CHAPTER THIRTEEN

Transcriptomic Approaches for Studying Quorum Sensing in *Vibrio cholerae*

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Contents

Abstract

Transcriptome analysis using RNA-sequencing (RNA-seq) has now become the standard approach to determine the transcriptional output of an organism. Various modifications to this technology have been developed over the years, usually aiming to improve the annotation of transcript borders, or to identify novel classes of RNAs, such as small regulatory RNAs (sRNAs) and antisense transcripts. RNA-seq has also led to the identification of dozens of new sRNAs in the major human pathogen, *Vibrio cholerae*. Several of these sRNAs function in the context of a cell-to-cell communication process, called quorum sensing (QS). QS is key for pathogenicity and biofilm formation of *V. cholerae* and the

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sRNAs involved typically act by base pairing with multiple target mRNAs to control gene expression at the posttranscriptional level. In this chapter, we describe the use of RNA-seq technologies for the discovery and characterization of regulatory RNAs in *V. cholerae* and discuss their relevance to QS and collective functions, such as biofilm formation. We further outline possible methods for the identification and validation of sRNA target genes, which can provide crucial information as to the physiological roles of an sRNA.

1. INTRODUCTION

The major human pathogen and causative agent of cholera disease, *Vibrio cholerae*, critically depends on collective group behaviors to colonize the host's small intestine and cause disease. These collective processes include the formation of biofilms to cope with the acidic environment of the human stomach, as well as the production of virulence factors (Almagro-Moreno, Pruss, & Taylor, 2015; Silva & Benitez, 2016). Both processes, biofilm formation and virulence gene expression, are only effective when conducted simultaneously by all members of the group. In *V. cholerae*, group behavior is controlled by quorum sensing (QS), a process of interspecies communication (Ng & Bassler, 2009). QS relies on the synthesis, release, and subsequent detection of small signaling molecules, called autoinducers (AIs). Microorganisms often produce multiple AI molecules, which determine the overall QS output (Papenfort & Bassler, 2016).

In *V. cholerae*, two AIs, i.e., CAI-1 ((S)-3-hydroxytridecan-4-one) and AI-2 ((2S,4S)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran borate), have been extensively studied. CAI-1 and AI-2 are produced by the CqsA and LuxS synthases, respectively, and both AIs are detected by membrane-bound receptor proteins (Chen et al., 2002; Higgins et al., 2007; Hurley & Bassler, 2017; Neiditch, Federle, Miller, Bassler, & Hughson, 2005; Ng et al., 2011; Surette, Miller, & Bassler, 1999). Whereas CAI-1 is recognized by CqsS, AI-2 is sensed by the LuxPQ protein complex (Fig. 1). At low cell density (LCD), when autoinducers are scarce, CqsS and LuxQ function as kinases transferring a phosphate group to the phosphorelay protein LuxU, which channels the phosphate to the response regulator LuxO (Bassler, Wright, & Silverman, 1994). Phosphorylated LuxO, together with the alternative sigma factor σ^{54}, promotes the expression of four homologous sRNAs, called Qrr1–4 (Lenz et al., 2004). Qrr1–4 are Hfq-dependent sRNAs (see below) that act in *trans* to destabilize, among others, the transcript coding for HapR, which is the main repressor of LCD functions in *V. cholerae*

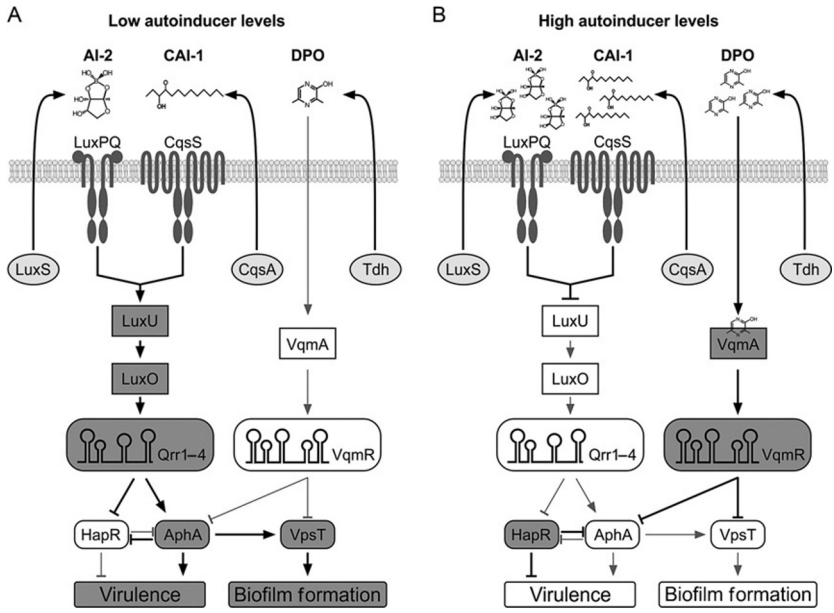

Fig. 1 Quorum Sensing pathways of *V. cholerae*. The autoinducers CAI-1 and AI-2 are synthesized by the CqsA and LuxS enzymes and sensed by the membrane-associated CqsS and LuxPQ receptors, respectively. The DPO autoinducer requires the Tdh (threonine dehydrogenase) enzyme for synthesis and binds to the VqmA receptor protein. (A) When autoinducer concentrations are low, CqsS and LuxPQ function as kinases and phosphorylate LuxU. LuxU-P channels the phosphate group to LuxO, and LuxO-P activates the transcription of the Qrr1–4 sRNAs. The Qrr sRNAs act at the posttranscriptional level to inhibit *hapR* and induce *aphA*. Under this condition virulence gene expression and biofilm formation are activated. (B) When autoinducer concentrations increase, interaction of CAI-1 and AI-2 with CqsS and LuxPQ, respectively, changes the receptors to phosphatases reducing LuxO-P levels and blocking *qrr1–4* expression. Under these conditions, AphA expression is inhibited and HapR is produced. The VqmA–DPO complex promotes expression of the VqmR sRNA. VqmR blocks VpsT production and therefore biofilm formation. In addition, HapR and AphA antagonize each other at the transcriptional level. Active factors are shown in *black*, inactivate (repressed) factors are depicted in *gray*.

(Fig. 1, left side). In addition, the Qrr2–4 sRNAs base pair with and stabilize the *aphA* mRNA encoding a key transcriptional activator of biofilm formation and virulence factor production (Rutherford, van Kessel, Shao, & Bassler, 2011; Shao & Bassler, 2012). Thus, at LCD, AphA is produced, while HapR is not.

Activation of biofilm formation by AphA relies on the induction of another transcriptional activator, called VpsT, which drives the expression

of polysaccharide synthesis genes and other protein component for biofilm assembly (Casper-Lindley & Yildiz, 2004; Teschler et al., 2015; Yang, Frey, Liu, Bishar, & Zhu, 2010). Also, VpsT requires the second messenger c-di-GMP (bis-(3′–5′)-cyclic dimeric guanosine monophosphate) for full activity, thereby linking interspecies- (QS) and intracellular signaling processes (Krasteva et al., 2010). Activation of virulence genes in *V. cholerae* is a multistep process and requires several transcriptional regulators acting downstream of AphA (Almagro-Moreno et al., 2015). Specifically, AphA in concert with AphB activates the production of the membrane-bound regulators TcpP and TcpH, which together with another transmembrane regulator, ToxRS, drive the expression of ToxT. ToxT induces the expression of *ctxAB* and *tcpA*, which code for the cholera toxin and the toxin-coregulated pilus, respectively. CtxAB and TcpA are both critical for pathogenicity of *V. cholerae*. Of note, the Qrr sRNAs also inhibit the expression of factors required for Type VI secretion (Shao & Bassler, 2014), which can also affect interspecies competition during host colonization (Logan et al., 2018; Zhao, Caro, Robins, & Mekalanos, 2018).

Continued growth and accumulation of AIs in the environment trigger *V. cholerae*'s transition into a high cell density (HCD) mode of gene expression (Fig. 1, right side). Binding of CAI-1 and AI-2 to CqsS and LuxPQ, respectively, alters the receptors' function from kinases to phosphatases, leading to dephosphorylation of LuxU and LuxO (Hurley & Bassler, 2017). Unphosphorylated LuxO is inactive and consequently expression of the Qrr sRNAs ceases. Reduced production of the Qrrs results in increased HapR production and inhibition of AphA synthesis. This pattern of gene expression is reinforced by the reciprocal repression of HapR and AphA, in which both transcription factors function to repress the promoter of the other regulator (Rutherford et al., 2011). With HapR being produced, virulence gene expression and biofilm formation of *V. cholerae* are inhibited.

Recently, we identified another QS pathway in *V. cholerae*, which is independent of the canonical QS signal transduction cascade. This new system relies on the 3,5-dimethylpyrazin-2-ol (DPO) AI molecule (Papenfort et al., 2017), which is synthesized during the catabolic degradation of L-threonine by threonine dehydrogenase (Tdh) (Fig. 1). DPO is recognized by the cytoplasmic LuxR-type receptor protein, VqmA, and the active DPO–VqmA complex induces the expression of the VqmR sRNA. VqmR base pairs with and inhibits translation of the *vpsT* mRNA, encoding a major activator of biofilm formation in *V. cholerae* (Papenfort, Forstner, Cong, Sharma, & Bassler, 2015). Consequently, VqmR is a repressor of biofilm formation. VqmR also regulates other

target mRNAs in *V. cholerae*, some of which also relate to QS and collective behaviors (Papenfort, Forstner, et al., 2015).

It is interesting to note that both QS pathways of *V. cholerae*, depending on either AI-2/CAI-1 or DPO, use sRNAs at crucial positions of their pathways (Fig. 1). It has been speculated that RNA regulators employ different regulatory dynamics when compared to transcription factors (Beisel & Storz, 2010; Nitzan, Rehani, & Margalit, 2017), which could also be relevant for the dynamics underlying QS regulation in *V. cholerae*. Indeed, in the related *Vibrio harveyi* species, regulation by the Qrr sRNAs can have different consequences depending on the nature of the RNA duplex formed between the sRNAs and the targets (Feng et al., 2015). In general, sRNAs can act catalytically (the target mRNA is degraded, while the sRNA remains stable in the cell), or by coupled degradation in which both target mRNA and sRNA are degraded after RNA duplex formation (Masse, Escorcia, & Gottesman, 2003). In few cases, sRNAs have also been reported to function by sequestration (Feng et al., 2015; Moller, Franch, Udesen, Gerdes, & Valentin-Hansen, 2002; Tu, Long, Svenningsen, Wingreen, & Bassler, 2010). Here, the sRNA and the target mRNA are believed to form a stable RNA duplex that is not subject to degradation by cellular ribonucleases.

Both, the Qrr sRNAs and VqmR, regulate multiple target genes, and it is evident that sRNAs, like transcription factors, can function as global regulators of gene expression (Hor, Gorski, & Vogel, 2018; Papenfort & Vogel, 2009). Similarly, QS is a multifaceted process affecting hundreds of genes with diverse physiological implications (Ball, Chaparian, & van Kessel, 2017). Therefore, genome-wide profiling technologies are required to study QS and the role of regulatory RNAs in this process. The advent of next-generation sequencing (NGS) technologies has turned transcriptome analysis and global gene expression profiling on its head and now has also been applied to study QS of *V. cholerae* and other microorganisms. In this chapter, we will revisit and update NGS-based technologies for studying QS in *V. cholerae* with an emphasis on sRNA-mediated gene regulation. We describe how to predict and validate of sRNA-target pairs, and outline possible future directions in this field of research.

2. DISCOVERY OF SMALL REGULATORY RNAS USING DIFFERENTIAL RNA-SEQ

Global expression analysis using RNA-sequencing (RNA-seq) has revolutionized the way scientists interpret and understand the transcriptional output of a cell. In general, RNA-seq has replaced hybridization-based

technologies such as microarrays and tilling arrays and comes with the major advantage of single-nucleotide resolution data allowing researchers to study individual transcripts. Various different technologies allowing RNA-seq are currently available (e.g., *Solexa*, *SOLiD*, and *454*; for a comprehensive overview, see Lowe, Shirley, Bleackley, Dolan, & Shafee, 2017), some of which also allow single-molecule sequencing (e.g., nanopore sequencing and SMRT sequencing).

RNA-seq has also had a major impact on the discovery and characterization of prokaryotic regulatory RNAs (Barquist & Vogel, 2015; Hor et al., 2018). For example, RNA-seq has been used to identify RNA ligands associated with the Hfq RNA chaperone in *Salmonella enterica* (Chao, Papenfort, Reinhardt, Sharma, & Vogel, 2012; Sittka et al., 2008), *Neisseria meningitidis* (Heidrich et al., 2017), *Sinorhizobium meliloti* (Torres-Quesada et al., 2014), and *Escherichia coli* (Bilusic, Popitsch, Rescheneder, Schroeder, & Lybecker, 2014). Homologs of *hfq* are present in approximately 50% of all sequenced bacterial genomes (Sun, Zhulin, & Wartell, 2002) and typically Hfq plays a major role in posttranscriptional gene regulation in these species. The current model suggests that Hfq acts as a matchmaker promoting base pairing of sRNAs with their cognate targets; however, Hfq is also relevant for sRNA stability and can function independent of sRNAs to control mRNA turnover and translation (Santiago-Frangos & Woodson, 2018; Vogel & Luisi, 2011; Woodson, Panja, & Santiago-Frangos, 2018). Not surprisingly, mutation of *hfq* is frequently associated with complex phenotypic alterations and oftentimes impairs the virulence of pathogenic microbes (Chao & Vogel, 2010), including *V. cholerae* (Ding, Davis, & Waldor, 2004).

The identification of sRNAs using Hfq as a bait has been an important strategy to categorize sRNAs by their function, i.e., Hfq-dependent sRNAs are likely to engage base pairing with *trans*-encoded target mRNAs. However, additional classes of sRNAs exist in bacteria (Hor et al., 2018; Waters & Storz, 2009) and therefore other, unbiased approaches are required to determine the output of regulatory RNAs expressed from a bacterial genome. One such powerful approach is differential RNA-seq (dRNA-seq; Fig. 2). The dRNA-seq method takes advantage of the different chemical nature of 5′ ends present in bacterial RNAs. Specifically, the cellular RNA pool consists of primary transcripts carrying a triphosphate at the 5′ end and processed transcripts, such as transfer RNAs and ribosomal RNAs, which carry a 5′ monophosphate, or in fewer cases, a 5′ hydroxyl group. To perform a dRNA-seq experiment (see below for details), RNA is isolated using conventional methods, and the RNA is split in two. One sample remains untreated

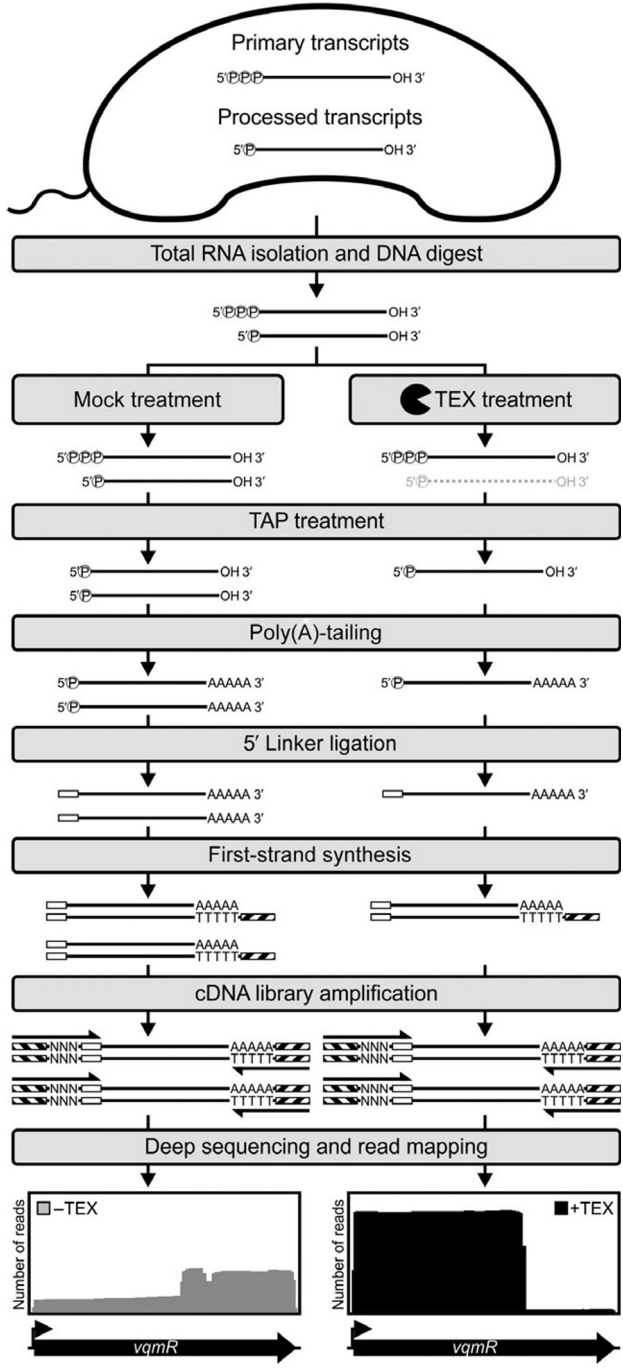

Fig. 2 See legend on next page.

(allowing the detection of all transcripts), while the other sample is treated with terminator exonuclease (TEX). TEX specifically degrades transcripts carrying a 5′ monophosphate and thus leads to the relative enrichment of primary transcripts. This strategy led to the identification of dozens of new regulatory RNAs in various species (Sharma & Vogel, 2014), which included many sRNAs acting independent of Hfq. Of note, dRNA-seq also promotes the identification of processed sRNAs (Chao et al., 2017, 2012; Kroger et al., 2012; Papenfort, Espinosa, Casadesus, & Vogel, 2015; Papenfort et al., 2009), for which there is no enrichment in the TEX-treated cDNA libraries.

2.1 RNA Preparation and DNA Digest

High quality RNA is key for reliable RNA-seq results. Therefore, the isolation of total RNA from bacterial cells is a critical step in every RNA-seq experiment. Most RNA isolation protocols use either organic extraction methods or column-based filter systems. Both have advantages and disadvantages. However, since the column-implemented silica membranes from RNA isolation kits often fail to recover small RNA fragments, we recommend the use of organic extraction protocols for transcriptome analyses. These protocols are based on five steps: Cell lysis, phase separation, RNA precipitation, washing, and elution. In the first step, cell pellets are homogenized in a phenol- and guanidine thiocyanate-containing solution to keep the RNA in solution and to inactivate RNases, respectively. The addition of chloroform separates the mixture into a lower organic phase, a solid middle phase and an aqueous, RNA-containing upper phase. RNA precipitation is performed by addition of isopropanol to the upper

Fig. 2 Workflow of the dRNA-seq experiment. Total RNA is isolated from *V. cholerae* and copurified cellular DNA is digested. RNA samples are split in two. One half is treated with water (mock), whereas the other half is treated with 5′-P-dependent terminator exonuclease (TEX), which specifically degrades processed transcripts carrying a 5′ monophosphate group (*gray, dashed line*). Next, samples are treated with tobacco acid pyrophosphatase, which converts the 5′ triphosphate groups (5′ PPP) of primary transcripts to 5′ phosphate ends (5′ P). An RNA-linker (*white box*) is ligated to the transcript's 5′ ends, and 3′ ends (3′ OH) are polyadenylated using *E. coli* poly(A) polymerase. First-strand cDNA synthesis is performed using an oligo(dT)-adapter (*striped box*). After second-strand synthesis, libraries are amplified by PCR using barcoded oligonucleotides (barcode sequences are shown as "NNN"). The cDNA libraries are sequenced and reads are mapped onto a reference genome. Primary transcripts are enriched in the +TEX-treated samples, which is exemplified by the two coverage plots for reads mapping to the *vqmR* gene (*bottom*).

phase. Subsequent washing of the pelleted RNA is required to remove residual salts, before nuclease-free water is used to dissolve the RNA. Despite careful implementation of the protocol, it is inevitable that low amounts of genomic DNA are copurified. Consequently, residual genomic DNA should be removed prior to ±TEX treatment, using DNase I. Enzymes and salts are removed in subsequent separation-, precipitation-, and washing steps. Finally, the integrity of the clean, DNA-free RNA samples should be confirmed to avoid sequencing of degraded RNA fragments. The RNA integrity number (RIN) is a reliable algorithm for RNA quality assessment and is easily calculated from Agilent 2100 Bioanalyzer measurements (Schroeder et al., 2006). To proceed with subsequent treatments, RNA samples should score a RIN of ≥7 on a scale from 1 (highly degraded) to 10 (highest integrity).

2.1.1 Equipment

- 2100 Bioanalyzer (Agilent)
- 5PRIME Phase Lock Gel tubes (Quantabio)
- Cooling centrifuge
- Heating block with shaking function (e.g., ThermoMixer, Eppendorf)
- RNA Nano Kit (Agilent)
- Spectrophotometer (e.g., NanoDrop 2000)

2.1.2 Buffers and Reagents

- 75% (vol./vol.) Ethanol
- Chloroform
- Ethanol–sodium acetate (3 *M*, pH 5.2) solution with 30:1 ratio
- ExtraZol (e.g., 7Bioscience)
- Propan-2-ol (Isopropanol)
- Nuclease-free water
- Phenol/Chloroform/Isoamyl alcohol solution (25:24:1 vol./vol.; e.g., Carl Roth)
- Stop-Mix (95% [vol./vol.] ethanol, 5.0% [vol./vol.] phenol)
- TURBO DNase (2 U/μL) & 10× Reaction Buffer (Thermo Fisher Scientific)

2.1.3 Procedure

1. Inoculate two or three independent clones of each strain and cultivate overnight in appropriate medium. Next day, dilute overnight cultures 1:1000 in fresh media and incubate at desired conditions.

2. Harvest cells (we recommend ≈4 OD equivalents, $\approx 4 \times 10^9$ cells) at selected growth phase(s) and immediately apply Stop-Mix (20% final conc. of the collected culture volume) to stop transcription and translation. Snap-freeze and store the mixture at −80°C, or directly continue to step 3.
3. Thaw RNA samples on ice and spin cells at 4°C (4000 rpm, 15 min for 15- or 50-mL tubes and 13,000 rpm, 2 min for 1.5- or 2-mL tubes, respectively). Discard the supernatant.
4. Suspend the cell pellet in 1 mL ExtraZol and incubate the lysates at RT for 5 min. Transfer the cell lysates to Phase Lock Gel tubes. Add 250 μL chloroform and shake vigorously for 10 s. Incubate at RT for 5 min prior to centrifugation (12°C, 13,000 rpm, 15 min). Transfer the RNA-containing aqueous phase (≈500 μL) to a fresh 1.5-mL reaction tube.
5. Precipitate the RNA using 500 μL of isopropanol, invert tubes 10 times, and let stand at RT for 30 min prior to centrifugation (4°C, 13,000 rpm, 30 min).
6. Remove the supernatant and wash (do not suspend) the RNA pellet with 350 μL of 75% ice-cold ethanol and centrifuge (RT, 13,000 rpm, 10 min). Repeat this step for a total of two washing steps.
7. Carefully remove any residual ethanol and air-dry pellets at RT for 10 min. Add 30 μL of nuclease-free water and dissolve RNA in a heating block at 60°C with vigorous shaking for 5 min. Determine the RNA concentration using a spectrophotometer.
8. Store RNA at −80°C or continue directly to step 9.
9. Use nuclease-free water to dilute 20 μg of RNA in a total volume of 89 μL. Add 10 μL of 10 × Reaction Buffer and 1 μL of TURBO DNase. Mix well and incubate at 37°C for 30–45 min to digest cellular DNA.
10. To remove the DNase enzyme, transfer the mixture to Phase Lock Gel tubes, add 100 μL PCI solution, and shake vigorously for 10 s. Centrifuge (12°C, 13,000 rpm, 15 min) and transfer the aqueous phase (≈100 μL) to a fresh 1.5-mL reaction tube.
11. For RNA precipitation, add 300 μL of the EtOH–NaOAc 30:1 solution, invert 10 times, and incubate at −20°C for 1 h to overnight. Collect RNA by centrifugation (4°C, 13,000 rpm, 30 min).
12. Wash, dry, and dissolve RNA by repeating steps 6 and 7.
13. Check RNA integrity using a RNA Nano chip on a 2100 Bioanalyzer. The electrophoresis profile should look similar to the one shown in Fig. 3A and the RIN needs to be ≥7.

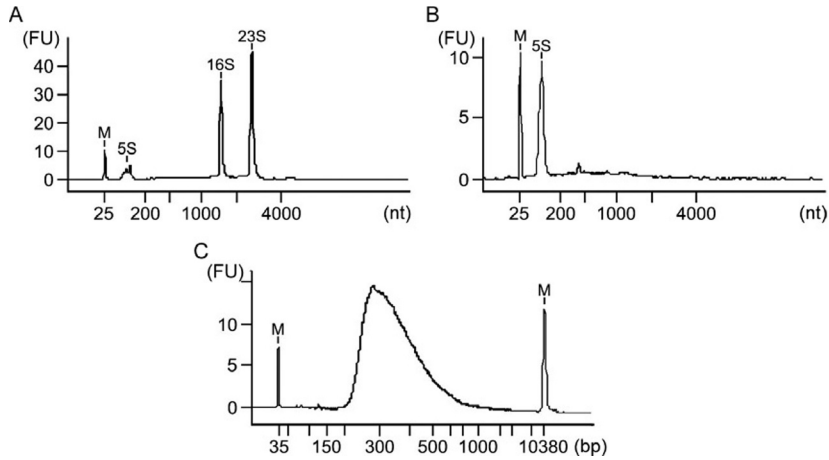

Fig. 3 Quality assessment after different steps of library preparation using a 2100 Bioanalyzer. Electrophoresis profiles obtained from (A) DNA-free total RNA, (B) 16S and 23S rRNA-depleted RNA, (C) a sequencing-ready cDNA library. Marker peaks are indicated (M).

2.1.4 Notes

1. Briefly spin the Phase Lock Gel tubes before use to collect gel at the bottom of the tubes.
2. Use nuclease-free water for the preparation of ethanol and NaOAc solutions.

2.2 Enrichment of Primary Transcripts and Generation of 5′ P Ends for Linker Ligation

The bacterial RNA pool consists of primary and processed transcripts. Primary transcripts are marked by a triphosphate group at the 5′ end (5′ PPP), whereas processed RNAs possess a 5′ monophosphate (5′ P). Less frequently, multiple processing of transcripts generates RNA species with a 5′ hydroxyl group (5′ OH). The goal of the dRNA-seq approach is to distinguish between primary and processed transcripts. For this purpose, the original RNA sample is split into two parts: One remains untreated, while the other part is treated with 5′-P-dependent TEX, an enzyme that specifically depletes transcripts carrying a 5′ P group. Thus, in the TEX-treated samples, processed transcripts such as the abundant rRNAs and tRNAs are degraded, and primary transcripts including mRNAs and sRNAs are relatively enriched. The TEX procedure is followed by treatment of both samples

with tobacco acid pyrophosphatase (TAP), which converts the 5′ triphosphate ends to monophosphate and thus allows RNA linker ligation. Finally, the RNA samples are subjected to poly(A) tailing and cDNA libraries are produced. Preparation of cDNA libraries and RNA-seq of both TEX-treated and untreated samples results in a typical enrichment pattern (Fig. 2), allowing for the global annotation of transcriptional start sites (TSS) and revealing putative sRNA genes. For example, the coverage plot of a +TEX-treated library (Fig. 2, bottom) shows a distinct enrichment of reads toward the 5′ end of the *vqmR* gene, compared to the −TEX sample. Of note, processed transcripts that harbor a 5′ OH end are not substrates of TAP and will therefore not appear in the final libraries. To include these noncanonical processed RNAs in dRNA-seq experiments, samples require treatment with a T4 polynucleotide kinase prior to TAP treatment.

2.2.1 Equipment

- 2100 Bioanalyzer (Agilent)
- 5PRIME Phase Lock Gel tubes (Quantabio)
- Cooling centrifuge
- Heating block with shaking function (e.g., ThermoMixer, Eppendorf)
- RNA Nano Kit (Agilent)
- Spectrophotometer (e.g., NanoDrop 2000)

2.2.2 Buffers and Reagents

- 75% (vol./vol.) Ethanol
- EDTA solution (0.5 *M*, pH 8.0)
- Ethanol–sodium acetate (3 *M*, pH 5.2) solution with 30:1 ratio
- GlycoBlue (15 mg/mL; Thermo Fisher Scientific)
- Nuclease-free water
- Phenol/Chloroform/Isoamyl alcohol solution (25:24:1 vol./vol.; e.g., Carl Roth)
- SUPERase In RNase Inhibitor (20 U/μL; Thermo Fisher Scientific)
- Terminator 5′-Phosphate-Dependent Exonuclease (1 U/μL) and 10× reaction buffers A and B (Epicentre)
- Tobacco Acid Pyrophosphatase (10 U/μL) and 10× reaction buffer (Epicentre)

2.2.3 Procedure

1. Transfer equal amounts of DNA-free RNA into two reactions tubes ("-TEX" and "+TEX"). We use 7 μg of RNA and adjust the reaction volume to 37.5 μL using nuclease-free water.

2. Denature RNA at 90°C for 2 min. Immediately put samples back on ice and incubate for 5 min.
3. Add 0.5 μL of SUPERase In RNase Inhibitor and 5.0 μL of the 10× TEX reaction buffer. Add 7 μL of TEX to the "+TEX" sample and treat the "−TEX" sample with the same volume of nuclease-free water. Incubate the samples at 30°C for 30 min.
4. Place tubes on ice and add 0.5 μL of the EDTA solution to stop the enzymatic reaction.
5. Add 49.5 μL of nuclease-free water to adjust to a total volume to 100 μL and transfer the mixture to Phase Lock Gel tubes. Add 100 μL of PCI solution per tube, shake vigorously for 10 s, and separate phases by centrifugation (12°C, 13,000 rpm, 15 min).
6. Transfer the aqueous phase to a fresh reaction tube and add 2 μL of GlycoBlue to increase precipitation efficiency and to visualize the RNA pellet during the following steps.
7. For RNA precipitation, add 300 μL of the EtOH–NaOAc solution, invert 10×, and incubate at −20°C overnight. Collect RNA pellet by centrifugation (4°C, 13,000 rpm, 30 min).
8. Remove the supernatant and wash (do not suspend) the RNA pellet with 100 μL of 75% ice-cold ethanol and centrifuge (4°C, 13,000 rpm, 10 min). Repeat this step for a total of two washing steps.
9. Carefully remove any residual ethanol and air-dry pellets at RT. Add 11 μL of nuclease-free water and dissolve RNA in a heating block at 60°C with vigorous shaking for 5 min.
10. Determine RNA concentration using a spectrophotometer. The RNA concentrations of the "+TEX" samples should be lower than in the "−TEX"-treated samples, due to the removal of processed transcripts.
11. Denature the remaining 10 μL of the ±TEX-treated RNA at 90°C for 1 min and immediately place samples back on ice for 5 min.
12. Prepare a TAP master-mix. Per sample calculate the following volumes: 2.0 μL of 10× TAP buffer, 0.5 μL TAP enzyme, 0.5 μL SUPERase In RNase Inhibitor, and 7 μL nuclease-free water. Add 10 μL of the mix to each sample and mix well by pipetting. Incubate at 37°C for 1 h.
13. Add 80 μL nuclease-free water to adjust the total volume to 100 μL. Transfer the mixture to Phase Lock Gel tubes and add 100 μL of PCI solution. Shake vigorously for 10 s and separate phases by centrifugation (12°C, 13,000 rpm, 15 min). Transfer the aqueous phase to a fresh reaction tube and add 0.8 μL of GlycoBlue.

14. Precipitate, wash, dissolve, and measure RNA concentration, as described in steps 7–10 except dissolve in 20 μL of nuclease-free water.
15. Check TEX treatment using the RNA Nano Kit and a 2100 Bioanalyzer (Agilent).

2.2.4 Notes

1. Use nuclease-free water for the preparation of ethanol and NaOAc solutions.
2. Briefly spin Phase Lock Gel tubes before use to collect gel matrix at the bottom of the tube
3. The ±TEX samples can also be analyzed by visual inspection using polyacrylamide gels (Borries, Vogel, & Sharma, 2012).

2.3 cDNA Library Preparation

The reads obtained from dRNA-seq experiments require strand-specific information to allow mapping onto a reference genome in the correct orientation (sense or antisense). To this end, the TEX- and TAP-treated transcripts are polyadenylated at the 3′ end and RNA linkers are ligated to the 5′ ends. Oligo(dT)-adapter primers bind to the complementary 3′ poly(A) stretches and a reverse transcriptase is used to perform first-strand cDNA synthesis. The cDNA libraries are amplified in a PCR reaction using barcoded primers. These barcodes are specific nucleotide sequences allowing massive parallel sequencing on an appropriate sequencing platform and subsequent sample-specific in silico assignment of reads (demultiplexing). For a more detailed protocol of the cDNA library preparation, the reader is referred to Berezikov et al. (2006) and Borries et al. (2012).

2.3.1 Equipment

- 2100 Bioanalyzer (Agilent)
- Agencourt AMPure XP beads (Beckman Coulter)
- DNA 1000 Kit (Agilent)
- Magnetic rack
- PCR thermocycler

2.3.2 Buffers and Reagents

- *E. coli* Poly(A) Polymerase (5 U/μL) and 10× reaction buffer (New England Biolabs)
- High-fidelity DNA polymerase (e.g., Q5 Polymerase, New England Biolabs) and reaction buffer

- Illumina primers for PCR amplification
- Illumina sequencing adapter for 5′ and oligo(dT)-adapter primer for 3′ end, e.g., TrueSeq Sense primer (5′ → 3′ orientation):
  AATGATACGGCGACCACCGAGATCTACACTCTTTCCCT ACACGACGCTCTTCCGATCT
  Antisense primer: NNNNNN = barcode sequence for multiplexing (5′ → 3′ orientation)
  CAAGCAGAAGACGGCATACGAGAT-NNNNNN-GTGACT GGAGTTCAGACGTGTGCTCTTCCGATC(dT25)
- M-MLV reverse transcriptase (200 U/μL) and 5 × reaction buffer (Thermo Fisher Scientific)
- T4 RNA Ligase (10 U/μL) and 10 × reaction buffer (New England Biolabs)
- dNTPs

2.3.3 Procedure

- cDNA libraries are generated as previously described (Berezikov et al., 2006; Borries et al., 2012), but omitting RNA size-fractionation prior to cDNA synthesis. Briefly, equal amounts of ± TEX- and TAP-treated RNA are incubated with poly(A) polymerase and a 5′ Illumina linker is ligated to the 5′ ends. Next, first-strand synthesis is performed using an oligo(dT)-adapter primer and the M-MLV reverse transcriptase. The incubation steps are conducted at 42°C for 20 min and 5 min at 55°C. Finally, cDNA libraries are amplified by PCR using a high-fidelity polymerase and Illumina adapter primers. The DNA is subsequently purified using AMPure XP beads (1.8 × sample volume). The quality of cDNA libraries is assessed on a DNA chip in a Bioanalyzer, and deep sequencing is performed on an Illumina HiSeq 2000 platform following standard protocols.

2.3.4 Notes

1. If adapter dimers are prominent in the electrophoresis profile of the DNA chip, a size selection step is recommended to avoid extensive sequencing of these self-ligated fragments.

3. IDENTIFICATION OF sRNA TARGET GENES

Determining the target spectrum of an sRNA is an important step in understanding its physiological roles. Given that the vast majority of Hfq-dependent sRNAs regulate multiple *trans*-encoded mRNAs

(Papenfort & Vogel, 2009; Waters & Storz, 2009), the search for putative targets requires a global view on gene expression and often involves transcriptome profiling. Such analyses have been particularly useful in the case of Hfq-dependent sRNAs because RNA-duplex formation is typically associated with changes in transcript abundance (Mohanty & Kushner, 2018), which can be measured by RNA-seq. Thus, sRNA overexpression followed by global transcriptome analysis is a promising strategy to discover target mRNAs. However, especially constitutive overexpression of an sRNA is prone to cause pleiotropic effects. For example, the regulation of a transcription factor as a primary sRNA target could indirectly affect the expression of dozens of downstream genes. Further, in some cases, constitutive sRNA overexpression from a multicopy plasmid is toxic (Sharma, Darfeuille, Plantinga, & Vogel, 2007; Sharma et al., 2011) or could increase the frequency of suppressor mutations. To circumvent these limitations, an inducible promoter (e.g., the arabinose-regulated P_{BAD} promoter) is used to tightly regulate sRNA expression from a multicopy plasmid. The rationale behind this approach is to strongly induce sRNA expression for 10–15 min (pulse-expression), which is sufficient to score changes in transcript levels of primary targets, but will not affect the regulation of downstream genes. Of note, sRNA pulse-expression followed by RNA-seq will not automatically reveal all primary targets of an sRNA because certain sRNA–mRNA interactions do not result in rapid transcript decay. Moreover, some targets may not be transcribed under the conditions tested. Fig. 4 illustrates the sRNA pulse-expression method, which we will explain in more detail in the following sections.

3.1 Generation of Inducible sRNA Overexpression Plasmids

For an sRNA pulse-expression experiment as described above a suitable sRNA expression plasmid should be generated. We recommend the use of mid- to high-copy plasmids, e.g., carrying pBR322 or p15a origins of replication (Sambrook, 2001). Further, the plasmid requires an inducible promoter, tightly controlling sRNA expression. The arabinose-dependent P_{BAD} promoter has been established as the promoter of choice for sRNA pulse-expression experiments (Papenfort et al., 2006) and is therefore used in our experiments. It is crucial, that the full-length sRNA sequence is inserted at the TSS position of the P_{BAD} promoter, since transcription of an extended or truncated sRNA variant possibly impairs or alters its regulatory potential. The Gibson assembly method (Gibson et al., 2009) allows the rapid and precise insertion of an sRNA gene into a linearized plasmid backbone carrying the P_{BAD} promoter and is described below.

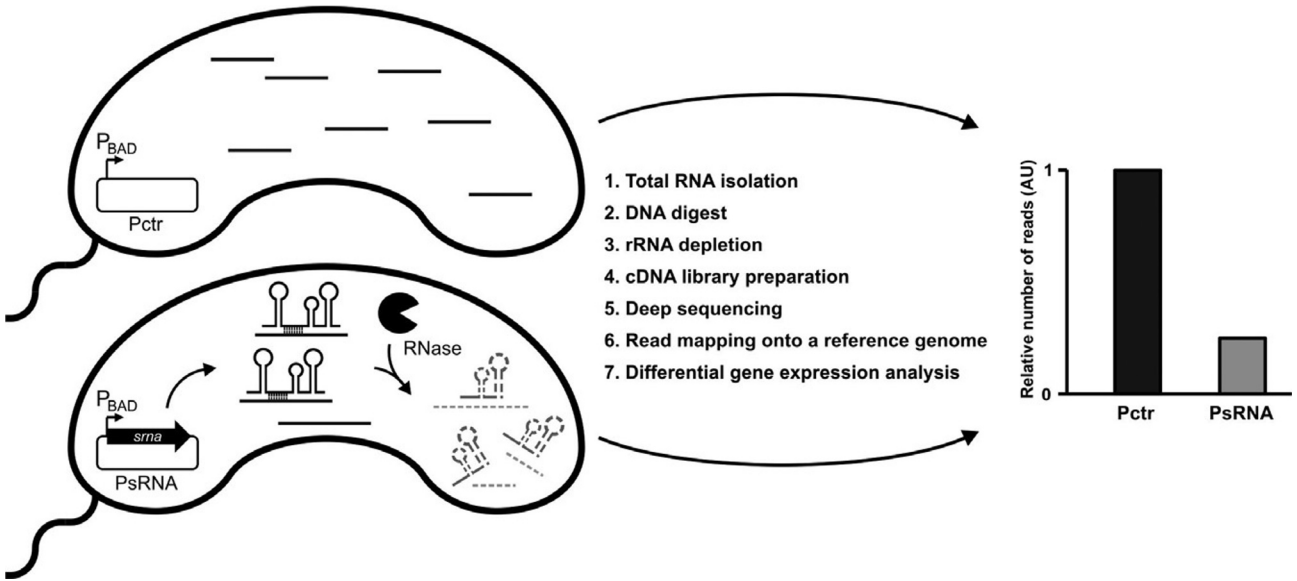

Fig. 4 Identification of sRNA targets using sRNA pulse-expression and RNA-seq. An empty control plasmid (Pctr) and an L-arabinose inducible P_{BAD} sRNA overexpression plasmid (PsRNA) are introduced into *V. cholerae* strains. Both strains are treated with L-arabinose triggering sRNA expression of the PsRNA-carrying strain. The sRNAs engage RNA duplex formation with target mRNAs, which typically results in altered transcript levels. After 10–15 min of induction, total RNA is isolated and prepared for RNA-seq. Transcripts with significantly changed read counts in the samples overexpressing the sRNA, compared to the Pctr samples, are potential sRNA targets.

3.1.1 Equipment

- Standard horizontal agarose gel electrophoresis system
- Electrophoresis power supply
- Gel documentation system
- PCR purification kit (e.g., DNA Clean & Concentrator Kit, Zymo Research)
- PCR thermocycler
- Plasmid DNA purification kit (e.g., HiYield Plasmid Mini DNA Kit, e.g., Südlabor)
- Spectrophotometer (e.g., NanoDrop 2000, Thermo)

3.1.2 Buffers and Reagents

- Appropriate antibiotics for plasmid selection
- dNTPs
- *Dpn*I restriction enzyme (20 U/μL; New England Biolabs)
- HiFi DNA Assembly Master Mix (New England Biolabs)
- High-fidelity DNA polymerase (e.g., Q5 Polymerase, New England Biolabs) and reaction buffer
- DNA oligonucleotides

3.1.3 Procedure

1. Linearize an expression plasmid carrying the inducible P_{BAD} promoter, by PCR. The primer sequences should be designed so that the −1 position of the P_{BAD} TSS is located at one end of the linearized product.
2. Amplify the sRNA of interest by PCR, using DNA oligonucleotides with 15–20 nts of sequence overlap to the ends of the linearized backbone.
3. Run PCR products on an agarose gel to ensure correct and specific amplification.
4. *Dpn*I digest all PCR products that are derived from a plasmid DNA template.
5. Purify DNA using a commercially available kit and determine the PCR product concentrations with a spectrophotometer.
6. Assemble the two fragments using the HiFi DNA Assembly Master Mix according to manufacturer's instructions. Transform the mixture into competent *E. coli* cells and plate on selective LB-agar plates.
7. Make sure that the fragments are assembled correctly via isolation and sequencing of the plasmid DNA.

8. Conjugate the sRNA expression plasmid and an empty control vector into *V. cholerae* cells. To avoid endogenous sRNA regulation, the plasmids should be introduced into an sRNA mutant strain.

3.1.4 Notes

1. For all enzymatic reactions, DNA purification, and plasmid DNA isolation, follow the manufacturers' protocols.
2. Make sure that the sRNA gene is inserted into the plasmid in the correct orientation. The DNA oligonucleotide that binds at the 5′ end of the sRNA should carry an extended sequence complementary to the end of the linearized plasmid, which displays the −1 position of the TSS of the inducible promoter.

3.2 sRNA Pulse-Expression, RNA Preparation, and DNA Digest

The timing of sRNA induction is of considerable importance, since putative target genes need to be expressed at this particular time point. If the target transcripts are not expressed, they will not be represented in the transcriptomic data. Profiling the expression of the sRNA of interest under various conditions is often helpful to identify a "sweet spot" for sRNA induction and can easily be monitored on Northern blots (see Section 4.1). Assuming that the sRNA is highest expressed when its function is needed the most, the time point that shows the highest sRNA production might also be most suitable for sRNA pulse-expression. Further, the experiment should be performed in an sRNA deletion background to avoid target regulation by the chromosomally expressed sRNA copies. For example, the VqmR sRNA is highly expressed in stationary phase and the *vpsT* mRNA is a known target of VqmR (Figs. 1 and 5). Thus, during stationary growth phase *vpsT* levels are low in *V. cholerae* wild-type cells and an additional pulse-expression of VqmR may only have mild effects. In contrast, *vpsT* levels in a *V. cholerae vqmR* mutant are increased and will be strongly affected upon VqmR overexpression. After 10–15 min of P_{BAD}-controlled sRNA induction, cells are collected and transcription is stopped. Isolation of total RNA and the depletion of genomic DNA is accomplished as described in Section 2.1. If one or more targets of the tested sRNA are already established, the experimental approach can be validated using the isolated RNA and Northern blot or qRT-PCR analyses.

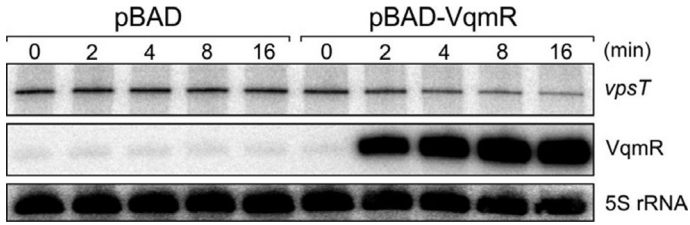

Fig. 5 Northern blot analysis of *vpsT* mRNA after VqmR pulse-expression. *V. cholerae* cells carrying either an empty control plasmid (P_{BAD}) or an L-arabinose inducible VqmR overexpression plasmid (P_{BAD}-VqmR) were cultivated to $OD_{600} = 0.2$. Cultures were treated with L-arabinose and expression levels of *vpsT* and VqmR prior induction (0 min) and 2, 4, 8, and 16 min after induction were analyzed on a Northern blot. 5S rRNA served as loading control.

3.2.1 Equipment

- 5PRIME Phase Lock Gel tubes (Quantabio)
- Cooling centrifuge
- Spectrophotometer (e.g., NanoDrop 2000)
- Heating block with shaking function (e.g., ThermoMixer, Eppendorf)

3.2.2 Buffers and Reagents

- 75% (vol./vol.) Ethanol
- Appropriate antibiotics (for plasmid maintenance)
- Inducer (e.g., L-arabinose when using the P_{BAD} promoter)
- Chloroform
- ExtraZol (7Bioscience)
- Nuclease-free water
- Phenol/Chloroform/Isoamyl alcohol solution (25:24:1 vol./vol.; e.g., Carl Roth)
- Propan-2-ol (Isopropanol)
- Stop-Mix (95% [vol./vol.] ethanol, 5.0% [vol./vol.] phenol)
- TURBO DNase (2 U/μL) & 10× Reaction Buffer (Thermo Fisher Scientific)

3.2.3 Procedure

1. Dilute overnight cultures of three independent clones each of the strains harboring the sRNA overexpression plasmid and the empty control plasmid, respectively, 1:1000 in fresh media (add appropriate antibiotics to maintain plasmid).

2. At an appropriate OD, treat all cultures with inducer to gain maximal sRNA expression (e.g., 0.2% final conc. of L-arabinose to induce a P_{BAD} promoter). After 10–15 min of induction, immediately proceed with step 3.
3. Harvest cells, isolate the RNA, and deplete chromosomal DNA by following steps 2–13 described in Section 2.1.3.

3.2.4 Notes

1. To test for potential "leakiness" of the inducible promoter, additional RNA samples should be collected immediately before addition of the inducer. Northern Blot analysis (see Section 4.1) can be used to probe cellular sRNA levels.
2. This method aims to identify only primary targets of the sRNA of interest. Thus, induced sRNA expression should not exceed 15 min.

3.3 Ribosomal RNA Depletion and cDNA Library Preparation

Ribosomal RNA (rRNA) constitutes approximately 70%–80% of the bacterial RNA pool (Westermann, Gorski, & Vogel, 2012). However, in most transcriptomic studies, rRNAs are of minor interest and are therefore depleted to enrich other RNA species (e.g., mRNAs and sRNAs) prior to cDNA library construction. Several methods have been developed to remove bacterial rRNAs, for example, size-fractionation using gel electrophoresis (Liu et al., 2009), coimmunoprecipitation of protein-bound RNAs (Sittka et al., 2008), or TEX treatment (Section 2.2). Further, there are a variety of commercially available kits, which are based on species-specific probes, which hybridize to rRNA sequences. These probes attach to magnetic beads and are easily separated in the presence of a magnet. In our experience, the Ribo-Zero Illumina Kit is suitable to remove the predominant 16S and 23S rRNA transcripts of *V. cholerae*. Of note, due to the rapid improvement of sequencing depth and the drop of sequencing costs, rRNA depletion has become an optional rather than an essential step. Nevertheless, the decision for or against rRNA depletion should be made carefully and involves multiple factors. For example, desired sequencing coverage and overall costs should be considered.

RNA sequences need to be converted into cDNA libraries in order to be applicable to most NGS platforms. To distinguish between sense and antisense reads after sequencing, the construction of cDNA libraries requires a strand-specific protocol. There are several established protocols that maintain strand-specific information, for example, poly(A)-tailing and 5′-end

linker ligation (as described in Section 2.3), first-strand sequencing (Croucher et al., 2009), bisulfite-induced C to U conversions prior cDNA synthesis (He, Vogelstein, Velculescu, Papadopoulos, & Kinzler, 2008), or the incorporation of dUTPs during second-strand cDNA synthesis (Parkhomchuk et al., 2009). The latter, so-called dUTP-method, is also applied in the commercially available NEBNext Ultra II Directional RNA Library Prep Kit, which we describe here. The kit's protocol includes a fragmentation step prior to first-strand synthesis increasing the distribution of reads along transcripts. The fragments are primed with random hexamers, which enable first-strand synthesis. In contrast to standard library construction protocols, dUTP nucleotides are used instead of dTTPs for second-strand synthesis. After dA-tailing and adapter ligation, the second strand is degraded using uracil-DNA glycosylase. The first-strand cDNA sequences are recovered, enriched by PCR, and finally sequenced on an Illumina sequencing platform.

3.3.1 Equipment

- 2100 Bioanalyzer (Agilent)
- Agencourt AMPure XP beads (Beckman Coulter)
- Centrifuge
- Magnetic rack (e.g., Life technologies)
- NEBNext Multiplex Oligos for Illumina (New England Biolabs)
- NEBNext Ultra II Directional RNA Library Prep Kit (New England Biolabs)
- PCR thermocycler or heating block
- Ribo-Zero rRNA Removal Kit for Bacteria (Illumina)
- RNA Nano Kit (Agilent)
- Spectrophotometer (e.g., NanoDrop 2000)
- Vortex mixer

3.3.2 Buffers and Reagents

- Ethanol (100% and 70% [vol./vol.])
- Nuclease-free water

3.3.3 Procedure

1. Deplete rRNA using the Ribo-Zero Kit, following the manufacturer's instructions. Briefly, 1–5 μg of DNA-free total RNA is mixed with probes that specifically hybridize to microbial rRNAs. The probes bind to magnetic beads and are easily separated from the rRNA-depleted RNA fraction. To clean up the recovered RNA, an ethanol precipitation step

is required. Analyze rRNA-depleted RNA samples on a 2100 Bioanalyzer platform using the RNA Nano Kit. The electrophoresis profiles should look similar to Fig. 3B.

2. cDNA libraries are prepared using the NEBNext Ultra II Directional RNA library Prep Kit for Illumina sequencing according to the manufacturer's protocol. In brief, 1–100 ng rRNA depleted RNA is fragmented to sizes of ≈200 nt. These fragments are randomly primed which allows first-strand cDNA synthesis. During second-strand synthesis, dUTP is incorporated and the cDNA products are purified using AMPure XP beads. After end repair and dA-tailing, NEBNext adaptors are ligated and the USER enzyme (Uracil-Specific Excision Reagent) cleaves at the uracil-incorporation sites. AMPure XP beads are used for size-selection and purification of the cDNA libraries. The libraries are amplified using NEBNext oligos for multiplexing and purified in a final cleanup step with AMPure XP beads. Quality of cDNA libraries is assessed on a DNA chip in a 2100 Bioanalyzer. Fig. 3C illustrates an example of a typical cDNA library size distribution.

3.3.4 Notes

1. The probes of the Ribo-Zero Kit do not hybridize to the 5S rRNA of *V. cholerae* (Fig. 3B).
2. If peaks at ≈80 bp (primers) or 128 bp (adapter dimer) appear in the Bioanalyzer traces of a cDNA library, the final cleanup step using AMPure XP beads should be repeated.

4. VALIDATION OF sRNA-TARGET INTERACTIONS

RNA-seq data obtained from sRNA overexpression experiments as described in Section 3.2 typically reveal multiple putative target transcripts of the tested sRNA (Wagner & Romby, 2015). To validate and characterize a direct sRNA-mediated regulation of a transcript, a combination of biochemical, genetic, and computational methods is required.

4.1 Northern Blots

Northern blotting is a well-established method for sensitive and highly specific detection of transcripts using radioactively labeled probes. In contrast to other methods (such as qRT-PCR), processing of a transcript can be easily visualized. Further, the transferred RNA is covalently bound to a membrane, which allows frequent reprobing of the same Northern blot.

Prior to RNA transfer onto a membrane, RNA needs to be size-separated using denaturating agarose or polyacrylamide gels and electrophoresis. Agarose gels are preferred for separation of large transcripts (>1200 nts); however, they provide only limited resolution for smaller fragments. In contrast, polyacrylamide gels provide a superior resolution in the range of <20 to approximately 1200 nts, depending on the acrylamide–bisacrylamide concentration of the gel (Rio, Ares Jr, Hannon, & Nilsen, 2010). Consequently, polyacrylamide gels are favored for Northern blot analyses of sRNAs, which are typically between 50 and 350 nts in size, and putative target transcripts that do not exceed 1200 nts. For example, the Northern blot shown in Fig. 5 shows that the VqmR sRNA (151 nt in length) promotes the decay of the ≈750 nt *vpsT* mRNA. Expression levels of larger transcripts (e.g., transcribed operons) can either be monitored by Northern blotting using agarose gel electrophoresis, or via qRT-PCR. Due to its minimal input requirements of RNA template, qRT-PCR can be the method of choice to quantify poorly expressed transcripts. In this section, we explain the Northern blot procedure using polyacrylamide gels. For more detailed information about Northern blotting using agarose gels and qRT-PCR analyses, we refer the reader to published protocols (Lan, Tang, Un San Leong, & Love, 2009; Rio, 2015).

4.1.1 Equipment

- Vertical electrophoresis system (e.g., VWR, PerfectBlue Dual Gel System Twin L)
- Tank blotter (e.g., VWR, PerfectBlue Electroblotter)
- Microcentrifuge
- Ambion MAXIscript T7 In Vitro Kit (Thermo Fisher Scientific)
- Centrifuge
- Electrophoresis power supply
- Transparent sheet protectors
- Gel documentation system
- Heating block
- Hybridization oven
- Hybridization tubes
- Microspin G-25/G-50 Columns (GE Healthcare)
- Nylon membrane (e.g., Amersham Hybond-XL Membrane, GE Healthcare)
- PCR purification kit (e.g., DNA Clean & Concentrator, Zymo Research)

- PCR thermocycler
- Phosphorimager (e.g., Typhoon FLA 7000, GE Healthcare Life Sciences)
- Spectrophotometer (e.g., NanoDrop 2000)
- Storage phosphor screen and exposure cassette
- UV light chamber
- Whatman paper

4.1.2 Buffers and Reagents

- 2 × RNA gel loading buffer (98%[v/v] formamide, 0.02% [w/v] xylene cyanol, 0.02% [w/v] bromophenol blue, 2 m*M* EDTA)
- 10 × TBE buffer
- 19:1 Acrylamide:Bisacrylamide solution (40% [w/v])
- Alpha-^{32}P-UTP (10 mCi/mL, for riboprobes)
- APS (10% [w/v])
- DNA marker (e.g., 50 bp DNA Ladder, New England Biolabs)
- DNA polymerase (e.g., Q5 Polymerase, New England Biolabs) and 5 × reaction buffer
- dNTPs
- Gamma-^{32}P-ATP (10 mCi/mL)
- Hybridization buffer (e.g., Roti-Hybri.Quick, Carl Roth)
- Nuclease-free water
- SSC buffers (5 ×, 1 ×, 0.5 ×)
- DNA oligonucleotides
- SDS solution (10% [w/v])
- T4 Polynucleotide Kinase (10 U/μL) and 10 × reaction buffer (New England Biolabs)
- TEMED
- Urea

4.1.3 Procedure

1. Prepare a denaturing 7 *M* urea gel for RNA gel electrophoresis (Table 1). For probing of transcripts up to 1200 nts (e.g., mRNAs) use 4% PAA gels; for sRNA detection 6%–8% PAA gels are recommended.
2. Immediately pour the gel and insert the comb. After approximately 10 min the gel is polymerized and fixed in a vertical electrophoresis unit. Fill up the apparatus with 1 × TBE to avoid drying of the gel.
3. For RNA size estimation on Northern Blots, size markers need to be labeled radioactively. Start with diluting 5 μL of a DNA marker (0.5 μg/μL) with 7 μL water, denature the mixture at 95°C for 5 min,

Table 1 Composition of Denaturing Urea Gels With Different PAA Concentrations

	4% PAA 7 *M* Urea Gel	**6% PAA 7 *M* Urea Gel**
19:1 Acrylamide solution	10 mL	15 mL
Urea	42 g	42 g
10 × TBE	10 mL	10 mL
10% APS	800 μL	800 μL
TEMED	106 μL	106 μL
H_2O	ad 100 mL	ad 100 mL

and place it on ice. Add 2 μL 10 × T4 PNK reaction buffer, 1 μL T4 PNK (10 U/μL), and 3–5 μL gamma-^{32}P-ATP. Incubate for 1 h at 37°C. Bring mixture to a total volume of 50 μL and purify the labeled marker using a Microspin G-50 Column according to the manufacturer's instructions. Dilute the cleanedup marker with 150 μL 1 × RNA loading buffer.

4. Prepare 10 μg of total RNA for each sample using a spectrophotometer and adjust volumes with nuclease-free water. Add the same volume of 2 × RNA loading dye. Use ≈1 μL of a freshly labeled marker and adjust the volume to 10 μL with 1 × RNA loading buffer. Boil all samples (including the marker) for 5 min at 95°C and immediately put back on ice.
5. Remove the comb from the gel and use a pipette to flush the pockets with 1 × TBE to allow proper loading. Carefully load the samples into the wells without disturbing the gel matrix and run the gel at 250–300 V for approximately 2–3 h.
6. Build the blotting "sandwich" in the following order (bottom to top): three layers of Whatman paper, released gel (note 2), nylon membrane, three layers of Whatman paper. Make sure that the Whatman paper and the membrane are wetted with 1 × TBE before use and that trapped air bubbles between the layers are removed (for example, by rolling a glass pipette across the surface). Place the transfer sandwich in the correct orientation into the tank electroblotter and fill up with 1 × TBE. Move the chamber to 4°C and start transfer 50 V for 1 h at 50 V.
7. Disassemble the blotting sandwich, quickly dry the membrane between two Whatman paper, and cross-link the RNA to the membrane using UV light (320 nm, 120 mJ). Make sure that the RNA site is facing the UV lamp.

8. Wet the membrane in 15 mL hybridization buffer in a hybridization tube at 42°C (for oligonucleotide probes) or 63°C (for riboprobes) for at least 30 min, before adding the labeled probe.
9. Riboprobes are generated using the Ambion MAXIscript T7 In Vitro Kit following manufacturer's instructions. Oligoprobes are prepared by mixing 1 μL DNA oligonucleotide (10 μ*M* stock solution), 2 μL 10 × T4 PNK reaction buffer, 1 μL T4 PNK (10 U/μL), and 13 μL nuclease-free water, followed by incubation at 37°C for 1–1.5 h. To remove unincorporated labeled nucleotides, the ribo- and oligonucleotide probes are cleaned up using Microspin G-50 and G-25 Columns, respectively. Both probes can be stored at −20°C.
10. Boil the cleaned-up probe for 5 min and quick chill on ice. Start hybridization by adding 2 or 4 μL of an oligo- or riboprobe, respectively, into the hybridization buffer. Perform hybridization for 2–12 h.
11. Discard hybridization buffer and perform three washing steps (15 min each) using SSC buffers of different concentrations in the following order: 5 ×, 1 ×, and 0.5 × SSC. Always use a washing volume of approximately 50 mL and add SDS to a final concentration of 0.1% (w/v). All washing steps are performed at 42°C.
12. Dry Northern blot membrane between two Whatman papers, move it to a sheet protector and seal the foil. Expose the blot to a storage phosphor screen in an exposure cassette.
13. Detect the signals using a phosphorimager (e.g., Typhoon FLA 7000, GE Healthcare Life Sciences).

4.1.4 Notes

1. The total volume of each sample that is loaded on the gel should not exceed 30 μL. If the RNA concentration is insufficient to prepare 10 μg of total RNA, we recommend to either load less RNA (e.g., 5 μg total RNA) or to increase the RNA concentration via precipitation methods or by using a vacuum concentrator system.
2. After electrophoresis, release the gel from one glass plate by slowly turning one spacer. Use a flexible material with a sticky surface to remove the gel from the second glass plate and transfer it to a Whatman paper.
3. Mark the orientation of the membrane with a pencil.
4. Oligoprobes are easily prepared and sufficient to detect abundant transcripts, e.g., most sRNAs. The generation of riboprobes requires a PCR template to perform the T7 in vitro transcription. In vitro transcription incorporates multiple alpha-^{32}P-UTPs, which allows the

detection of weakly expressed transcripts (e.g., mRNAs of transcription factors) on a phosphor screen.

5. Northern Blot membranes can be reprobed several times. Therefore, previous probes often need to be removed (stripped), which can be achieved by applying boiling distilled water onto the membrane and adding SDS solution to a final concentration of ≈1% (w/v). Seal the container with a lid and incubate on a rocking platform for 20 min. Discard the solution and repeat the "stripping" step. Seal the membrane into a foil and apply an erased phosphor screen to check for removal of the previous probe(s) using a phosphorimager.

4.2 Generation of Plasmid-Based Posttranscriptional Reporters

Transcripts that are regulated in response to sRNA overexpression cannot automatically be classified as direct sRNA targets. For example, an sRNA could inhibit the synthesis of a transcription factor, which would also result in decreased transcript levels of the genes that are controlled by this regulator. Methods like Northern blots and qRT-PCR do not allow to discriminate between direct and indirect sRNA targets. Thus, reporter assays have been established that specifically score for sRNA-mediated posttranscriptional regulation of a selected transcript (Corcoran et al., 2012). Our posttranscriptional reporter assay is based on the constitutive expression of the sRNA and the putative target transcript, which is translationally fused to a fluorescent reporter gene (e.g., *sfgfp*). Specifically, the P_{tac} promoter drives the sRNA expression from a mid-copy plasmid, whereas a P_{tetO} promoter on a low-copy pXG10 plasmid backbone (carrying a pSC101* origin of replication; Corcoran et al., 2012) controls the expression of the translational reporter fusion (Fig. 6). In the case of a direct sRNA–mRNA interaction, translation of the reporter fusion is altered, leading to a decreased (repressed targets, Fig. 6, right) or increased (activated targets) fluorescence signal. Considering that sRNA/mRNA duplex formation frequently occurs at or close to the ribosome-binding site (Waters & Storz, 2009), we recommend to narrow down the transcript sequence to the 5′ UTR and the first 60 bases of the coding sequence. Shortening of the putative target sequence can further reduce the number of "false-positive" results. To reduce *Vibrio*-specific background noise (e.g., through autoregulatory feedback loops and other sRNAs), which could affect the frequency or stability of sRNA–mRNA interactions, these assays can be performed in a heterologous host, such as *E. coli*.

4.2.1 Equipment

- Standard horizontal agarose gel electrophoresis system
- Clear bottom 96-well Microplate (e.g., Greiner 96 Flat Black)
- Electrophoresis power supply
- Gel documentation system
- Microplate reader (e.g., Tecan Spark 10 *M*)
- PCR purification kit (e.g., DNA Clean & Concentrator, Zymo Research)
- PCR thermocycler
- Spectrophotometer (e.g., NanoDrop 2000)

4.2.2 Buffers and Reagents

- 1 × PBS
- Appropriate antibiotics for plasmid cloning
- dNTPs
- *Dpn*I restriction enzyme (20 U/μL; New England Biolabs)
- HiFi DNA Assembly Master Mix (New England Biolabs)
- High-fidelity DNA polymerase (e.g., Q5 Polymerase, New England Biolabs) and reaction buffer
- DNA oligonucleotides

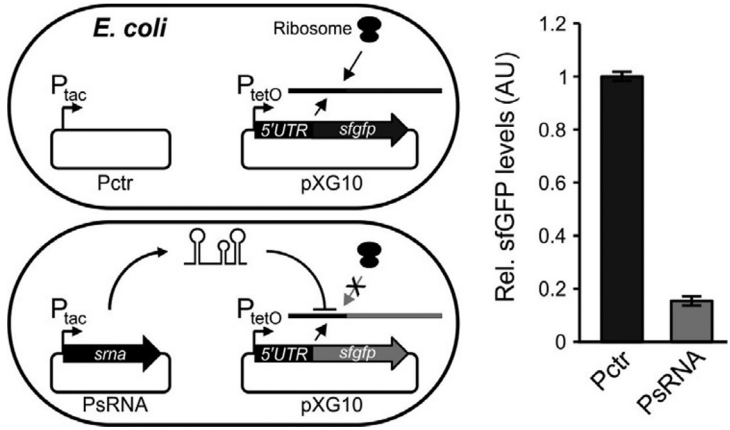

Fig. 6 A posttranscriptional reporter system to investigate direct sRNA-target interactions. *E. coli* cells carrying a pXG10-based target gene fusion were cotransformed with either an empty control plasmid (Pctr) or an sRNA overexpression plasmid (PsRNA). For inhibited target genes translation of the target gene fusion is reduced in the presence of the sRNAs. Thus, sfGFP levels are decreased compared to the cells that carry the control plasmid, and this effect can be measured, e.g., in a microplate reader or by Western blot analysis.

4.2.3 Procedure

1. The sRNA overexpression plasmid can be generated following steps 1–7 of Section 3.1.3; however, the template plasmid should harbor a constitutive promoter. We use a P_{tac} promoter implemented in a plasmid backbone. Additionally, transform an empty vector control.
2. The second plasmid is based on a pXG10 backbone that constitutively expresses a start codon-lacking *sfgfp* gene (starting at the second codon) from a P_{tetO} promoter. To construct a translational reporter fusion of a gene of interest, open the backbone by PCR between the −1 site of the TSS and the first nucleotide of the *sfgfp* gene. Next, the 5′ UTR and the coding sequence of the first 20 amino acids of the putative target gene is amplified from genomic DNA by a set of two primers that carry 15–20 nt overhangs to the ends of the linearized plasmid backbone. Follow steps 3–7 of Section 3.1.3 and transform the plasmid into *E. coli* cells that already carry the sRNA overexpressing plasmid or the empty vector control.
3. Inoculate a single colony of the *E. coli* strains harboring the translational reporter plasmid and either the sRNA overexpression plasmid (PsRNA), or the empty control plasmid (Pctr) and incubate for ~12 h. Make sure to additionally inoculate an *E. coli* strain that lacks any fluorescent proteins (autofluorescence control).
4. Dilute the cultures 1:1000 in fresh media and let the cells grow to an OD_{600} of 0.5–1. Harvest 200 μL of each culture by centrifugation (4°C, 13,000 rpm, 1 min) and wash cell pellets with 1 mL PBS.
5. Repeat the previous step and suspend each pellet in 500 μL PBS. Transfer 3 × 150 μL to 96-well plates (technical triplicates) and measure absorbance at 600 nm and sfGFP fluorescence in a microplate reader.
6. Correct the fluorescence over OD_{600} and subtract the values of the autofluorescence control.

4.2.4 Notes

1. Make sure that both plasmids carry compatible origins of replication (e.g., p15A and pSC101*).
2. Aerobic conditions can promote fluorescence measurements, since oxygen is required for chromophore formation.

4.3 In Silico Prediction of sRNA–Target mRNA Interactions

Hfq-dependent sRNAs usually act through imperfect base pairing with *trans*-encoded target mRNAs. This challenges the development of computational tools, which can predict sRNA–mRNA duplexes at a genome-wide

scale. Algorithms like CopraRNA (Wright et al., 2013), IntaRNA (Mann, Wright, & Backofen, 2017), and TargetRNA2 (Kery, Feldman, Livny, & Tjaden, 2014) allow for the identification of putative sRNA-binding sites in candidate mRNA sequences. Nonetheless, experimental validation of these computationally generated predictions is usually required. After target identification, e.g., by RNA-seq (Section 3), and posttranscriptional reporter experiments (Section 4.2), the exact base pairing site should be located. Here, we recommend the easy-to-use web interface of the RNAhybrid algorithm, which determines the energetically most favorable hybridization site between an sRNA and its target sequence. For example, Fig. 7 shows the predicted duplex formation between VqmR and the *vpsT* mRNA (Papenfort, Forstner, et al., 2015).

4.3.1 Procedure

1. Open the RNAhybrid Web tool at the following URL: https://bibiserv2.cebitec.uni-bielefeld.de/rnahybrid and go to "Submission."
2. Copy and paste the 5′ UTR sequence and the sequence of the first 20 codons of the putative target gene as "target sequence(s)." Next, copy and paste (parts of) the sRNA of interest in the 5′–3′ direction as "miRNA sequence(s)." Convert both sequences into the FASTA format and click next.
3. Select desired parameters. We usually choose a value of 3 for "hits per target" and check the "Generate graphics" box. Click "nothing" as a source of approximate *P*-value estimation. Click next and start calculation.

4.4 Compensatory Base Pair Exchange Experiments

Once an sRNA–mRNA duplex formation has been predicted, it needs to be validated experimentally. To this end, we use the two plasmid posttranscriptional reporter assay described in Section 4.2 and introduce mutations into

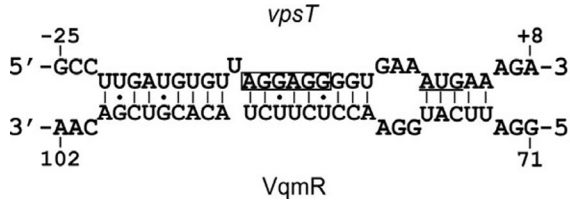

Fig. 7 Predicted base pairing between VqmR and the *vpsT* mRNA. *Numbers* indicate the distances from the translational start site for the *vpsT* mRNA, and the distance from the 5′ end for the VqmR sRNA. The ribosome-binding site is *boxed* and the AUG start codon is *underlined*.

the sRNA and mRNA sequences. Specifically, one base pair of the putative base pairing region is altered to the complementary base. An sRNA–mRNA interaction is confirmed, when the mutagenesis of one nucleotide impairs posttranscriptional regulation, while the introduction of a complementary nucleotide restores regulation.

4.4.1 Equipment

- Standard horizontal agarose gel electrophoresis system
- Black 96-well microplate
- Gel documentation system
- Microplate reader (e.g., Tecan Spark 10 *M*)
- PCR purification kit (e.g., DNA Clean & Concentrator, Zymo Research)
- PCR thermocycler
- Plasmid DNA purification kit (e.g., HiYield Plasmid Mini DNA Kit, SLG)

4.4.2 Buffers and Reagents

- 1 × PBS
- Appropriate antibiotics for plasmid selection
- dNTPs
- *Dpn*I restriction enzyme (20 U/μL; New England Biolabs)
- High-fidelity DNA polymerase (e.g., Q5 Polymerase, New England Biolabs) and 5 × reaction buffer
- DNA oligonucleotides

4.4.3 Procedure

1. Select one base pair of a predicted sRNA–mRNA duplex for site-directed mutagenesis that potentially disrupts the sRNA–mRNA interaction. For example, the G at position −13 of the mRNA shown in Fig. 8A that is predicted to base pair with the C at position 67 of the sRNA is altered to a C. In the reciprocal approach, the C67 of the sRNA is mutated into a G. The implementation of these point mutations into the mRNA and sRNA sequences of the plasmids generated in Section 4.2 is described in the following steps.
2. For each plasmid, design two complementary DNA oligonucleotides that are between 25 and 40 nt in length ($T_m > 70°C$) and carry the desired mutation in the middle. Additionally, a C or G should define the 3′ end of each primer.

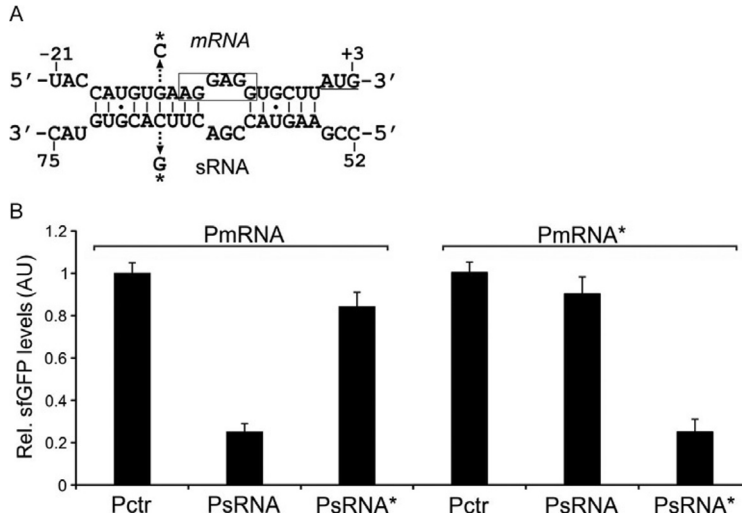

Fig. 8 Compensatory base pair exchange experiments. (A) Predicted RNA duplex formation of a hypothetical sRNA-target pair. The sRNA sequesters the ribosome-binding site (*boxed*) directly upstream of the AUG start codon (*underlined*). *Arrows* and *asterisks* indicate the mutations, which were tested in (B). (B) The posttranscriptional reporter system described in Section 4.2 shows a direct interaction between the target mRNA (PmRNA) and the sRNA (PsRNA). Altering cytosine at position 67 of the sRNA (PsRNA*) inhibits posttranscriptional regulation by the sRNA (*left part*). A compensatory mutation changing guanine at position −13 in the mRNA sequence (PmRNA*) restores repression by PsRNA*, whereas the native sRNA does not inhibit translation (*right part* of the figure).

3. Amplify the plasmids by PCR using the corresponding primer set and run products on an agarose gel.
4. Perform *Dpn*I digest, purify DNA using a commercially available kit, and transform the plasmid DNA into competent *E. coli* cells. The nicks in the mutated plasmid are repaired in vivo.
5. Isolate the plasmid DNA and confirm mutagenesis using Sanger sequencing.
6. Transform the translational reporter plasmid (PmRNA) and its mutated variant (PmRNA*) into competent *E. coli* cells.
7. Introduce a control plasmid (Pctr), the sRNA overexpressing plasmid (PsRNA), and its mutated variant (PsRNA*) into both strains generated in step 6 (resulting in six strains total).
8. Analyze sfGFP levels of these strains as described in Section 4.2.3 (step 3–6). The bar graphs shown in Fig. 8B illustrate the characteristic regulation found in compensatory base pair exchange experiments.

4.4.4 Notes

1. G-C base pairs are a more stable when compared to A-U and G-U pairs and thus make higher contributions to RNA duplex formation. Therefore, G-C base pairs are preferred targets for compensatory base pair exchanges.
2. It may be necessary to alter more than a single nucleotide to disrupt interaction of certain sRNA–mRNA pairs.

5. SUMMARY AND OUTLOOK

In this chapter, we provide a workflow from initial sRNA discovery, to target mRNA identification and validation highlighting the advantages of RNA-seq. dRNA-seq has been employed to explore the primary transcriptomes of bacteria and archaea with single-nucleotide resolution and has boosted the number of TSS and sRNAs in these organisms (Sharma & Vogel, 2014). In *V. cholerae*, dRNA-seq analysis led to the discovery of 107 new regulatory RNAs, including the VqmR sRNA (Papenfort, Forstner, et al., 2015). It has now been established that VqmR is the centerpiece of a novel QS pathway relying on the DPO autoinducer molecule and the VqmA transcription factor (Papenfort et al., 2017). DPO signaling is independent of the AI-2 and CAI-1 autoinducers; however, when detected by *V. cholerae* all three autoinducers repress biofilm formation (Fig. 1). In addition, LuxPQ and CqsS receptors are required to sense AI-2 and CAI-1, respectively. Phosphorylation of LuxO is also affected by two additional sensors, called CqsR and VpsS (Jung, Chapman, & Ng, 2015). Future experiments will show which signaling molecules affect the activity of these sensors and if there are additional sRNAs involved in QS of *V. cholerae*.

In past few years, RNA-seq based methods have largely replaced microarrays to investigate the regulatory networks of bacterial sRNA. Here, we describe the use of sRNA pulse-expression followed by RNA-seq analysis as a tool to identify sRNA target genes. However, this approach only reports on target transcripts with changing stability upon sRNA overexpression. In contrast, certain targets are only translationally inhibited by the sRNA. For example, pulse-expression of the Qrr4 sRNA in *V. harveyi* did not reveal a change in *luxO* transcript stability (Shao, Feng, Rutherford, Papenfort, & Bassler, 2013), although the Qrrs bind to the *luxO* mRNA and block translation (Feng et al., 2015; Tu et al., 2010). To detect these cases of translational inhibition, the pulse-expression RNA-seq approach can be coupled

with ribosome profiling analysis, a method that yields genome-wide profiles of protein synthesis (Guo et al., 2014; Wang et al., 2015).

Besides sRNA pulse-expression, several alternative methods have been described to discover target genes of bacterial sRNAs. For instance, in the MAPS (MS2 affinity purification coupled with RNA-sequencing) approach an sRNA of interest is fused to a MS2 aptamer tag, purified from cell lysates and bound interaction partners are identified using RNA-seq (Carrier, Lalaouna, & Masse, 2016) (see chapter "On the prowl: An in vivo method to identify RNA partners of a sRNA" by Carrier, Morin, & Massé). A related method is GRIL-seq (Global small noncoding RNA target identification by ligation and sequencing), which is based on the in vivo coexpression of an RNA ligase that fuses the sRNA to its targets. The generated chimeric RNAs can be analyzed by RNA-seq and the method comes with the advantage that the sRNA of choice does not require genetic manipulation, e.g., by adding an aptamer tag (Han, Tjaden, & Lory, 2016).

Most sRNAs require an RNA-binding "matchmaker" protein, e.g., Hfq, to facilitate base pairing of the sRNA with its targets, a feature that is harnessed in a related technology, called RIL-seq (RNA interaction by ligation and sequencing). Here, sRNA-target pairs are cross-linked on Hfq, trimmed by ribonucleases, and ligated using T4 RNA Ligase (Melamed et al., 2018, 2016). Generation of cDNA and sequencing of the chimeric RNA allow for the global identification of sRNA-target interactions under selected conditions. Of note, RIL-seq is not limited to Hfq and can also be applied to other RNA-binding proteins such as RNase E (Waters et al., 2017) (see chapter "Transcriptome-wide analysis of protein–RNA and RNA–RNA interactions in pathogenic bacteria" by Tree, Gerdes, & Tollervey). We are yet to apply similar technologies to *V. cholerae* to determine the global RNA interactome of this important pathogen, which could also involve other RNA chaperones mediating sRNA-target interactions, such as ProQ (Holmqvist, Li, Bischler, Barquist, & Vogel, 2018; Smirnov et al., 2016).

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