site to determine putative sRNA partners. However, prediction algorithms do not integrate all factors influencing base-pairing in vivo such as the involvement of RNA chaperones[8] favoring sub-optimal interactions or the co-expression of both partners. Due to their inherent limitations, the false positive rate of prediction tools remains high. Most experimental large-scale approaches are based on the co-purification of sRNA:mRNA couples interacting with a tagged RNA binding protein (RBP)[6,9]. For example, the RNA Interaction by Ligation and sequencing (RIL-seq) method identified RNA duplexes co-purified with RNA chaperones such as Hfq and ProQ in *Escherichia coli*[10,11]. A similar technology called UV-Crosslinking, Ligation And Sequencing of Hybrids (CLASH) was applied to RNase E- and Hfq-associated sRNAs in *E. coli*[12,13]. Despite the well-described roles of Hfq and ProQ in sRNA-mediated regulation in multiple bacteria[8,14,15], sRNA-based regulation seems to be RNA chaperone-independent in several organisms like *S. aureus*[16-18]. Even if the purification of RNA duplexes in association with RNases is feasible as demonstrated by Waters and coworkers[13], this remains tricky as RNases trigger their rapid degradation. Hence, the MS2-Affinity Purification coupled with RNA Sequencing (MAPS) approach[19,20] constitutes a solid alternative in such organisms.

Unlike above-mentioned methods, MAPS uses a specific sRNA as bait to capture all interacting RNAs and hence does not rely on the involvement of an RBP. The entire process is depicted in **Figure 1**. In brief, the sRNA is tagged at the 5' with the MS2 RNA aptamer that is specifically recognized by the MS2 coat protein. This protein is fused with the maltose binding protein (MBP) to be immobilized on an amylose resin. Therefore, MS2-sRNA and its RNA partners are retained

on the affinity chromatography column. After elution with maltose, co-purified RNAs are identified using high-throughput RNA sequencing followed by bioinformatic analysis (**Figure 2**). The MAPS technology ultimately draws an interacting map of all potential interactions occurring in vivo.

MAPS technology was originally implemented in the non-pathogenic Gram-negative bacterium *E. coli*[21]. Remarkably, MAPS helped identify a tRNA-derived fragment specifically interacting with both RyhB and RybB sRNAs and preventing any sRNA transcriptional noise to regulate mRNA targets in non-inducing conditions. Thereafter, MAPS has been successfully applied to other *E. coli* sRNAs like DsrA[22], RprA[23], CyaR[23] and GcvB[24] (**Table 1**). In addition to confirming previously known targets, MAPS extended the targetome of these well-known sRNAs. Recently, MAPS has been performed in *Salmonella* Typhimurium and revealed that SraL sRNA binds to *rho* mRNA, coding for a transcription termination factor[25]. Through this pairing, SraL protects *rho* mRNA from the premature transcription termination triggered by Rho itself. Interestingly, this technology is not restricted to sRNAs and can be applied to any type of cellular RNAs as exemplified by the use of a tRNA-derived fragment[26] and a 5'-untranslated region of mRNA[22] (**Table 1**).

MAPS method has been also adapted to the pathogenic Gram-positive bacterium *S. aureus*[19]. Specifically, the lysis protocol has been widely modified to efficiently break cells due to a thicker cell wall than Gram-negative bacteria and to maintain RNA integrity. This adapted protocol already unravelled the interactome of RsaA[27], RsaI[28] and RsaC[29]. This approach gave insights into the crucial role of these sRNAs in regulatory mechanisms of cell surface properties, glucose uptake, and oxidative stress responses.

The protocol developed and implemented in *E. coli* in 2015 has been recently described in great detail[30]. Here, we provide the modified MAPS protocol, which is particularly suitable for studying sRNA regulatory networks in Gram-positive (thicker cell wall) bacteria whether non-pathogenic or pathogenic (safety precautions).

PROTOCOL

1. Buffers and media

1.1 For MAPS experiments, prepare the following buffers and media:

- Buffer A (150 mM KCl, 20 mM Tris-HCl pH 8, 1 mM $MgCl_2$ and 1 mM DTT)
- Buffer E (250 mM KCl, 20 mM Tris-HCl pH 8, 12 mM maltose, 0.1% triton, 1 mM $MgCl_2$ and 1 mM DTT)
- RNA loading buffer (0.025*%* xylene cyanol and 0.025*%* bromophenol blue in 8 M urea)
- Brain Heart Infusion (BHI) medium (12.5 g of calf brain, 10 g of peptone, 5 g of beef heart, 5 g of NaCl, 2.5 g of Na_2HPO_4 and 2 g of glucose for 1 L)
- Lysogeny Broth (LB) medium (10 g of peptone, 5 g of yeast extract and 10 g of NaCl for 1 L)

1.2 For Northern blot assays, prepare the following buffers:

- Blocking solution (1x maleic acid and 10% blocking reagent)
- Hybridization solution (50% formamide, 5X SSC, 7% SDS, 1% blocking solution and 0.2% N-lauryl sarcosine, 50 mM sodium phosphate). Heat with agitation to dissolve.
CAUTION: Carefully follow the safety precautions related to each product.
- 1 M sodium phosphate (58 mM sodium phosphate dibasic and 42 mM sodium phosphate monobasic)
- Saline, sodium citrate (SSC) buffer, 20x concentrate (3 M NaCl and 300 mM trisodium citrate)

2. Safety issues

2.1 Carry out all steps involving viable pathogenic bacteria in a level 2 containment lab.

NOTE: Only cell extracts can be taken outside after lysis (step 5).

2.2 Put on a lab coat and gloves.

2.3 Ensure that the wrists are covered.

2.4 Clean the biological safety cabinet (Class II) with a disinfectant solution.

2.5 Dispose solid wastes exposed to bacteria in the appropriate biomedical bin.

2.6 Treat flasks containing contaminated liquids with a disinfectant solution. Then, discard it in a sink.

2.7 Carefully wash hands and wrists with soap and remove the lab coat before leaving the level 2 containment lab.

3. Plasmid construction

NOTE: For cloning purposes, it is crucial to first identify the boundaries of the endogenous sRNA. The pCN51-P3 and pCN51-P3-MS2 plasmids are described in Tomasini et al. (2017)[27]. The P3 promoter allows high expression of the sRNA in a cell-density-dependent manner (i.e., when bacteria enter the stationary phase of growth). Many staphylococcal sRNAs accumulate during this growth phase.

3.1 Amplify the sRNA sequence by PCR using a high-fidelity DNA polymerase and a PCR machine. Carefully follow manufacturer's instructions and read Garibyan and Avashia (2013)[31] for more details.

3.2 Use the following templates to design the specific primers:
5'-CGCCTGCAG**CGTACACCATCAGGGTACGTTTTTCAGACACCATCAGGGTCTGTTT**(N)-3' and 5'-CGCGGATCC(N)-3' for forward and reverse primers, respectively.

NOTE: These oligonucleotides enable to fuse the MS2 sequence (in bold) to the 5' end of the sRNA of interest. *Pst*I and *Bam*HI restriction sites (underlined) are added at the 5' and 3' extremities of the MS2-sRNA construct to clone the amplicon into the pCN51-P3 plasmid[27]. (N) corresponds to the gene-specific sequences (15-20 nucleotides).

3.3 Digest 1 µg of pCN51-P3 plasmid and 1 µg of the MS2-sRNA PCR product with 2 U of *Pst*I and 1 U of *Bam*HI in the appropriate buffer according to manufacturer's recommendations.

3.4 Incubate 1 h at 37 °C and purify DNA using a PCR purification kit (see **Table of Materials**).

3.5 Mix the digested pCN51-P3 plasmid (300 ng) and MS2-sRNA amplicon (molar ratio for vector:insert = 1:3) in a 1.5 mL tube. See Revie et al. (1988)[32] to maximize ligation efficiency. Add 1 µL of the Ligase Buffer and 10 U of T4 Ligase in each tube. Adjust the volume to 10 µL with ultrapure water.

3.6 Incubate at 37 °C for at least 2 h.

3.7 Add 5 µL of ligation mixture to 50 µL of frozen DH5α chemically competent *E. coli* cells. Read Seidman et al. (2001)[33] to learn more about plasmid transformation and chemically competent cells.

3.8 Incubate 30 min on ice.

3.9 Heat shock (45 s at 42 °C) the transformation tube using a heat block or water bath.

3.10 Add 900 µL of LB medium and incubate at 37 °C for 30 min.

3.11 Plate 100 µL of the bacterial suspension on a LB agar plate supplemented with ampicillin (100 µg/µL).

NOTE: the pCN51-P3 vector encodes an ampicillin resistance gene, which enables to select only *E. coli* clones carrying the pCN51-P3-MS2-sRNA plasmid.

3.12 Extract the pCN51-P3-MS2-sRNA plasmid from an overnight bacterial culture (5 mL) grown in the presence of ampicillin (100 µg/µL) using a plasmid DNA miniprep kit (see table of materials).

3.13 Verify the construct by Sanger sequencing[34] using the following primer, 5'-TCTCGAAAATAATAGAGGG-3'.

3.14 Transform the pCN51-P3-MS2-sRNA plasmid into DC10B chemically competent *E. coli* cells. Repeat steps 3.7 to 3.11.

3.15 Extract the pCN51-P3-MS2-sRNA plasmid (see step 3.12) and transform 1-5 µg of plasmid DNA into HG001 electrocompetent *S. aureus* cells using an electroporation apparatus. Carefully follow manufacturer's instructions. Read Grosser and Richardson (2016)[35] to learn more about methods for preparing electrocompetent *S. aureus*.

CAUTION: This step involves handling of pathogenic bacteria (see step 2).

3.16 Add 900 µL of BHI medium and incubate at 37 °C for 3h.

3.17 Centrifuge 1 min at 16,000 x *g*. Discard the supernatant.

3.18 Resuspend the pellet in 100 µL of BHI and plate the bacterial suspension on BHI agar plates supplemented with erythromycin (10 µg/µL).

NOTE: The pCN51-P3 vector also encodes an erythromycin resistance gene, which enables to select only *S. aureus* clones carrying the pCN51-P3-MS2-sRNA plasmid.

4. Bacteria harvesting

CAUTION: This step involves handling of pathogenic bacteria (see step 2).

4.1 Grow one colony of strains carrying either pCN51-P3-MS2-sRNA or pCN51-P3-MS2[27] plasmids in 3 mL of BHI medium supplemented with erythromycin (10 µg/µL) in duplicates.

4.2 Dilute each overnight culture in 50 mL (≈1/100) of fresh BHI medium supplemented with erythromycin (10 µg/µL) to reach an OD_{600nm} of 0.05. Use 250 mL sterilized flasks (5:1 flask-to-medium ratio).

NOTE: Medium and growth conditions should be set according to the expression pattern of the studied sRNA.

4.3 Grow cultures at 37 °C with shaking at 180 rpm for 6 h.

4.4 Transfer each culture into a 50 mL centrifuge tube.

4.5 Centrifuge at 2,900 x *g* during 15 min at 4 °C. Discard the supernatant.

4.6 Keep pellets on ice and directly perform mechanical cell lysis (step 5) or freeze and store pellets at -80 °C.

5. Mechanical cell lysis

CAUTION: Following steps must be performed on ice and buffers must be at 4 °C in a L2 laboratory. Use gloves and take all precautions to protect samples from RNases.

5.1 Resuspend pellets (step 4.6) in 5 mL of Buffer A.

5.2 Transfer the resuspended cells in 15 mL centrifuge tubes with 3.5 mg of silica beads (0.1 mm).

5.3 Insert tubes in a mechanical cell lysis instrument (see **Table of Materials**). Run a cycle of 40 s at 4.0 m/s.

NOTE: If one cycle is not enough to break cells, let the device cool for 5 min while keeping samples on ice. Then, repeat another cycle of 40 s at 4.0 m/s. The efficiency of cell lysis can be tested by plating the supernatant on BHI-agar plate.

5.4 Centrifuge at 15,700 x *g* for 15 min. Recover the supernatant and keep it on ice.

6. Column preparation

CAUTION: Be careful not to allow the amylose resin to dry. If needed, seal the column with an end-cap. Prepare all the solutions before starting the affinity purification.

6.1 Put a chromatography column in a column rack (see **Table of Materials**).

6.2 Remove the column tip and wash the column with ultrapure water.

6.3. Add 300 µL of amylose resin.

6.4 Wash the column with 10 mL of Buffer A.

6.5 Dilute 1,200 pmol of MBP-MS2 protein in 6 mL of Buffer A and load it into the column.

6.6 Wash the column with 10 mL of Buffer A.

7. MS2-affinity purification (Figure 1)

7.1 Load the cell lysate into the column.

NOTE: Keep 1 mL of the cell lysate (Crude extract, CE) to extract total RNA (step 8) and perform Northern blot (step 9) and transcriptomic (step 10) analysis.

7.2 Collect the flow-through fraction (FT) in a clean collection tube.

7.3 Wash the column 3 times with 10 mL of Buffer A. Collect the wash fraction (W).

7.4 Elute the column with 1 mL of Buffer E and collect the elution fraction (E) in a 2 mL microtube.

7.5 Keep all collected fractions on ice until RNA extraction (step 8) or freeze them at -20 °C for later use.

8. RNA extraction of collected fractions (CE, FT, W and E)

8.1 Use 1 mL of each fraction (including FT and W) for RNA extraction.

8.2 Add 1 volume of phenol. Mix vigorously.

CAUTION: Phenol is volatile and corrosive, pay attention and work safely under a fume hood.

8.3 Centrifuge at 16,000 x *g* for 10 min at 20 °C.

8.4 Transfer the upper phase in a clean 2 mL microtube.

8.5 Add 1 volume of chloroform/isoamyl alcohol (24:1) and repeat steps 8.3 to 8.4.

CAUTION: Work safely under a fume hood.

8.6 Add 2.5 volumes of cold ethanol 100% and 1/10 volume of 3 M sodium acetate (NaOAc) pH 5,2.

8.7 Precipitate overnight at -20 °C.

NOTE: Precipitation can also be performed in an ethanol/dry ice bath during 20 min or at -80 °C during 2 h.

8.8 Centrifuge at 16,000 x *g* for 15 min at 4 °C. Slowly remove ethanol with a pipette while being careful not to disturb the pellet.

CAUTION: The RNA pellet is not always visible and is sometimes loose in presence of ethanol.

8.9 Add 500 µL of 80% cold ethanol.

8.10 Centrifuge at 16,000 x *g* for 5 min at 4 °C.

8.11 Discard ethanol by pipetting it slowly. Dry the pellet using a vacuum concentrator, 5 min on run mode.

8.12 Resuspend the pellet in an appropriate volume (15-50 µL) of ultrapure water. Freeze the pellet at -20 °C for later use.

8.13 Assess RNA quantity (260 nm) and quality (260/280 and 260/230 wavelength ratios) using a spectrophotometer/fluorometer (see **Table of Materials**). Carefully follow manufacturer's

instructions.

NOTE: 3-4 µg are generally obtained in the elution fraction (E). This mainly depends on tested conditions.

9. Analysis of MS2-affinity purification by Northern blot[36]

9.1 Dilute 5 µg of RNA of CE, FT, W fractions and 500 ng of E fraction in 10 µL of ultrapure water and mix with 10 µL of RNA loading buffer.

9.2 Incubate 3 min at 90 °C.

9.3 Load samples into wells of an 1% agarose gel supplemented with 20 mM of guanidium thiocyanate and run the gel at 100-150 V in TBE 1x buffer at 4 °C. Read Koontz (2013)[37] for more details.

9.4 Transfer RNAs on a nitrocellulose membrane by vacuum transfer for 1h or capillarity transfer overnight.

NOTE: The capillarity method is more efficient for large RNAs.

9.5 UV cross-link RNAs on the membrane (120 mJ at 254 nm) using an ultraviolet crosslinker.

9.6 Insert the membrane in a hybridization bottle with the RNA side facing up.

9.7 Add 10-20 mL of preheated hybridization solution. Incubate 30 min at 68 °C.

9.8 Discard the solution and add 10-20 mL of fresh hybridization solution supplemented with 1 µL of the sRNA-specific probe. Incubate overnight at 68 °C.

NOTE: The DIG-labelled RNA probe is synthetized using a DIG RNA labelling kit and following manufacturer's instructions. Alternatively, a radiolabelled probe can be used.

9.9 Wash the membrane with 10-20 mL of wash solution 1 (2x SSC and 0.1% SDS) for 5 min at 20 °C. Repeat once.

9.10 Wash the membrane with 10-20 mL of wash solution 2 (0.2x SSC and 0.1% SDS) for 15 min at 68 °C. Repeat once.

9.11 Incubate with 10-20 mL of blocking solution for at least 30 min at 20 °C.

9.12 Discard the solution and add 10-20 mL of the blocking solution supplemented with the polyclonal anti-digoxigenin antibody (1/1000), conjugated to alkaline phosphatase. Incubate 30 min at 20 °C.

9.13 Wash the membrane with 10-20 mL of the wash solution 3 (1x maleic acid and 0.3% Tween 20) for 15 min at 20 °C. Repeat once.

9.14 Incubate the membrane with 10-20 mL of the detection solution (0.1 M Tris HCl and 0.1 M NaCl pH 9.5) 5 min at 20 °C.

9.15 Put the membrane on a plastic film and soak it with the substrate (see **Table of Materials**). Incubate 5 min in the dark.

9.16 Seal the membrane in a plastic film. Put the membrane in an autoradiography cassette.

9.17 Expose the membrane to an autoradiography film in the dedicated dark room.

NOTE: The exposition time depends on the signal strength, from few seconds to minutes.

9.18 Reveal the exposed film in an automatic developing device.

10. Preparation of the samples for RNA sequencing

NOTE: This step only concerns RNAs extracted from E and CE fractions.

10.1 Add to each sample 10 µL of 10x DNase buffer and DNaseI (1 U/µg of treated RNAs). Add water for a final volume of 100 µL.

10.2 Incubate 1 h at 37 °C.

10.3 Extract and purify RNAs as previously described (steps 8.2 to 8.11).

10.4 Resuspend the RNA pellet in 20 µL of ultrapure water.

NOTE: The presence of remaining DNA can be checked using PCR and specific primers (e.g., 16S gene).

10.5 Assess RNA quantity and quality using a microfluidics-based electrophoresis analysis system (see **Table of Materials**).

NOTE: 1 µg is generally obtained in the elution fraction (E) after DNase treatment.

10.6 Remove ribosomal RNAs with a bacterial rRNA depletion kit.

NOTE: Large and abundant RNAs (i.e., rRNAs, RsaC sRNA) tend to non-specifically interact with the affinity column. 500 ng of extracted RNA are required to perform this step.

10.7 Again assess RNA quantity and quality using a microfluidics-based electrophoresis analysis system.

10.8 Prepare cDNA libraries with 10-20 ng of ribodepleted RNA using a cDNA library preparation kit and following manufacturer's instructions.

10.9 Sequence the obtained libraries using a sequencing instrument (e.g., single-end, 150 bp; see **Table of Materials**).

NOTE: 5-10 million reads per sample are generally enough.

11. RNAseq data analysis (Figure 2)

11.1 Download the FastQ sequencing files from the sequencing platform.

11.2 Access to the Galaxy instance of Roscoff biological station (https://galaxy.sb-roscoff.fr/) and log in.

NOTE: Every mentioned algorithm can be easily found using the search bar. A user guide is provided for each tool.

CAUTION: The version of required tools may differ from the public Galaxy server[38].

11.3 Click on **Get Data** icon and then **Upload File from your computer**. Upload FastQ sequencing file of each MS2 control and MS2-sRNA samples. Upload also FASTA reference genome file and GFF annotation file.

11.4 Run FastQC Read Quality reports (Galaxy Version 0.69).

NOTE: This tool provides a quality assessment of raw sequences (e.g., quality score, presence of adapter sequences).

11.5 Run Trimmomatic flexible read trimming tool (Galaxy Version 0.36.6) to notably remove adapter sequences and poor-quality reads. Indicate adapter sequences used for library preparation (e.g., TruSeq 3, single-ended). Add the following Trimmomatic operations: SLIDINGWINDOW (Number of bases=4; Average quality=20) and MINLEN (Min length of reads=20).

11.6 Run again FastQC Read Quality reports (Galaxy Version 0.69).

11.7 Run Bowtie2 - map reads against reference genome (Galaxy Version 2.3.2.2). Use the Genome Reference FASTA file from the history to map reads with default settings (very sensitive local).

NOTE: BAM file generated by Bowtie2 tool can be visualized using the Integrative Genomics Viewer (IGV). Associated BAI file will also be required.

11.8 Optionally, Run Flagstat which compiles stats for BAM dataset (Galaxy Version 2.0).

11.9 Run htseq-count - Count (Galaxy Version 0.6.1) which aligns reads overlapping features in the GFF annotation file. Use the Intersection (non-empty) mode.

11.10 Archive all raw counts files from htseq-count analysis into a single Zip file.

11.11 Run SARTools DESeq2 to compare data (Galaxy Version 1.6.3.0). Provide the Zip file containing raw counts files and the design file, a tab delimited file describing the experiment. Carefully follow provided instructions to generate the design file.

REPRESENTATIVE RESULTS

The representative results originate from the study of RsaC targetome in *S. aureus*[29]. RsaC is an unconventional 1,116 nt-long sRNA. Its 5' end contains several repeated regions while its 3' end (544 nt) is structurally independent and contains all predicted interaction sites with its mRNA targets. The expression of this sRNA is induced when manganese (Mn) is scarce, which is often encountered in the context of host immune response. Using MAPS technology, we identified several mRNAs interacting directly with RsaC, revealing its crucial role in oxidative stress (*sodA*, *ldh1* and *sarA*) and metal-related (*znuBC-zur* and *sufCDSUB*) responses.

Validation of the MS2-sRNA construct and experimental conditions

Before performing MAPS experiments, it is important to determine the optimum conditions of expression of the studied sRNA. If a non-native promoter is used, it will definitively help produce the MS2-sRNA construct when its targets are present. In addition, the MS2-sRNA construct should be carefully validated with regard to size, stability, expression and function. The MS2 aptamer was fused to the 5' end of either the full-length RsaC (MS2-$RsaC_{1116}$) or the shorter form (MS2-$RsaC_{544}$) corresponding to the 3' part of RsaC. Both constructs were expressed in vivo under the control of the quorum sensing dependent P3 promoter in *S. aureus* HG001 Δ*rsaC.* The deletion of *rsaC* gene avoids a competition between the endogenous RsaC and MS2-RsaC. The wild-type strain containing the same vector with the MS2 tag alone was used as control. This control allows subtracting unspecific interactions occurring with the MS2 tag.

To confirm the constructs and visualize their pattern of expression, bacterial cells were harvested after 2 h, 4 h and 6 h of growth in BHI medium at 37 °C. After RNA extraction, Northern blot analysis was performed using RsaC-specific DIG probe (**Figure 3A**). The level of endogenous RsaC (lanes 1-3) significantly increased after 6h of growth, justifying the selection of this time point for MAPS experiments. Importantly, the levels of MS2-$RsaC_{544}$ (lanes 7-9) and MS2-$RsaC_{1,116}$ (lanes 10-12) were comparable to endogenous RsaC at 6 h. Hence, they should mimic the endogenous expression pattern of RsaC. A larger but minor form of RsaC was distinguishable and might be due to an inefficient end of transcription. This phenomenon is frequently observed

when a MS2-sRNA is expressed under the control of a strong promoter from a plasmid[21]. No shorter forms resulting from aberrant transcription termination or degradation were observed. The addition of the MS2 aptamer at the 5' of sRNAs could also disrupt their proper folding and affect their functions. This step is critical for highly structured sRNAs as RsaC. Hence MS2-sRNA activity should be tested and compared to endogenous sRNA when possible. A previously known target or an observable phenotype can help to monitor it. For example, the impact of RsaC on intracellular ROS accumulation was used to validate MS2-$RsaC_{544}$ and MS2-$RsaC_{1,116}$ constructs[29].

Analysis of collected fractions during affinity purification

RNAs were extracted from CE, FT and E fractions in WT strain expressing MS2 tag alone and Δ*rsaC* strain expressing MS2-$RsaC_{544}$ construct. We showed using Northern blot analysis that the 1,116 nt-long endogenous RsaC was enriched in the elution fraction but turned out to interact non-specifically with the affinity column (**Figure 3B**, lanes 2-3). We observed the same phenomenon with MS2-$RsaC_{1,116}$ (data not shown). This is certainly due to its length and complex secondary structure. Therefore, only a less structured and shorter form (544 nt) of RsaC corresponding to its 3' part was used to perform MAPS experiments. In **Figure 3B**, the MS2-$RsaC_{544}$ was highly enriched in the elution fraction demonstrating that it was successfully retained by the MS2-MBP fusion protein (lane 6). A larger but minor form of MS2-$RsaC_{544}$ was observed as in **Figure 3A**. Here, the stringency and number of washes can be adjusted to either reduced non-specific binding or, on the contrary, to limit loss of true interacting partners.

Validation of putative mRNA targets after MAPS analysis

Following bioinformatic analysis, putative mRNA targets are listed according to the Fold-change between MS2-sRNA and MS2 control, obtained using DeSeq2 (**Figure 2**). For instance, MS2-$RsaC_{544}$ MAPS data[29] suggested that *sodA* mRNA, coding for a superoxide dismutase in *S. aureus*, is a main target (best hit, higher Fold-change). A Northern blot analysis, performed with a *sodA*-specific DIG probe after MS2-affinity purification, shows that *sodA* was efficiently co-enriched with MS2-$RsaC_{544}$ compared to the MS2 control (**Figure 3C**).

A global transcriptomic analysis is systematically performed on the CE fraction. The comparison of MAPS data and this transcriptomic analysis helps adjust the enrichment ratio and reveals a potential target hierarchy. Indeed, a poorly expressed mRNA, which is highly enriched after MS2-affinity purification has certainly a greater binding affinity than a highly enriched and highly expressed mRNA.

It is important to note that all candidates identified by MAPS must be individually validated using in vitro and/or in vivo experiments such as Electrophoresis Mobility Shift Assays (EMSA) or reporter gene assays (see Jagodnik et al. (2017)[39] for more details).

FIGURE AND TABLE LEGENDS

Figure 1. Schematic illustration of the MAPS protocol adapted to *S. aureus*. From plasmid construction to data analysis.

Figure 2. MAPS analysis workflow and processed data. Each step, check point and file format are represented (see also step 11). FastQ format is a text file consisting of the DNA sequences and corresponding quality scores. BAM format is a compressed file containing aligned sequences. Tabular format is a tab-delimited text file with counts for each gene. The results chart illustrates the kind of data obtained after bioinformatic analysis. Presented results are fictitious and do not originate from any study. For further details, basic tutorials are available on Galaxy Project website (https://galaxyproject.org/).

Figure 3: Constructs validation and MAPS controls. A. Northern blot analysis of endogenous RsaC sRNA and related MS2 constructs. WT strain carries the pCN51-P3-MS2 (control) and Δ*rsaC* mutant strain carry either the pCN51-P3-MS2, pCN51-P3-MS2-$RsaC_{544}$ or pCN51-P3-MS2-$RsaC_{1,116}$. Samples were taken after 2 h, 4 h and 6 h of growth in BHI at 37 °C. Northern blot assays were performed using a RsaC-specific DIG probe. **B**. Northern blot analysis of MS2-affinity purification fractions using a RsaC-specific DIG probe. The co-purification was performed using WT strain + pCN51-P3-MS2 (control) and Δ*rsaC* mutant strain + pCN51-P3-MS2-$RsaC_{544}$. Cells were harvested after 6h of growth in BHI at 37 °C. Crude extract (CE), flow-through (FT), elution (E). C. Northern blot analysis of MS2-affinity purification fractions (CE and E) using a *sodA*-specific DIG probe. See (**B**) for details.

Table 1. MAPS technology revealed the targetome of several RNAs in various organisms.

DISCUSSION

A modified protocol for Gram-positive bacteria

The initial protocol of MAPS was developed to study sRNA interactome in the model organism *E. coli*[20,30]. Here, we describe a modified protocol which is suitable for the characterization of sRNA-dependent regulatory networks in the opportunistic human pathogen *S. aureus* and is certainly transposable to other Gram-positive bacteria, pathogenic or not.

Particular attention was paid to the cell lysis step. The French press has been replaced by a mechanical cell lysis instrument. This method is effective to break Gram-positive cells and to limit risks associated to the handling of pathogenic strains. To improve the yield of the MS2-affinity purification, the quantity of MS2-MBP immobilized on the amylose resin has been drastically increased. This has required to adjust the stringency and number of washes.

Unlike the initial protocol, MAPS is here performed in duplicate, from two biological replicates. A workflow has been developed to implement statistical analysis (**Figure 2**), which definitively increases the robustness of obtained data.

MS2 construct and its expression

This MAPS protocol was established to identify the targetome of staphylococcal sRNAs. The MS2-sRNA construct is expressed from a low-copy number plasmid (pCN51, 20 to 25 copies/cell) and under the control of the quorum-sensing dependent P3 promoter, mainly induced during stationary phase. This expression pattern corresponds to studied sRNAs in *S. aureus* (i.e. RsaA, RsaI and RsaC) and ensures a rather strong MS2-sRNA synthesis. However, we cannot exclude

that in other cases, the P3 promoter may not be appropriate and does not reflect the natural physiological state of bacteria when the sRNA is induced. Another way to control MS2-sRNA production is to use chemically inducible promoters (e.g., tetracycline-inducible promoters). These tools allow a pulse expression of the MS2-sRNA construct, but this setting does not guarantee that RNA targets will be concomitantly expressed. Another drawback is that expression from a plasmid can lead to the overproduction of the studied sRNA, even more emphasized with high copy number vector. This might potentially result in artefactual interactions or the disruption of associated RNA chaperone functions. The most appropriate alternative is to insert a MS2 tag at the 5' end of the endogenous sRNA gene. Thus, the MS2-sRNA would be chromosomally encoded and under the control of its native promoter. This should better mimic the endogenous expression of studied sRNA and avoid bias due to the overproduction of the studied sRNA. In any case, the crucial step is to carefully verify the length, stability and functionality of the MS2-sRNA construct, notably using Northern blot assays with total RNA extracted from cultures grown under conditions that trigger the endogenous sRNA production and function. Undeniably, the use of an improper/unfunctional MS2-sRNA construct can drastically affect generated results, supporting the significance of controls described above. Finally, the MS2-sRNA is always produced in a Δ*sRNA* background to maximize the enrichment of mRNA targets. Moreover, experiments can be performed in host strains with specific mutations in RNases (e.g., deletion mutants or temperature-sensitive mutants) to avoid mRNA targets degradation by sRNA-dependent recruitment of RNases.

The experimental validation of candidates identified by MAPS is still required

One point which needs to be considered is that MAPS provides a list of putative RNA targets. However, some RNAs may be indirectly enriched via interaction with a shared mRNA target or an RNA binding protein. Consequently, revealed candidates must be confirmed by other experimental approaches. EMSA and footprinting experiments are commonly used to visualize the direct binding of an sRNA to putative RNA targets in vitro. Then, in vitro toeprinting assays help monitor the impact of an sRNA on translation initiation of its mRNA target. Finally, Northern blot and/or gene reporter assays (*lacZ* or *gfp*) complement this experimental validation in vivo. All these approaches are described in Jagodnik et al. (2017)[39].

Unlike RIL-seq and CLASH technologies, MAPS does not directly provide information on the interaction sites. Nevertheless, a peak of mapped reads is frequently observed in a restricted region of the RNA target (e.g., the 3' end of *glyW-cysT-leuZ* operon[21]), facilitating the identification of the pairing site. Alternatively, several prediction tools can be used to predict the putative binding site on the identified target such as IntaRNA[40].

MAPS scrutinizes the targetome of a particular sRNA

MAPS technology is suitable for the study of sRNA targetome in Gram-negative bacteria such as *E. coli* and *S.* Typhimurium, but also now with this modified protocol in Gram-positive bacteria like *S. aureus*. Basically, MAPS offers a snapshot of RNA:sRNA duplexes formed at a given time and in specific growth conditions. Unfortunately, the list of mRNA targets could be incomplete. For instance, a cognate mRNA target could be missed due to inappropriate experimental conditions, justifying the above-mentioned precautions.

Compared to other commonly used methods such as RIL-seq or CLASH, MAPS uses a specific sRNA to co-purify all interacting RNA targets. Here, sRNA:RNA interaction are not diluted among other RBP-associated duplexes, allowing theoretically to characterize its targetome more deeply. However, it seems that RIL-seq/CLASH and MAPS methods revealed different sets of putative sRNA targets[12,41], suggesting that these experimental approaches are complementary. This discrepancy could be explained by variations in growth conditions and/or bias generated by each method.

Accordingly, the purpose of the study should influence the choice of the method. For a global analysis of sRNA targetomes and when an RBP is involved in sRNA-mediated regulation, RIL-seq and CLASH methods should be preferred. Both approaches provide an overview of regulatory networks relying on a particular RBP and, consequently, uncover a wide variety of sRNAs and associated targets. On the contrary, for the study of a specific sRNA or when no RBP is known/involved, MAPS represents an appropriate option.

ACKNOWLEDGMENTS

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DISCLOSURES

The authors have nothing to disclose.

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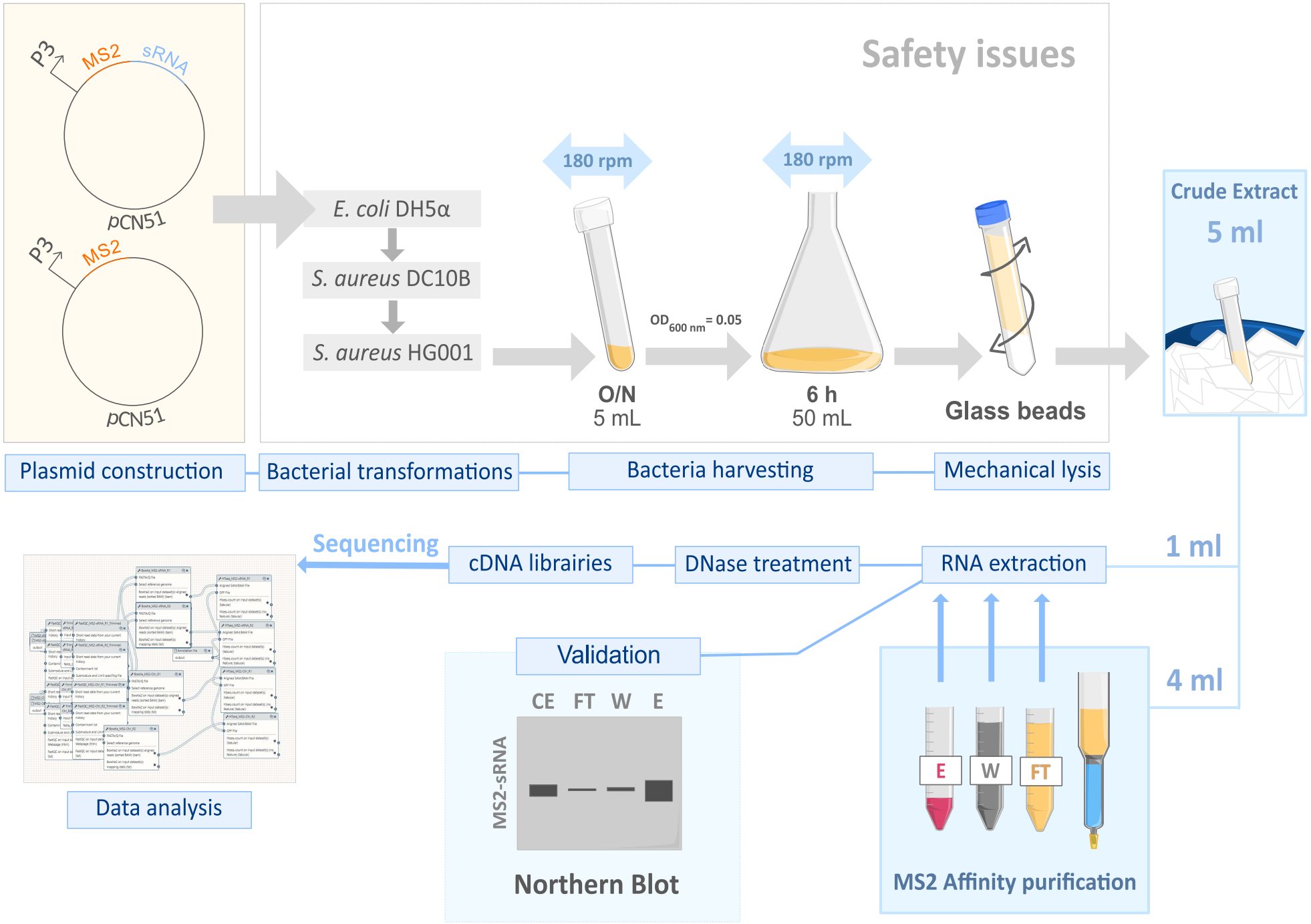
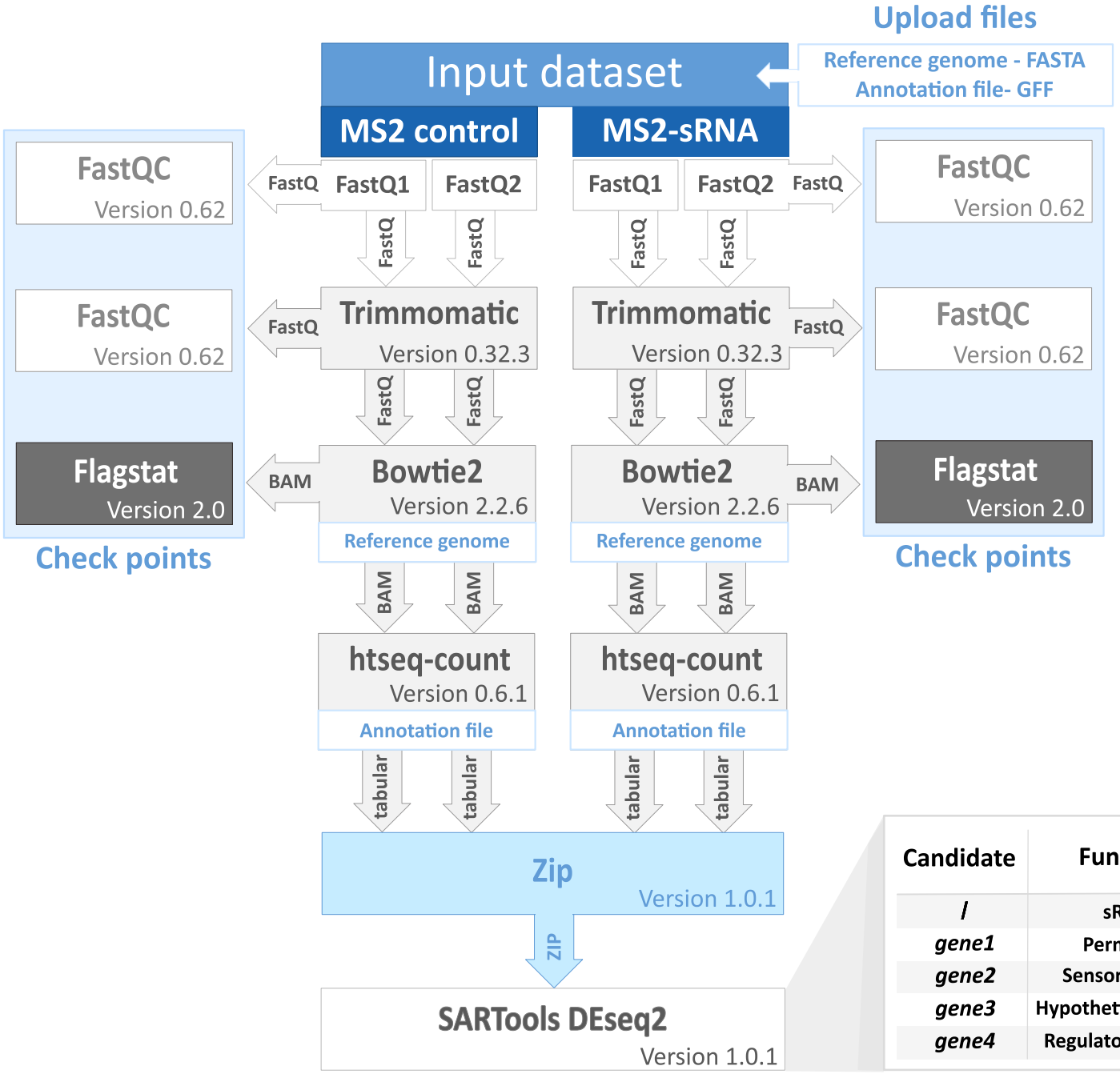


Figure 1



Candidate	Function	Fold Change MS2-sRNA / MS2	P-value
/	sRNA	10.2	10^{-23}
gene1	Permease	6.4	10^{-15}
gene2	Sensor protein	4.8	10^{-11}
gene3	Hypothetical protein	3.7	10^{-08}
gene4	Regulatory protein	3.1	10^{-07}

Figure 2

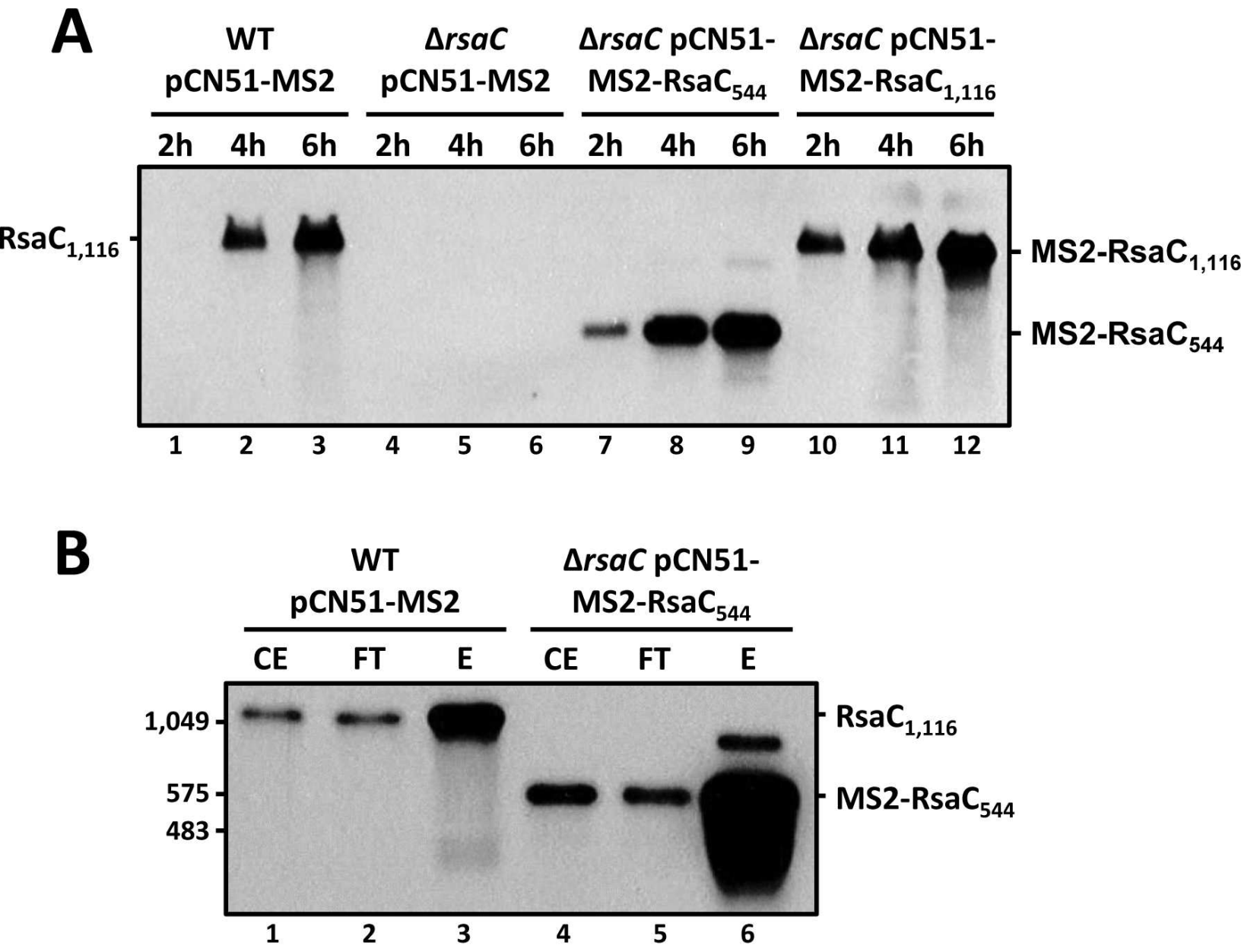


Figure 3

Table 1. MAPS technology revealed the targetome of several RNAs in various organisms

RNA	Type	Reference
Escherichia coli		
RyhB	sRNA	Lalaouna *et al.* (2015)[21]
RybB	sRNA	Lalaouna *et al.* (2015)[21]
3'ETS^{leuZ}	tRNA-derived fragment	Lalaouna and Massé (2015)[26]
DsrA	sRNA	Lalaouna *et al.* (2015)[22]
hns	mRNA (5'UTR)	Lalaouna *et al.* (2015)[22]
CyaR	sRNA	Lalaouna *et al.* (2018)[23]
RprA	sRNA	Lalaouna *et al.* (2018)[23]
GcvB	sRNA	Lalaouna *et al.* (2019)[24]
Salmonella Typhimurium		
SraL	sRNA	Silva *et al.* (2019)[25]
Staphylococcus aureus		
RsaA	sRNA	Tomasini *et al.* (2017)[27]
RsaC	sRNA	Lalaouna *et al.* (2019)[29]
RsaI	sRNA	Bronesky *et al.* (2019)[28]

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
1.5 mL microcentrifuge tube	Sarstedt	72.690.001	
15 mL centrifuge tubes	Falcon	352070	
2 mL microcentrifuge tube	Starstedt	72.691	
2100 Bioanalyzer Instrument	Agilent	G2939BA	RNA quantity and quality
250 mL culture flask	Dominique Dutscher	2515074	Bacterial cultures
50 mL centrifuge tubes	Falcon	352051	Culture centrifugation
Absolute ethanol	VWR Chemicals	20821.321	RNA extraction and purification
Allegra X-12R Centrifuge	Beckman Coulter		Bacterial pelleting
Ampicilin (amp)	Sigma-Aldrich	A9518-5G	Growth medium
Amylose resin	New England BioLabs	E8021S	MS2-affinity purification
Anti-dioxigenin AP Fab fragment	Sigma Aldrich	11093274910	Northern blot assays
Autoradiography cassette	ThermoFisher Scientific	50-212-726	Northern blot assays
BamHI	ThermoFisher Scientific	ER0051	Plasmid construction
BHI (Brain Heart Infusion) Broth	Sigma-Aldrich	53286	Growth medium
Blocking reagent	Sigma Aldrich	11096176001	Northern blot assays
CDP-Star	Sigma Aldrich	11759051001	Northern blot assays (substrate)
Centrifuge 5415 R	Eppendorf		RNA extraction and purification
Chloroform	Dominique Dutscher	508320-CER	RNA extraction and purification
DIG-RNA labelling mix	Sigma-Aldrich	11277073910	Northern blot assays
DNase I	Roche	4716728001	DNase treatment
Erythromycin (ery)	Sigma-Aldrich	Fluka 45673	Growth medium
FastPrep device	MP Biomedicals	116004500	Mechanical lysis
Guanidium Thiocyanate	Sigma-Aldrich	G9277-250G	Northern blot assays
Hybridization Hoven Hybrigene	Techne	FHB4DD	Northern blot assays
Hybridization tubes	Techne	FHB16	Northern blot assays
Isoamyl alcohol	Fisher Scientific	A/6960/08	RNA extraction and purification
LB (Lysogeny Broth)	Sigma-Aldrich	L3022	Growth medium
Lysing Matrix B Bulk	MP Biomedicals	6540-428	Mechanical lysis
MicroPulser Electroporator	BioRad	1652100	Plasmid construction
Milli-Q water device	Millipore	Z00QSV0WW	Ultrapure water
NanoDrop spectrophotometer	ThermoFisher Scientific		RNA/DNA quantity and quality
Nitrocellulose membrane	Dominique Dutsher	10600002	Northern blot assays

Phembact Neutre	PHEM Technologies	BAC03-5-11205	Cleaning and decontamination
Phenol	Carl Roth	38.2	RNA extraction and purification
Phusion High-Fidelity DNA Polymerase	New England Biolabs	M0530	Plasmid construction
pMBP-MS2	Addgene	65104	MS2-MBP production
Poly-Prep chromatography column	BioRad	7311550	MS2-affinity purification
PstI	ThermoFisher Scientific	ER0615	Plasmid construction
Qubit 3 Fluorometer	Invitrogen	15387293	RNA quantity
RNAPro Solution	MP Biomedicals	6055050	Mechanical lysis
ScriptSeq Complete Kit	Illumina	BB1224	Preparation of cDNA librairies
Spectrophotometer Genesys 20	ThermoFisher Scientific	11972278	Bacterial cultures
SpeedVac Savant vacuum device	ThermoFisher Scientific	DNA120	RNA extraction and purification
Stratalinker UV Crosslinker 1800	Stratagene	400672	Northern blot assays
T4 DNA ligase	ThermoFisher Scientific	EL0014	Plasmid construction
TBE (Tris-Borate-EDTA)	Euromedex	ET020-C	Northern blot assays
ThermalCycler T100	BioRad	1861096	Plasmid construction
Tween 20	Sigma Aldrich	P9416-100ML	Northern blot assays
X-ray film processor	hu.q	HQ-350XT	Northern blot assays
X-ray films Super RX-N	FujiFilm	4741019318	Northern blot assays

Editorial Comments:

• Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.

Author's response: According to Editor's comment, we have carefully reviewed the manuscript.

• Avoid punctuating the title.

Author's response: We have changed the title: "A modified protocol for MS2-affinity purification coupled with RNA sequencing in Gram-positive bacteria"

• **Protocol Language:** Please ensure that all text in the protocol section is written in the imperative voice/tense as if you are telling someone how to do the technique (i.e. "Do this", "Measure that" etc.) Any text that cannot be written in the imperative tense may be added as a "Note", however, notes should be used sparingly and actions should be described in the imperative tense wherever possible.
1) Some examples NOT in the imperative: 2.1-2.6

Author's response: We have rephrased several sentences in the protocol section to follow the editor's instructions.

• **Protocol Detail:** Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video. **Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) to your protocol steps.** There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

Author's response: We have taken into account the Editor's comments and added more details and/or references to our protocol.

Some examples:
1) 3.1: Mention PCR steps or cite references. Mention primers used and reaction conditions. Mention enzyme concentrations. How is the sRNA acquired?

Author's response: We have indicated the primers used to amplify the sRNA for cloning into the pCN51-P3 plasmid. We have cited a reference describing all PCR steps.

2) 3.2: How is the digestion performed? Mention all enzyme concentrations.

Author's response: We have now indicated all enzymes concentrations and explain how to digest both the pCN51-P3 plasmid and the MS2-sRNA construct (see 3.3 to 3.4).

3) 3.3: how?

Author's response: A ligation protocol has been added (see 3.5 to 3.6).

4) 3.5: how are the clones identified?

Author's response: We have indicated that the pCN51-P3 encodes an ampicillin resistance gene, enabling the selection of *E. coli* strains carrying this vector (see 3.11). In the same vein, the erythromycin resistance gene allows the selection of transformed *S. aureus* cells (see 3.18).

5) 3.6: Needs more detail or a reference if you aren't planning to film this.

Author's response: We have added a reference and the primer used to sequence the cloning cassette (see 3.13).

6) 3.8: how is it extracted?

Author's response: We have indicated that a commercial kit can be used to extract plasmids (see 3.12).

7) 5.1: What is the composition of Buffer A?

Author's response: The composition of Buffer A is already specified in Buffers and media section (see 1.1)

8) 8.13: How? Mention wavelength.

Author's response: Wavelengths are now mentioned (see 8.13)

9) 9.6: References.

Author's response: We have added details and a reference.

10) This list is not exhaustive, use it as a guide to identify the types of specific details needed.

Author's response: We have reviewed the whole protocol section.

• **Protocol Highlight:** After you have made all of the recommended changes to your protocol (listed above), please re-evaluate the length of your protocol section. Please highlight ~2.5 pages or less of text (which includes headings and spaces) in yellow, to identify which steps should be visualized to tell the most cohesive story of your protocol steps.

1) The highlighting must include all relevant details that are required to perform the step. For example, if step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be included in the highlighting.
2) The highlighted steps should form a cohesive narrative, that is, there must be a logical flow from one highlighted step to the next.
3) Please highlight complete sentences (not parts of sentences). Include sub-headings and spaces when calculating the final highlighted length.
4) Notes cannot be filmed and should be excluded from highlighting.

Author's response: We have re-evaluated the length of our protocol section and highlighted in yellow the steps that should be filmed. Unfortunately, we cannot include the DNase treatment due to length restrictions (10.1 to 10.4). This step is required to prepare samples for RNA sequencing and could be mentioned in the conclusion of the movie.

• **Discussion:** JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form (3-6 paragraphs): 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.

Author's response: We have revised the Discussion section according to both Editor and Reviewer 1 comments.

• **References:**
1) Please spell out journal names.

Author's response: Journal names are now spelled out.

• **Commercial Language:**JoVE is unable to publish manuscripts containing commercial sounding language, including trademark or registered trademark symbols (TM/R) and the mention of company brand names before an instrument or reagent. Examples of commercial sounding language in your manuscript are FastPrep, Poly-Prep, NanoDrop
1) Please use MS Word's find function (Ctrl+F), to locate and replace all commercial sounding language in your manuscript with generic names that are not company-specific. All commercial products should be sufficiently referenced in the table of materials/reagents. You may use the generic term followed by "(see table of materials)" to draw the readers' attention to specific commercial names.

Author's response: We have replaced the commercial sounding language by generic names.

• **Table of Materials:** Please remove the registered trademark symbols TM/R from the table of reagents/materials.

Author's response: We have removed trademark symbols.

• If your figures and tables are original and not published previously or you have already obtained figure permissions, please ignore this comment. If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."

Author's response: Our figures and tables were not published previously.

Comments from Peer-Reviewers:

Reviewers' comments:

Reviewer #1:

The authors describe the adaptation of the MS2-Affinity Purification coupled with RNA Sequencing (MAPS) technology to be used in the Gram-positive bacterium Staphylococcus aureus. MAPS was previously applied to characterize several small RNAs in the Gram-negative bacterium E. coli and the protocol was published. Yet, a detailed protocol of using the MAPS technology in Gram-positive bacteria will be useful to the scientists in the field.

The paper could be improved by the following:

* The authors emphasize in the title and in several places in the text that the described protocol is specifically for pathogenic bacteria, but pathogenic bacteria can be Gram-positive or Gram-negative and therefore this is non-relevant. Please remove.

Author's response: We have removed it.

* The authors should highlight what are the improvements or adaptations from the previous protocol in publications Lalaouna et al., 2017; PMID: 27876680 and Lalaouna et al., 2018; PMID: 30502950).

Author's response: We have now discussed the modifications from the previous protocol implemented in *E. coli*. The major differences are based on the specific manipulations of pathogenic bacteria and the lysis of the Gram-positive bacteria. We have also increased the amount of MS2-MBP and added appropriate statistical analyses (two biological repeats).

* Page 8, line 342: The authors can add which rRNA depletion kit they were using.

Author's response: The rRNA depletion kit is indicated in the Table of Materials.

* Page 10, lines 406-7: The authors can clarify what is the purpose of finding the optimum conditions of expression of the MS2-sRNA construct, taking into consideration that the construct is under the control of an inducible promoter (e.g. P3 promoter).

Author's response: It is important to determine the optimum conditions of induction of the studied sRNA. The goal is to produce the MS2-sRNA construct when its targets are expressed. We have rephrased this confusing sentence.

* Page 10, line 408: "The MS2 aptamer was fused to the 5' end of short (MS2-RsaC544; 3' part)" is confusing, please revise.

Author's response: This sentence has been rephrased. Indeed, we have constructed two MS2-RsaC constructs, the long comprising the full-length RNA and the short containing the 3' part of the RNA with the pairing sites.

* Page 10, line 422: The authors state that "this phenomenon is often observed…". A reference would be useful.

Author's response: A reference has been added.

* Page 11, line 445-6: The authors can describe in more details how the putative mRNA targets list is generated.

Author's response: We have indicated that the ranking is obtained using DeSeq2 tool comparing the enrichment ratio between RNAs pulled down with the MS2-RNA and the MS2 control.

* Page 12, lines 488-509: Can the authors expand their discussion on the option of tagging the endogenous sRNA gene?

Author's response: We have added further details on the possibility of tagging the endogenous sRNA gene to allow native sRNA expression and avoid its over-expression due to the plasmid copy number.

* Page 13, lines 535-540: I am not convinced that the MAPS is superior to the RIL-seq or CLASH when studying one sRNA as the authors state in line 540. On the other hand, the authors can emphasize more the advantage of using MAPS to study an sRNA when the RNA chaperone that facilitates the regulation is unknown, or if the sRNA is acting independently of any RNA chaperone.

Author's response: We agree with the reviewer. Our intention was not to claim that MAPS is superior than RIL-seq or CLASH. These technologies are clearly complementary. We have rephrased the paragraph to take into account the comments.

* The discussion can be improved by adding two more sections:
A. Discuss the bioinformatic analyses that can be done on the sRNA target list resulted from the MPAS such as: finding the seed sequence of the sRNA using MEME.

Author's response: The prediction of the pairing site is now discussed. We have notably mentioned IntaRNA algorithm, a powerful tool to predict seed sequences.

B. Is it feasible to add a UV crosslinking step in the protocol, allowing the MAPS technology to identify unknown protein binding partners of the sRNAs in vivo? This might be one of the advantages of MAPS for studying a role of Hfp-independent sRNAs.

Author's response: Indeed, this is feasible and the first publication using a MS2-sRNA identified Hfq as a binding partner (Said et al. 2009). However, this option has already been discussed in previous reviews (e.g. Lalaouna et al. 2017). Here, we chose to focus only on the identification of RNA:RNA interactions.

* Figure 3C: Show a Northern blot of negative control target (non RsaC mRNA target).

Author's response: We agree that the reviewer's comment is appropriate. We already demonstrated the specificity of RsaC-*sodA* interaction in Lalaouna *et al.* (2019) NAR. Here the purpose of the control was to show that a validation based on a candidate approach is feasible.

* Figures 1 and 2: Increase the font size.

Author's response: We have modified both figures.

Reviewer #2:
Manuscript Summary:
This manuscript describes a protocol to decipher the targetome of bacterial regulatory sRNAs in vivo. Regulation by RNA is ubiquitous in bacteria and impacts most, if not all, cell processes. However, this level of gene regulation remains unexplored in many bacterial species. The identification of the typically multiple mRNA targets is key to understand the regulatory function of an sRNA, but this determination is a challenge and commonly the bottleneck of the functional characterization pipeline. Therefore, this is a timely and necessary protocol for scientists facing the primary characterization of the thousands of regulatory RNAs expressed by bacteria. Description of the protocol is extremely thorough, accurate and careful. Therefore, I strongly recommend publication without any concern.

Author's response: We thank the reviewer 2 for emphasizing the benefits of our manuscript.

Reviewer #3:
Manuscript Summary:
This manuscript describes the rationale and approach for identifying mRNA targets for small RNAs of interest in Staphylococcus aureus using the MS2-affinity purification coupled with RNA sequencing (MAPS) technique. The manuscript is well-written and the protocol is very clear. It is an important technique and the protocol will be very useful to the research community. I have only a few minor comments.

Major Concerns:
None

Author's response: We would like to thank the reviewer 3 for these useful recommendations. We have taken all his/her minor concerns into account.

Minor Concerns:
Line 70: change "relied" to "rely"
Lines 73-74: The sentence is confusing, especially "co-expression of both partners hinting that this interaction can really occur in the bacterial cell." The meaning is unclear.
Line 91: "the sRNA is tagged in 5'" change to "the sRNA is tagged at the 5'"
line 103: change "confirm" to "confirming"
Line 120: change "details" to "detail"
Line 164: change "accumulates" to "accumulate"
What are the highlighted lines for?

Author's response: Only highlighted lines will be filmed.

Line 210: change "them" to "pellets"
Line 268: by "projection" do you mean "splashes"?
Line 448: change "specific sodA-specific" to "sodA-specific"
Line 503: delete "allow to"
Line 503: change "cases" to "case"
Line 505: after "Northern blot assays" add "and growth under conditions that test for sRNA function."
Lines 505-506: The meaning of the sentence "Undeniably, these construct features can

drastically affect generated results." is unclear. Is it meant to underscore the importance of functional testing of tagged sRNAs?

Author's response: We have modified this sentence: "Undeniably, the use of an improper MS2-sRNA construct can drastically affect generated results"

Line 508-509: Perhaps delete "use of specific RNase mutants can be envisaged." and replace with "experiments can be performed in host strains with specific mutations in RNases."

Author's response: We have rephrased this sentence for a better understanding.

Line 535: Clarify the sentence, perhaps "The purpose of the study should determine the choice of method."

Author's response: We have clarified this sentence as suggested.