

Experimental approaches for the discovery and characterization of regulatory small RNA

Cynthia Mira Sharma and Jörg Vogel

Following the pioneering screens for small regulatory RNAs (sRNAs) in *Escherichia coli* in 2001, sRNAs are now being identified in almost every branch of the eubacterial kingdom. Experimental strategies have become increasingly important for sRNA discovery, thanks to increased availability of tiling arrays and fast progress in the development of high-throughput cDNA sequencing (RNA-Seq). The new technologies also facilitate genome-wide discovery of potential target mRNAs by sRNA pulse-expression coupled to transcriptomics, and immunoprecipitation with RNA-binding proteins such as Hfq. Moreover, the staggering rate of new sRNAs demands mechanistic analysis of target regulation. We will also review the available toolbox for wet lab-based research, including *in vivo* and *in vitro* reporter systems, genetic methods and biochemical co-purification of sRNA interaction partners.

Address

RNA Biology Group, Max Planck Institute for Infection Biology, Charitéplatz 1, D-10117 Berlin, Germany

Corresponding author: Vogel, Jörg (vogel@mpiib-berlin.mpg.de)

Current Opinion in Microbiology 2009, 12:536–546

This review comes from a themed issue on
Genomics
Edited by Carmen Buchrieser and Stewart Cole

Available online 14th September 2009

1369-5274/\$ – see front matter
© 2009 Elsevier Ltd. All rights reserved.

DOI 10.1016/j.mib.2009.07.006

Introduction

A couple of decades after the first discovery of a chromosomally encoded small regulatory RNA (sRNA) [1], and eight years after the first genome-wide searches for sRNA genes in *Escherichia coli* [2–4], this field of research has come to full blossom. New sRNAs are being identified and characterized in a wide range of bacterial species; novel technologies as well as modifications of long-standing standard techniques are used to study sRNAs at the genome-wide level. What bacterial sRNAs look like and how they target the activity of mRNAs or proteins to regulate metabolism, stress responses or bacterial virulence were covered in depth by several recent reviews (e.g. [5,6,7,8]). In addition, we recently reviewed along with a historical perspective the approaches that were traditionally used to identify sRNAs

[9] and functionally characterize their targets [10]. Bio-computational analyses have become an integral part of many sRNA studies. Because the present review has its focus on experimental approaches, we refer the reader to recent reviews of algorithms and websites for sRNA and targets searches, and any sRNA discoveries that were primarily facilitated by biocomputation [11,12,13]. This review is organized in two main sections, the first of which describes how sRNAs can be identified based on their expression in the cell, whilst the other describes methods for functional characterization and validation of interaction partners of sRNAs.

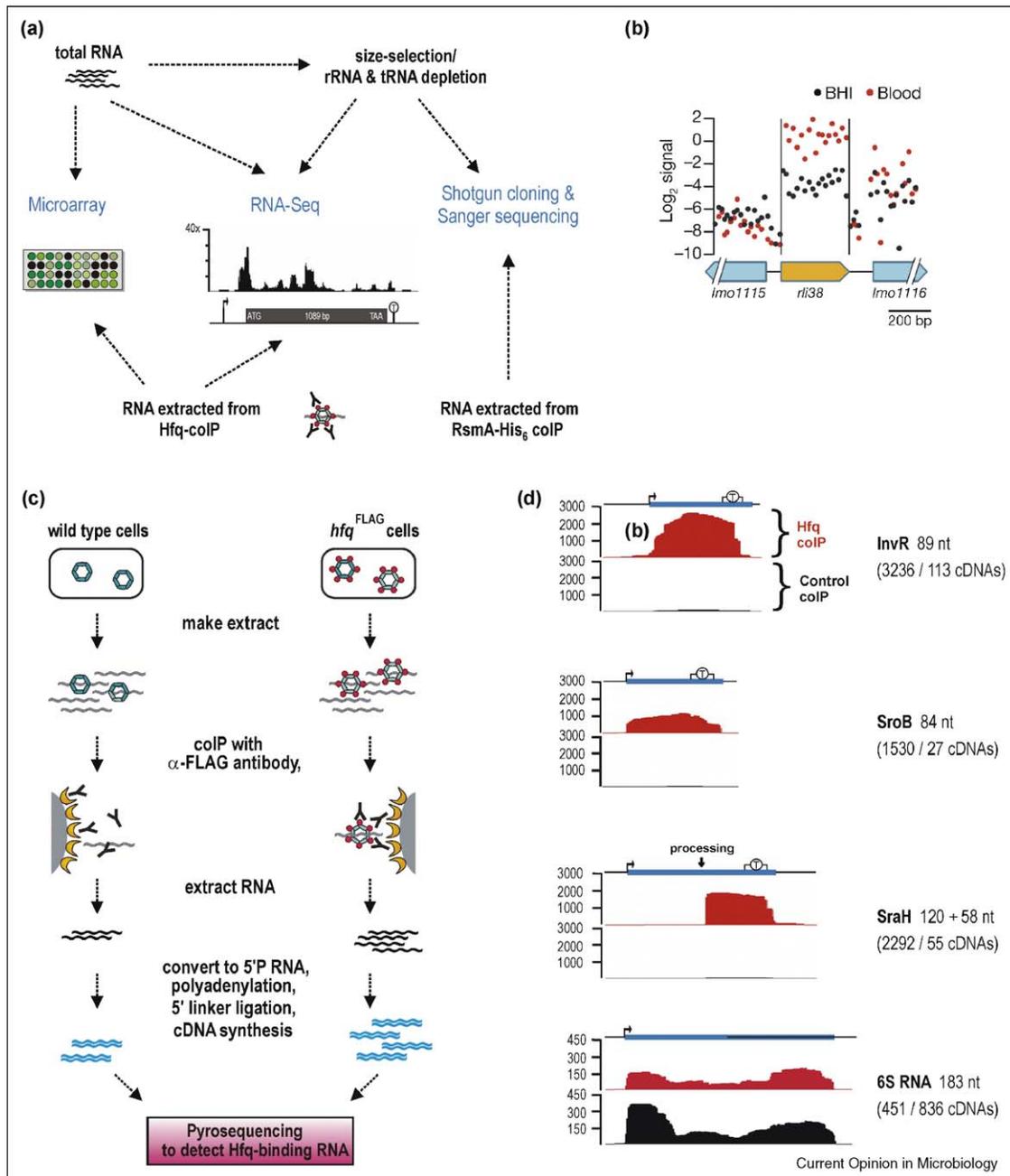
Genome-wide detection of expressed sRNAs

Notwithstanding exceptions (e.g. RNAlII, SgrS [7]), the regulatory sRNAs known to date are typically 50–250 nucleotides in length, untranslated and sufficiently abundant to be detectable by a variety of methods. Historically, several sRNAs were discovered because they produced strong signals when total bacterial RNA was labelled with isotopes or dyes, and run out on denaturing gels [9]. Such visualization is useful to get a first glimpse at the abundant sRNAs of a given organism, especially those bound to proteins of interest; recent examples include the discovery of 6S RNA species in *Bacillus subtilis* [14] or new Hfq-associated sRNAs of *Listeria monocytogenes* [15]. Multi-copy plasmid libraries of chromosomal DNA fragments provide an indirect means to find sRNA genes through scoring for a phenotype or target regulation of interest [9], and brought about several serendipitous discoveries, for example, of the prototypical MicF sRNA [1], post-transcriptional activators of *rpoS* mRNA [5] and the unexpected trapping of MicM sRNA by a polycistronic mRNA [16,17]. Collectively, these methods are well suited to identify individual sRNAs with an *a priori* defined function. In contrast, the global detection of sRNAs is commonly facilitated by cDNA sequencing (RNomics, RNA-Seq) and microarrays (Figure 1A). We will describe these approaches and associated studies (see Table 1 for overview) in more detail below, and discuss some experimental twists that may increase their coverage and sensitivity.

Microarrays

Microarrays have been the most common method for transcriptome analysis, and have successfully been used to discover novel sRNAs. They generally come in two flavors: low-density arrays with oligonucleotide or double-strand (PCR fragments) DNA probes for a defined set of

Figure 1



Detection of sRNAs using microarrays or RNA-Seq. **(A)** sRNAs can be identified using microarrays, shotgun-cloning and Sanger sequencing of size-fractionated RNA, or by high-throughput transcriptomic analysis of total RNA or size-selected RNA via RNA-Seq. Analysis of RNA extracted after coIP of RNA-binding proteins can provide a global picture of sRNAs and mRNAs bound by a protein of interest such as Hfq or CsrA/RsmA. **(B)** Transcriptional tiling map of *Listeria* detects the expression of sRNA genes, such as *rli38*. Furthermore, hybridization of RNA from different growth conditions shows differential expression of the sRNA. Figure taken from [22*]. **(C)** Strategy to identify sRNA and mRNA-binding partners of the widespread RNA chaperone Hfq. RNA can be co-immunoprecipitated with Hfq in extracts from a wild-type and chromosomal *hfq*^{FLAG} strain using an anti-FLAG antibody. The extracted RNA is converted into 5' monophosphate RNA, and subsequently into cDNA, followed by direct pyrosequencing. Figure adapted from [34*]. **(D)** Read distribution of exemplar Hfq-dependent and Hfq-independent sRNAs in *Salmonella*. Read distribution for RNA-Seq results following Hfq-coIP (red) for the Hfq-dependent *InvR*, *SroB* and *SraH* sRNAs, or the Hfq-independent *6S* sRNA, compared to reads obtained from control coIP (black). Vertical axes indicate the number of cDNA sequences that were obtained for each locus; a bent arrow indicates the sRNA promoter, a circled 'T' the transcriptional terminator. Figure adapted from [34*].

Table 1

Experimental sRNA screens in diverse bacteria

Bacterium	Experimental component	Reference
Gram-negative bacteria		
<i>Escherichia coli</i>	Oligonucleotide arrays	[3]
<i>Escherichia coli</i>	RNomics	[28]
<i>Escherichia coli</i>	Hfq co-immunoprecipitation, microarrays	[27]
<i>Escherichia coli</i>	Direct cloning	[30]
<i>Salmonella typhimurium</i>	Hfq co-immunoprecipitation, deep sequencing	[34*,44]
<i>Prochlorococcus marinus</i>	Microarrays	[21]
<i>Vibrio cholerae</i>	Deep sequencing	[37]
<i>Aquifex aeolicus</i>	RNomics	[29]
<i>Pseudomonas aeruginosa</i>	RNomics	[42]
<i>Sinorhizobium meliloti</i>	Microarrays	[97]
<i>Caulobacter crescentus</i>	Tiling array	[20]
<i>Burkholderia cenocepacia</i>	Deep sequencing	[38]
Gram-positive bacteria		
<i>Bacillus subtilis</i>	Microarray	[25]
<i>Bacillus anthracis</i>	Deep sequencing	[39*]
<i>Listeria monocytogenes</i>	Hfq co-immunoprecipitation	[15]
<i>Listeria monocytogenes</i>	Tiling array	[22*]
<i>Streptomyces coelicolor</i>	sRNA cloning	[98]

mRNA, tRNA, rRNA and sRNA regions, and high-density (tiling) arrays carrying up to hundreds of thousands of DNA oligonucleotides systematically covering the sense and antisense strand of a genome, including the intergenic regions (IGRs) from which most known sRNAs are expressed.

The first tiling array [18] covered IGRs of >40 bp (with one probe every six bases), in addition to strand-specific probes for all annotated regions of the *E. coli* chromosome. Whilst the initial study was focused on sensitivity and reproducibility issues of mRNA profiling, the tiling array came into full play for sRNA discovery by bolstering predictions based on comparative genome analysis [3], and the profiling of IGR-derived transcripts under various growth conditions [19]. Tiling arrays have since been used for global discovery of sRNAs in *Caulobacter crescentus* [20], marine cyanobacteria (*Prochlorococcus* [21]) and the pathogens, *L. monocytogenes* [22*] (see Figure 1B) and *Mycobacterium leprae* [23]. These studies revealed intriguing new features of sRNA biology, for example, an uncompromised sRNA expression in a cyanobacterial strain that had lost the gene encoding Hfq protein [21], or sRNAs of virulent bacteria that are up-regulated during [22*,23] and required for successful host infection [22*].

Low-density arrays have provided an affordable means to validate predicted sRNAs, for example, in isolates of *Staphylococcus aureus* [24] or in the sporulation-network of *Bacillus subtilis* [25], and can be easily produced 'in house' at a core facility. In contrast, tiling arrays are typically made by commercial providers, and require considerably higher set-up and production costs. However, their high probe density enable unbiased sRNA

searches and allow a rough estimate of the 5' and 3' termini of sRNAs, and help distinguish sRNAs encoded by free-standing genes from processed UTRs.

Issues of probe labelling, which could potentially be hampered by the intrinsic structure of small stable RNAs, have been addressed by improving protocols for higher detection sensitivity and lower noise from cross-hybridization [3,19,20]. This includes a new protocol alternative to the common labelling of bacterial RNA or cDNA with fluorescent dyes, and which is based on very sensitive recognition of RNA:DNA hybrids following direct hybridization of RNA to tiling arrays [26,27].

RNomics and RNA-Seq (next-generation sequencing)

The discovery of new RNA classes through sequencing of cDNA prepared from size-fractionated or total cellular RNA has like no other approach helped unravel the diversity and importance of small RNAs in all kingdoms of life. The first such studies in bacteria, in *E. coli* and *Aquifex aeolicus*, enriched RNA species of a size range of 50–500 nt [28,29] or 15–50 nt [30] by gel extraction. In these so-called RNomics approaches [31], the RNA was reverse transcribed, cloned in a plasmid vector and up to 3000 cDNA inserts [28] were analyzed by conventional Sanger sequencing. These studies identified a number of abundant new sRNAs in *E. coli* whose relative representation in the cDNA libraries prepared from different growth conditions often reflected their expression over growth [28]. Exceptions include the extremely abundant yet highly structured 6S RNA which was hardly recovered in *E. coli* [28] but constituted almost half of the cDNA sequences in *A. aeolicus* [29]. Both of the *E. coli* studies detected parallel transcriptional output of genes by detecting stable small-sized RNA species that originated

from 5' or 3' UTRs of annotated ORFs, including riboswitch regions [28,30].

High-throughput sequencing techniques have now obviated both the time-consuming cDNA cloning step and the costly Sanger sequencing. Using any of the currently available technologies, that is, 454 pyrosequencing, SOLEXA or SOLiD [32[•]], millions of cDNAs can be analyzed directly once the RNA is reverse transcribed into cDNA and amplified by means of added adapters. Although the term is quite misleading, such cDNA analysis is often referred to as RNA-Seq (RNA sequencing) [33[•]]. The first such approach to discover bacterial sRNAs used pyrosequencing of RNA associated with Hfq protein in *Salmonella* [34[•]], and will be covered in the next section. Pyrosequencing also pioneered the profiling of bacterial transcriptomes of *Sinorhizobium meliloti* and marine microbial communities [35,36], and these studies identified new sRNA candidates in passing. Similar to RNomics, Liu *et al.* [37] enriched by gel extraction the 14–200 nt fraction to discover new sRNA species in *Vibrio cholerae* by pyrosequencing; a new protocol for the depletion of the small-sized 5S rRNA and tRNAs by oligonucleotide-guided RNase H treatment was introduced.

Pyrosequencing was the first method to offer sufficiently long reads for unequivocal genome mapping, yet the read lengths obtained on the SOLEXA or SOLiD platforms have since increased to >50 bp. The latter technologies were used for transcriptome analyses of *Burkholderia cenocepacia* strains [38] and *Bacillus anthracis* [39[•]] grown under relevant environmental conditions; in both studies, abundant ribosomal RNA species were depleted with the MICROBEexpress kit (Ambion) to increase the coverage of interesting transcripts. The *Bacillus anthracis* study showed that SOLEXA and SOLiD were equally suited to profile bacterial transcriptomes in a quantitative manner. Similar to detection on tiling arrays, sRNA genes were often revealed through short blocks of transcripts in IGRs, yielding 13 new putative sRNAs in *Burkholderia* [38] and hundreds of candidates, including antisense RNAs and UTR-derived species, in *Vibrio* [37].

Appropriate cDNA synthesis permitting, the most salient feature of RNA-Seq is that it can detect sRNAs with single-nucleotide resolution at the 5' and 3' ends of transcripts. For example, the RNA is ligated to a 5' adaptor, followed by polyA (or polyC) tailing at the 3' end, and cDNA synthesis is initiated with a 3' adaptor primer anchored to the tail [34[•]]. Moreover, we have developed a new approach, termed differential RNA-Seq (dRNA-Seq), which cares about the 5' group of RNA and thus permits to differentiate primary transcripts with native 5' ends from processed species. The dRNA-Seq approach has unravelled transcription initiation sites

at the genome-wide level, and discovered an unexpected large number of new sRNAs and antisense transcripts, in the human pathogen, *Helicobacter pylori* (Sharma *et al.*, submitted). In principle, it is applicable to all bacterial species where native transcripts carry a 5' tri-phosphate.

Co-purification of sRNAs with proteins

As part of their biological activity, many sRNAs stably associate — and can therefore be co-purified — with cellular proteins. As paradigm, CsrA protein of *E. coli* was serendipitously purified as a ribonucleoprotein complex; cDNA cloning identified the associated ~360 nt CsrB sRNA as the founding member of widespread sRNAs that act to antagonize CsrA/RsmA proteins [40].

The bacterial Sm-like protein, Hfq, has been the most common bait for sRNA discovery (Figure 1A). Hfq is required for both intracellular stability and target mRNA pairing of sRNAs in many bacteria; almost half of the known *E. coli* and *Salmonella* sRNAs might associate with Hfq [41]. In a pioneering global study in *E. coli* [27], cellular extracts were incubated with polyclonal antisera against Hfq to enrich RNA by co-immunoprecipitation (coIP), followed by hybridization to tiling arrays. The procedure enriched (as compared to a control reaction) one-third of the *E. coli* sRNAs known at the time. Studies in *Listeria* [15] and in *Pseudomonas aeruginosa* [42] identified individual abundant Hfq-associated RNAs by direct RNA sequencing or conventional RNomics.

To overcome the limited availability of high-density microarrays and species-specific Hfq antibodies, we recently established a generic approach, adding a triple FLAG tag epitope [43] to the chromosomal *hfq* gene of *Salmonella*, and analyzing Hfq-associated RNA after coIP with a commercial monoclonal anti-FLAG antibody by RNA-Seq (Figure 1C) [34[•]]. Examples of *Salmonella* sRNA profiles based on the analysis of ~350 000 cDNAs, comparing coIP with the FLAG-tagged Hfq to control IP in a wild-type strain, are shown in Figure 1D. Generally, the Hfq-binding sRNAs were covered over the entire length, detecting the native 5'–3' ends with single-nucleotide resolution. Some sRNAs, for example, ArcZ (a.k.a. RyhA or SraH), were enriched in their processed form, and again the sequencing precisely captured the site of processing.

As an extension of the above approach, we also investigated which type of RNA is associated with heterologous Hfq proteins upon their ectopic expression in a *Salmonella hfq* mutant [44]. Interestingly, these 'foreign' Hfq proteins including that of the archeon *Methanocaldococcus jannaschii* [45] stabilized some sRNAs with low expression. Thus, foreign Hfq proteins may catch sRNAs that are not normally detected under standard assay conditions, and perhaps also sRNAs in bacteria without Hfq [44].

We caution that irrespective of the final detection method the Hfq-coIP approach is not necessarily quantitative because of long cell breakage and lysate incubation steps. It may favour sRNAs that are intrinsically stable, although short-lived species such as GcvB sRNA (half-life <2 min; [28,46[•]]) were also recovered with high frequency [27,34[•],44]. Nonetheless, the combination of chromosomal epitope-tagging, coIP and RNA-Seq could facilitate sRNA discovery in many genetically tractable bacteria. Note that the FLAG epitope did not interfere with Hfq functions in *Salmonella*, including virulence control [34[•],47], but this will require prior testing in other species, especially when other bacterial RNA-binding proteins such as the global acting CsrA/RsmA-like or Csp-like proteins are to be studied.

Genomic SELEX

RNomics-based or microarray-based sRNA discovery methods require sRNAs to be expressed under the chosen assay condition. A genomic SELEX approach, which would in principle cover all sRNAs that are encoded by a given genome has been developed ([48] and references therein). A representative library of the *E. coli* genome was constructed from random sequences of 50–500 bp length. These fragments were *in vitro* transcribed with T7 RNA polymerase, incubated with Hfq, and selected for Hfq binding on filters. Intriguingly, the genomic SELEX approach detected many low abundance antisense RNAs yet it remains to be understood why known Hfq-associated sRNAs were hardly recovered [48].

Discovery of small RNA functions

A full understanding of the biological function of a given sRNA requires the identification of its cellular interaction partners. If current trends hold, these will be most likely *trans*-encoded mRNAs to which the sRNA base-pairs with short and imperfect complementarity [5,7^{••},8]. Diverse biocomputational [12[•],13[•]] and experimental tools have helped to predict and validate target mRNAs. Regardless of whether the target is repressed or activated, regulation is often visible at the protein level as scored by SDS gels or Western blot. Chromosomal epitope-tagging [43] helps to detect regulated proteins if no specific antibody is available. Chromosomal or plasmid-borne mRNA fusions to *lacZ* [49[•]], or *gfp* [50[•]], or luciferase [51] reporter genes are commonly used, too.

Target regulation often entails changes at the mRNA level, to be detected by a variety of methods, for example, Northern blot hybridization, primer extension, RNA protection and RT-PCR, at the single gene level. However, it is now appreciated that many sRNAs act on multiple targets, which recommends global methods involving microarrays, RNA-Seq or proteomics be used in the initial phase of sRNA characterization. These global methods, including the cataloguing of Hfq-associated mRNAs as potential targets [3,34[•]], will be outlined below.

We currently know few regulatory sRNA–protein interactions, for example, sRNAs targeting CsrA/RsmA proteins [7^{••}] or RNA polymerase [14]. However, this might as well reflect the current bias towards mRNA targets and the paucity of biocomputational algorithms to globally predict protein targets. The experimental methods covered here rely mostly on co-purification of proteins with sRNAs and subsequent analysis by mass spectrometry.

sRNA overexpression and deletion

Fortuitous overexpression of sRNA genes present in multi-copy plasmid libraries of genomic *E. coli* fragments underlay some of the early discoveries in the field [1,5,10,52]. Whereas overexpression may simply be achieved by increasing gene copy number, plasmid-based expression of sRNAs from a strong constitutive promoter, for example, P_{L_{laco}-1} [50[•],53], has also helped identify targets. Recent examples include MicA sRNA-mediated OmpA repression [54], and the activation of GlmS synthesis by the hierarchically acting *E. coli* GlmY and GlmZ sRNAs [55,56]. Constitutive expression of the GlmYZ sRNAs causes such strong activation that GlmS becomes easily visible in Coomassie-stained SDS gels [55,56]. However, only GlmZ directly regulates the *glmS* message, whereas GlmY regulation is indirect through GlmZ [56,57]. Needless to say that strong overexpression of sRNAs runs the risk of causing lethality, for example, *Salmonella* GcvB sRNA is toxic when constitutively expressed from a multi-copy plasmid [46[•]].

Reciprocally, scoring deregulated protein synthesis in a given sRNA deletion background has also been successful. Resolution of whole cell proteins in 1D or 2D SDS gels revealed repression of many ABC transporters by GcvB [46[•],58], and of OmpA or LamB synthesis by the *V. cholerae* VrrA [59] or *Salmonella* MicA [60[•]] sRNAs, respectively. However, the growth condition to be analyzed can matter, e.g., ArcZ sRNA represses some targets in early growth and another only in late stationary phase *Salmonella* [61].

Libraries of sRNA deletion mutants were constructed to screen for regulated targets in a systematic fashion. Disruption of a few dozen known sRNA loci in *Salmonella* [62] revealed the CRP-dependent CyaR sRNA as a repressor of OmpX synthesis (the existence of a regulatory sRNA had been hypothesized because of the strong Hfq association of *ompX* mRNA [27,34[•]]). At the level of physiology, analysis of sRNA mutant libraries revealed GcvB as a regulator of acid resistance in *E. coli* [63], and unravelled roles of the *Salmonella* IstR-1, OxyS and SroA sRNAs in host infection [64].

Pulse-expression of sRNA combined with transcriptome profiling

Constitutive overexpression of an sRNA is prone to report indirect regulations because of potential off-target and

pleiotropic effects on overall gene expression, especially if the primary targets include a transcriptional regulator such as RpoS [65]. Pulse-expression of sRNAs, for example, from the tightly controlled arabinose-inducible P_{BAD} promoter, has overcome some of these limitations [62,66[•],67[•],68[•],69[•],70,71]. The logic behind this approach is to overexpress sRNAs (for 10–15 min) long enough to induce the decay of direct target mRNAs, whilst keeping the expression pulse short enough to avoid significant alterations at the protein level which could cause secondary effects. Altered mRNA levels are scored on whole genome microarrays. Pulse-expression revealed that the iron starvation regulator, RyhB sRNA of *E. coli*, might directly control 18 loci, including large operons with documented functions in iron homeostasis [66[•]]. Likewise, the *Salmonella* RybB and *E. coli* OmrAB sRNAs were each found to repress large numbers of outer membrane protein-encoding mRNAs [68[•],70]. Many of such identified targets, including a case of translational activation [72], have meanwhile been verified by genetic or structural characterization of the underlying base-pairing mechanism [73]. Although pulse-expression has become the method of choice for the identification of target mRNAs, it requires targets to be transcribed under the assay condition. Whether short overexpression will also alter mRNA levels where wild-type regulation proceeds without concomitant mRNA decay, for example, Spot42 control of the *gal* operon mRNA [74], remains to be seen.

Genetic approaches

Although sRNA genes are small and 5–10 times less likely to be hit by a transposon than the average ORF, they have indeed been selected in phenotypic screens using transposon mutagenesis. For example, GlmZ was selected in a screen for transposon insertions that abolished the activity of a *glmS::lacZ* in a *yhbJ* mutant background [57]. Fortunately, only housekeeping sRNAs (e.g. the RNase P or SRP sRNA [7^{••}]) seem to be essential under standard conditions, although some sRNAs can be conditionally essential, for example, the IstR sRNA locus cannot be deleted in an *E. coli* strain with a constitutive SOS response because it represses the synthesis of an SOS-induced toxin, TisB [75].

For target discovery, chromosomal reporter gene fusions (see next section) lend themselves for activity screening by combination with plasmid libraries carrying cloned sRNA genes or genomic fragments; the latter recently identified the *E. coli* MicM (a.k.a. RybC or SroB) sRNA as a regulator of the DpiA/B two-component system [49[•]].

Experimental validation of targets and RNA interactions *in vivo*

To formally consider a regulated mRNA as a direct sRNA target, evidence for post-transcriptional regulation and, at best, for sRNA–mRNA base-pairing must be obtained. To validate targets *in vivo*, a two-plasmid system using

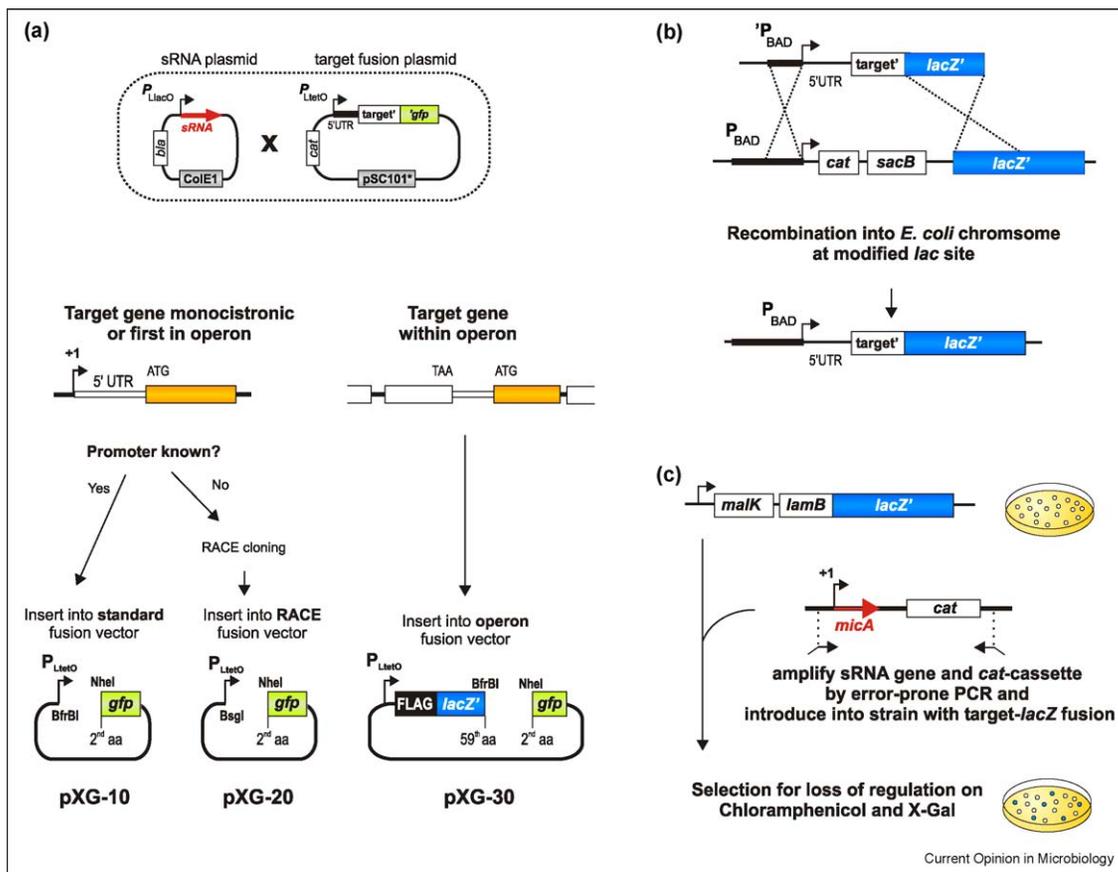
translational fusions to *gfp* was developed (Figure 2A) [50[•]]. Here, both the sRNA and the target mRNA fusions are co-expressed from constitutive promoters to primarily assay post-transcriptional regulation. Three standard vectors facilitate cloning of the 5' region of a putative target either from PCR-amplified genomic DNA, as a 5' RACE product if the transcription start site is unknown, or when the target site is located within a polycistronic mRNA. Owing to small plasmid size, point mutations to disrupt (and restore) regulations can be easily introduced in both sRNA and target. The system has reported the regulation of diverse targets in *E. coli* and *Salmonella* [50[•],56,62,69[•],76] and heterologous sRNA regulation in an *E. coli* host [50[•]].

A similar approach [49] combines 5' RACE-mediated cloning of 5' UTRs to a *lacZ* reporter gene, followed by chromosomal integration of the fusion downstream of the P_{BAD} promoter (Figure 2B). Both the *lacZ* [49[•]] and the above *gfp* reporters (upon integration in the chromosome; JH Urban and J Vogel, unpublished results) have permitted fast screening on MacConkey indicator plates or by FACS analysis, respectively, for potential sRNA regulators and genes encoding auxiliary factors. Construction of reporter fusions should take into account that sRNA-binding sites might as well be located in the coding sequence (CDS). For example, a standard fusion to the AUG start codon failed to report MicC-mediated repression of *ompD* mRNA (targeted at codon 23–26 [69[•]]). Likewise, regulation at the 3' end of mRNA, for example, of *gadX* by GadY sRNA [77], requires specialized fusion constructs.

To validate predicted RNA interactions, compensatory base-pair exchanges have become standard: a mutation in the interaction site of either the sRNA or at the opposite position in the target mRNA should abolish regulation, but regulation is sought to be restored by expression of both mutant RNAs in the same cell. Although point mutations often suffice, longer interactions may require the mutation of several nucleotides, as evident from the MicC interaction with the *ompD* versus *ompC* mRNAs [51,69[•]]. Studies of the SgrS [78] and MicA [60[•]] were dedicated to the identification of nucleotides critical for target regulation. This included a new technique to introduce point mutations in a chromosomal sRNA gene by error-prone PCR, followed by screening for loss of regulation of a *lacZ* fusion to the target gene of interest (Figure 2C) [60[•]]. Importantly, if regulation is lost, the mutant sRNA must be confirmed to have unaltered stability.

RACE approaches, commonly used to determine target sites of plant microRNAs, could also help map bacterial sRNA–mRNA interactions. 5' RACE detected the RNase III-cleaved duplex of IstR–*tisAB* [75]. Some sRNAs seem to linger on their targets upon base-pairing and thereby

Figure 2



Reporter gene systems for the identification of sRNA targets. **(A)** Principal approach for the validation of sRNA targets using the *gfp* reporter system described in [50^{*}]. Top: both, sRNA and a translational fusion of a putative target mRNA to *gfp* are expressed under control of the constitutive P_{LacO} or P_{LtetO} promoters, respectively, to assay post-transcriptional control. The sRNAs are cloned in a mid-copy vector (p15A or *ColE1* origin of replication; 20 or 70 copies/cell, respectively), and target fusions in a low-copy vector (*pSC101** origin of replication; 3–4 copies per cell). *E. coli* cells carrying both the sRNA and the fusion plasmid are assayed on agar plates, in liquid cultures (standard laboratory flasks, microtitre plates), by Western analysis, or by flow cytometry. Bottom: depending on the chromosomal context of a putative target gene (indicated in yellow) and whether the native promoter is known, different cloning protocols for the target fusions into one of three plasmids can be applied. This allows 5' RACE mapping a cloning of native +1 site as well as construction of operon fusion plasmids where translation of the target gene might be coupled to the upstream gene. Figure adapted from [50^{*}]. **(B)** Modified 5' RACE protocol to amplify PCR products of putative target genes and clone these downstream of P_{BAD} promoter as fusions to a *lacZ* reporter [49^{*}]. The obtained fragment is then recombined into the chromosome of a modified *E. coli* strain which carries a P_{BAD} -*cat*-*sacB* cassette upstream of *lacZ* at the 5' coding region using mini- λ -mediated recombination. Figure adapted from [49^{*}]. Recombinants are selected on sucrose for loss of the *cat*-*sacB* genes. **(C)** Lac-based screen for identifying mutations affecting sRNA-mediated target gene regulation [60^{*}]. A chromosomal *lamB*:*lacZ* fusion which is normally repressed by MicA sRNA yields white colonies when grown on X-Gal plates. Point mutations are introduced into a fragment of the *micA* gene (linked to a *cat* marker) by PCR-amplification under error-prone conditions. The resulting fragments are then recombined into the strain harbouring the *lamB*-*lacZ* reporter gene by λ -red recombination. Mutations in either the *micA* promoter or the sRNA coding region abolish down-regulation of the *lamB*-*lacZ* reporter, to be revealed by blue colony color on X-Gal plates.

stall exonucleolytic mRNA degradation from the 3' end. Interestingly, 3' RACE initiated at the 5' end of *Salmonella ompD* mRNA detected stalled processing after the induction of MicC sRNA, four to five nucleotides downstream of the MicC-*ompD* duplex; the RACE signal was strongly enhanced in a degradosome-deficient strain [69^{*}]. Because similar observations have been made for other sRNA targets (V Pfeiffer and J Vogel, unpublished), 3' RACE seems useful for a rough estimate of a target site location in parallel to biocomputational predictions.

In vitro approaches

In vitro experiments have become popular to both identify and validate sRNA-mRNA interactions, and to confirm translational regulation. These biochemical experiments include determination of binding affinities by gel-shift assays of *in vitro* transcribed RNAs. One such study included mutational analysis to reveal that only six nucleotides are critical in SgrS-*ptsG* mRNA pairing [78]. Most commonly, *in vitro* complex formation is coupled to RNA 'footprinting' with enzymatic or lead(II)-induced cleavage, or RNA modifications, to determine binding

sites [79**]. RNA probing has detected very short interactions such as RyhB–*sodB* [80] or MicC–*ompD* [69*], or the bipartite OxyS–*flhA* pairing [81]. Several *in vitro* studies determined sRNA and target cleavage by major nucleases such as RNase E or III [82,83].

30S ribosome toeprinting has been widely used to confirm that sRNA pairing to the ribosome-binding site of a target represses translational initiation (Ref. in [76]). Assaying 30S association also identified cases of translational repression [46*,83,84] or activation [85] at upstream sites in the 5' UTR, and translation inhibition in the proximal CDS within a 'five codon window' downstream of AUG [76].

The latest addition to the toolbox is a commercially available *in vitro* translation system (PURESYSTEM) using reconstituted 70S ribosomes, which has reported both translational repression [46*,86*] and activation [56]. Unlike in bacterial S30 extracts, *in vitro* transcribed RNA is stable for >30 min in the PURESYSTEM [46*]. In principle, the 70S translation system lends itself for high-throughput screening of predicted sRNA regulations; it can be easily combined with the above described *gfp* fusions [46*,50*,56,69*].

Co-purification of target proteins and mRNAs

Bacterial sRNAs are known to interact with proteins other than Hfq, either as part of their dedicated biological activity, for example, 6S RNA [7**,14] or CsrB [40], or potentially with RNase E in a recently discovered complex for sRNA-guided mRNA degradation [87]. A recent study discovered tryptophanase as the target of the ColE1 plasmid-encoded Rcd RNA; here, Rcd was cross-linked to sepharose, incubated with a lysate of *E. coli*, and the retained proteins were identified by mass spectroscopy [88]. The Schroeder lab tagged *E. coli* sRNAs *in vitro* with a streptomycin-binding aptamer, and recovered by affinity chromatography several interacting proteins including polymerase beta-subunit, Hfq and S1 [89*]. In addition, a critical evaluation of three commonly used aptamers (MS2, λ boxB, eIF4) has suggested that the MS2 aptamer is a suitable fusion partner for *in vivo* expressed sRNAs in *Salmonella* [90]. Collectively, the method sections of the above papers provide a rich source for everyone to embark on fishing expeditions for proteins associated with and/or targeted by sRNA.

The analysis of Hfq-bound RNA not only facilitated sRNA discovery but also identified potential mRNA targets *in vivo* [27,34*]. For example, we observed that Hfq-coIP significantly enriched ~700 *Salmonella* mRNAs [34*], including 15-fold enrichment of *ybfM* which is now known as a target of MicM sRNA [16*,17*]. Interestingly, a recent study based on sample-matched transcriptomics and proteomics [91] predicted that >20% of all annotated

Salmonella genes are regulated post-transcriptionally, primarily by Hfq.

Additionally, cDNA cloning and Sanger sequencing of RNA fragments that were co-purified with *Pseudomonas* RsmA protein identified 40 genes that are translationally regulated by RsmA and, thus, indirectly by the RsmA-antagonizing RsmY/Z sRNAs [92]. Other studies directly used sRNAs as a bait to capture interacting mRNAs. Specifically, *in vitro* transcribed, biotinylated RseX RNA bound to streptavidin [93], or RydC RNA in an immobilized complexes with Hfq [94], were used to fish *E. coli* mRNAs from cellular extracts for further analysis by cDNA cloning or on microarrays.

Conclusion

The staggering interest in sRNAs as regulators of bacterial gene expression keeps fuelling the creativity of researchers in their quest for new experimental strategies for discovery and functional characterization. Given the breathtaking speed of technological development, increasing availability and free falling prices of high-throughput sequencing, the days of microarrays seem numbered, and we expect a major transition in the way sRNA and gene expression will be analyzed in the coming years. We advise to keep a close eye on the eukaryotic field in which many new sequencing-based methods, including the mapping of small RNA interaction sites on proteins or target RNA molecules are being developed. Moreover, the increasing depths of RNA-Seq will provide us with large catalogues of sRNAs in virtually any bacterium, be it genetically tractable or not, as well as in environmental communities and in host-associated pathogens.

All of the approaches covered in this review have shortcomings, and wherever we failed to discuss these owing to space constraints, we refer the reader to the relevant publications. We have here focussed on proteins and mRNAs as the target but variations of the theme seem likely, as evident from the discovery of mRNA-mediated regulation of sRNA activity in chitobiose metabolism [16*,17*]. We believe that the modulation of protein activity by sRNAs is currently underrated owing to the lack of methods to reliably predict such regulations. In addition, the recent advances in understanding CRISPR-mediated immunity [95] suggests that DNA as a target of regulatory RNA activity is yet to be fully addressed.

There has been much focus on the prototypical translational repression of mRNAs by sRNAs binding to the narrow RBS region. However, the increasing number of validated binding sites elsewhere in the 5' UTR [5,46*,72,75,84] or the CDS [61,69*,76] needs to be considered when devising methods for target characterization. Moreover, the emerging role of RNase E as a major player in sRNA-mediated regulation [69*,82,87,96] requires the

development of suitable assays for target regulation by the induction of mRNA decay.

Acknowledgements

We thank K Papenfort for comments on the manuscript, and P Cossart and Y Chao for providing graphics. Work in the Vogel lab is supported by the Deutsche Forschungsgemeinschaft (DFG) Priority Program SPP1258 *Sensory and Regulatory RNAs in Prokaryotes*.

References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

- of special interest
 - of outstanding interest
1. Mizuno T, Chou MY, Inouye M: **A unique mechanism regulating gene expression: translational inhibition by a complementary RNA transcript (micRNA)**. *Proc Natl Acad Sci U S A* 1984, **81**:1966-1970.
 2. Argaman L, Hershberg R, Vogel J, Bejerano G, Wagner EG, Margalit H, Altuvia S: **Novel small RNA-encoding genes in the intergenic regions of *Escherichia coli***. *Curr Biol* 2001, **11**:941-950.
 3. Wassarman KM, Repoila F, Rosenow C, Storz G, Gottesman S: **Identification of novel small RNAs using comparative genomics and microarrays**. *Genes Dev* 2001, **15**:1637-1651.
 4. Rivas E, Klein RJ, Jones TA, Eddy SR: **Computational identification of noncoding RNAs in *E. coli* by comparative genomics**. *Curr Biol* 2001, **11**:1369-1373.
 5. Majdalani N, Vanderpool CK, Gottesman S: **Bacterial small RNA regulators**. *Crit Rev Biochem Mol Biol* 2005, **40**:93-113.
 6. Vogel J: **A rough guide to the noncoding RNA world of *Salmonella***. *Mol Microbiol* 2009, **71**:1-11.
 7. Waters LS, Storz G: **Regulatory RNAs in bacteria**. *Cell* 2009, **136**:615-628.
An excellent recent review that covers the function and regulatory principles of RNA-based regulation in bacteria.
 8. Romby P, Vandenesch F, Wagner EG: **The role of RNAs in the regulation of virulence-gene expression**. *Curr Opin Microbiol* 2006, **9**:229-236.
 9. Vogel J, Sharma CS: **How to find small non-coding RNAs in bacteria**. *Biol Chem* 2005, **386**:1219-1238.
 10. Vogel J, Wagner EG: **Target identification of regulatory sRNAs in bacteria**. *Curr Opin Microbiol* 2007, **10**:262-270.
 11. Livny J, Waldor MK: **Identification of small RNAs in diverse bacterial species**. *Curr Opin Microbiol* 2007, **10**:96-101.
See annotation to Ref. [13*].
 12. Pichon C, Felden B: **Small RNA gene identification and mRNA target predictions in bacteria**. *Bioinformatics* 2008, **24**:2807-2813.
See annotation to Ref. [13*].
 13. Tjaden B: **TargetRNA: a tool for predicting targets of small RNA action in bacteria**. *Nucleic Acids Res* 2008, **36**(Web Server issue):W109-W113.
Reviews of the current biocomputational toolbox for the prediction of bacterial sRNAs and mRNA targets.
 14. Trotochaud AE, Wassarman KM: **A highly conserved 6S RNA structure is required for regulation of transcription**. *Nat Struct Mol Biol* 2005, **12**:313-319.
 15. Christiansen JK, Nielsen JS, Ebersbach T, Valentin-Hansen P, Sogaard-Andersen L, Kallipolitis BH: **Identification of small Hfq-binding RNAs in *Listeria monocytogenes***. *RNA* 2006, **12**:1383-1396.
 16. Overgaard M, Johansen J, Møller-Jensen J, Valentin-Hansen P: **Switching off small RNA regulation with trap-mRNA**. *Mol Microbiol* 2009, **73**:790-800.
See annotation to Ref. [17*].
 17. Figueroa-Bossi N, Valentini M, Malleret L, Bossi L: **Caught at its own game: regulatory small RNA inactivated by an inducible transcript mimicking its target**. *Genes Dev* 2009 Jul 28. [Epub ahead of print] doi:10.1101/gad.541609
Along with Ref. [16*], this work reports the unexpected discovery of an sRNA being regulated by an mRNA.
 18. Selinger DW, Cheung KJ, Mei R, Johansson EM, Richmond CS, Blattner FR, Lockhart DJ, Church GM: **RNA expression analysis using a 30 base pair resolution *Escherichia coli* genome array**. *Nat Biotechnol* 2000, **18**:1262-1268.
 19. Tjaden B, Saxena RM, Stolyar S, Haynor DR, Kolker E, Rosenow C: **Transcriptome analysis of *Escherichia coli* using high-density oligonucleotide probe arrays**. *Nucleic Acids Res* 2002, **30**:3732-3738.
 20. Landt SG, Abeliuk E, McGrath PT, Lesley JA, McAdams HH, Shapiro L: **Small non-coding RNAs in *Caulobacter crescentus***. *Mol Microbiol* 2008, **68**:600-614.
 21. Steglich C, Futschik ME, Lindell D, Voss B, Chisholm SW, Hess WR: **The challenge of regulation in a minimal photoautotroph: non-coding RNAs in *Prochlorococcus***. *PLoS Genet* 2008, **4**:e1000173.
 22. Toledo-Arana A, Dussurget O, Nikitas G, Sesto N, Guet-Revillet H, Balestrino D, Loh E, Gripenland J, Tiensuu T, Vaitkevicius K et al.: **The *Listeria* transcriptional landscape from saprophytism to virulence**. *Nature* 2009.
An excellent and comprehensive study illustrating the power of high-density tiling arrays for transcriptome profiling and discovery of new regulatory RNA elements in a bacterial pathogen.
 23. Akama T, Suzuki K, Tanigawa K, Kawashima A, Wu H, Nakata N, Osana Y, Sakakibara Y, Ishii N: **Whole-genome tiling array analysis of *Mycobacterium leprae* RNA reveals high expression of pseudogenes and noncoding regions**. *J Bacteriol* 2009, **191**:3321-3327.
 24. Pichon C, Felden B: **Small RNA genes expressed from *Staphylococcus aureus* genomic and pathogenicity islands with specific expression among pathogenic strains**. *Proc Natl Acad Sci U S A* 2005, **102**:14249-14254.
 25. Silvaggi JM, Perkins JB, Losick R: **Genes for small, noncoding RNAs under sporulation control in *Bacillus subtilis***. *J Bacteriol* 2006, **188**:532-541.
 26. Hu Z, Zhang A, Storz G, Gottesman S, Leppla SH: **An antibody-based microarray assay for small RNA detection**. *Nucleic Acids Res* 2006, **34**:e52.
 27. Zhang A, Wassarman KM, Rosenow C, Tjaden BC, Storz G, Gottesman S: **Global analysis of small RNA and mRNA targets of Hfq**. *Mol Microbiol* 2003, **50**:1111-1124.
 28. Vogel J, Bartels V, Tang TH, Churakov G, Slagter-Jager JG, Hüttenhofer A, Wagner EG: **RNomics in *Escherichia coli* detects new sRNA species and indicates parallel transcriptional output in bacteria**. *Nucleic Acids Res* 2003, **31**:6435-6443.
 29. Willkomm DK, Minnerup J, Huttenhofer A, Hartmann RK: **Experimental RNomics in *Aquifex aeolicus*: identification of small non-coding RNAs and the putative 6S RNA homolog**. *Nucleic Acids Res* 2005, **33**:1949-1960.
 30. Kawano M, Reynolds AA, Miranda-Rios J, Storz G: **Detection of 5'- and 3'-UTR-derived small RNAs and cis-encoded antisense RNAs in *Escherichia coli***. *Nucleic Acids Res* 2005, **33**:1040-1050.
 31. Hüttenhofer A: **Experimental RNomics: a global approach to identify non-coding RNAs in model organisms**. In *Handbook of RNA Biochemistry*. Edited by Hartmann R, Bindereif A, Schön A, Westhof E. Wiley-VCH Verlag GmbH; 2005:643-654.
 32. MacLean D, Jones JD, Studholme DJ: **Application of 'next-generation' sequencing technologies to microbial genetics**. *Nat Rev Microbiol* 2009, **7**:287-296.
See annotation to Ref. [33*].
 33. Wang Z, Gerstein M, Snyder M: **RNA-Seq: a revolutionary tool for transcriptomics**. *Nat Rev Genet* 2009, **10**:57-63.
This review and Ref. [32*] are recent comprehensive reviews of high-throughput sequencing techniques, including applications to bacteria.

34. Sittka A, Lucchini S, Papenfort K, Sharma CM, Rolle K,
 • Binnewies TT, Hinton JC, Vogel J: **Deep sequencing analysis of small noncoding RNA and mRNA targets of the global post-transcriptional regulator, Hfq.** *PLoS Genet* 2008, **4**:e1000163.
 Introduces a generic method for the identification of sRNAs and the cataloging of potential target mRNAs by co-immunoprecipitation of epitope-tagged RNA-binding proteins (here, Hfq).
35. Mao C, Evans C, Jensen RV, Sobral BW: **Identification of new genes in *Sinorhizobium meliloti* using the genome sequencer FLX system.** *BMC Microbiol* 2008, **8**:72.
36. Shi Y, Tyson GW, DeLong EF: **Metatranscriptomics reveals unique microbial small RNAs in the ocean's water column.** *Nature* 2009, **459**:266-269.
37. Liu JM, Livny J, Lawrence MS, Kimball MD, Waldor MK, Camilli A: **Experimental discovery of sRNAs in *Vibrio cholerae* by direct cloning, 5S/tRNA depletion and parallel sequencing.** *Nucleic Acids Res* 2009, **37**:e46.
38. Yoder-Himes DR, Chain PS, Zhu Y, Wurtzel O, Rubin EM, Tiedje JM, Sorek R: **Mapping the *Burkholderia cenocepacia* niche response via high-throughput sequencing.** *Proc Natl Acad Sci U S A* 2009, **106**:3976-3981.
39. Passalacqua KD, Varadarajan A, Ondov BD, Okou DT, Zwick ME,
 • Bergman NH: **Structure and complexity of a bacterial transcriptome.** *J Bacteriol* 2009, **191**:3203-3211.
 An RNA-Seq study that uses Solexa and SOLiD technologies for bacterial transcriptome profiling and sRNA discovery.
40. Liu MY, Gui G, Wei B, Preston JF 3rd, Oakford L, Yuksel U, Giedroc DP, Romeo T: **The RNA molecule CsrB binds to the global regulatory protein CsrA and antagonizes its activity in *Escherichia coli*.** *J Biol Chem* 1997, **272**:17502-17510.
41. Brennan RG, Link TM: **Hfq structure, function and ligand binding.** *Curr Opin Microbiol* 2007, **10**:125-133.
42. Sonnleitner E, Sorger-Domenigg T, Madej MJ, Findeiss S, Hackermuller J, Huttenhofer A, Stadler PF, Blasi U, Moll I: **Detection of small RNAs in *Pseudomonas aeruginosa* by RNomics and structure-based bioinformatic tools.** *Microbiology* 2008, **154**:3175-3187.
43. Uzzau S, Figueroa-Bossi N, Rubino S, Bossi L: **Epitope tagging of chromosomal genes in *Salmonella*.** *Proc Natl Acad Sci U S A* 2001, **98**:15264-15269.
44. Sittka A, Sharma CM, Rolle K, Vogel J: **Deep sequencing of *Salmonella* RNA associated with heterologous Hfq proteins in vivo reveals small RNAs as a major target class and identifies RNA processing phenotypes.** *RNA Biol* 2009, **6**.
45. Nielsen JS, Boggild A, Andersen CB, Nielsen G, Boysen A, Brodersen DE, Valentin-Hansen P: **An Hfq-like protein in archaea: crystal structure and functional characterization of the Sm protein from *Methanococcus jannaschii*.** *RNA* 2007, **13**:2213-2223.
46. Sharma CM, Darfeuille F, Plantinga TH, Vogel J: **A small RNA regulates multiple ABC transporter mRNAs by targeting C/A-rich elements inside and upstream of ribosome-binding sites.** *Genes Dev* 2007, **21**:2804-2817.
 Together with Ref. [86*], this study including its associated supplementary material evaluates the use of an mRNA decay-free, 70S ribosome translation system to determine sRNA effects on mRNAs *in vitro*.
47. Pfeiffer V, Sittka A, Tomer R, Tedin K, Brinkmann V, Vogel J: **A small non-coding RNA of the invasion gene island (SPI-1) represses outer membrane protein synthesis from the *Salmonella* core genome.** *Mol Microbiol* 2007, **66**:1174-1191.
48. Lorenz C, von Pelchrzim F, Schroeder R: **Genomic systematic evolution of ligands by exponential enrichment (Genomic SELEX) for the identification of protein-binding RNAs independent of their expression levels.** *Nat Protoc* 2006, **1**:2204-2212.
49. Mandin P, Gottesman S: **A genetic approach for finding small RNAs regulators of genes of interest identifies RybC as regulating the DpiA/DpiB two-component system.** *Mol Microbiol* 2009, **72**:551-565.
 See annotation to Ref. [50*].
50. Urban JH, Vogel J: **Translational control and target recognition by *Escherichia coli* small RNAs in vivo.** *Nucleic Acids Res* 2007, **35**:1018-1037.
 Along with Ref. [49*], this study introduces general reporter (*lacZ*, *gfp*) systems for the investigation of post-transcriptional control of mRNAs.
51. Chen S, Zhang A, Blyn LB, Storz G: **MicC, a second small-RNA regulator of Omp protein expression in *Escherichia coli*.** *J Bacteriol* 2004, **186**:6689-6697.
52. Majdalani N, Chen S, Murrow J, St John K, Gottesman S: **Regulation of RpoS by a novel small RNA: the characterization of RprA.** *Mol Microbiol* 2001, **39**:1382-1394.
53. Lutz R, Bujard H: **Independent and tight regulation of transcriptional units in *Escherichia coli* via the LacR/O, the TetR/O and AraC/I1-I2 regulatory elements.** *Nucleic Acids Res* 1997, **25**:1203-1210.
54. Udekwi KI, Darfeuille F, Vogel J, Reimegard J, Holmqvist E, Wagner EG: **Hfq-dependent regulation of OmpA synthesis is mediated by an antisense RNA.** *Genes Dev* 2005, **19**:2355-2366.
55. Urban JH, Papenfort K, Thomsen J, Schmitz RA, Vogel J: **A conserved small RNA promotes discoordinate expression of the *glmUS* operon mRNA to activate *GlmS* synthesis.** *J Mol Biol* 2007, **373**:521-528.
56. Urban JH, Vogel J: **Two seemingly homologous noncoding RNAs act hierarchically to activate *glmS* mRNA translation.** *PLoS Biol* 2008, **6**:e64.
57. Kalamorz F, Reichenbach B, Marz W, Rak B, Görke B: **Feedback control of glucosamine-6-phosphate synthase *GlmS* expression depends on the small RNA *GlmZ* and involves the novel protein *YhbJ* in *Escherichia coli*.** *Mol Microbiol* 2007, **65**:1518-1533.
58. Urbanowski ML, Stauffer LT, Stauffer GV: **The *gcvB* gene encodes a small untranslated RNA involved in expression of the dipeptide and oligopeptide transport systems in *Escherichia coli*.** *Mol Microbiol* 2000, **37**:856-868.
59. Song T, Mika F, Lindmark B, Liu Z, Schild S, Bishop A, Zhu J, Camilli A, Johansson J, Vogel J *et al.*: **A new *Vibrio cholerae* sRNA modulates colonization and affects release of outer membrane vesicles.** *Mol Microbiol* 2008, **70**:100-111.
60. Bossi L, Figueroa-Bossi N: **A small RNA downregulates *Lamb* maltoporin in *Salmonella*.** *Mol Microbiol* 2007, **65**:799-810.
 Includes a genetic approach to identify critical residues in regulatory sRNAs.
61. Papenfort K, Said N, Welsink T, Lucchini S, Hinton JCD, Vogel J: **Specific and pleiotropic patterns of mRNA regulation by *ArcZ*, a conserved, Hfq-dependent small RNA.** *Mol Microbiol* 2009, in press
62. Papenfort K, Pfeiffer V, Lucchini S, Sonawane A, Hinton JC, Vogel J: **Systematic deletion of *Salmonella* small RNA genes identifies *CyaR*, a conserved CRP-dependent riboregulator of *OmpX* synthesis.** *Mol Microbiol* 2008, **68**:890-906.
63. Jin Y, Watt RM, Danchin A, Huang JD: **Small noncoding RNA *GcvB* is a novel regulator of acid resistance in *Escherichia coli*.** *BMC Genomics* 2009, **10**:165.
64. Santiviago CA, Reynolds MM, Porwollik S, Choi SH, Long F, Andrews-Polymeris HL, McClelland M: **Analysis of pools of targeted *Salmonella* deletion mutants identifies novel genes affecting fitness during competitive infection in mice.** *PLoS Pathog* 2009, **5**:e1000477.
65. Lease RA, Smith D, McDonough K, Belfort M: **The small noncoding *DsrA* RNA is an acid resistance regulator in *Escherichia coli*.** *J Bacteriol* 2004, **186**:6179-6185.
66. Massé E, Vanderpool CK, Gottesman S: **Effect of *RyhB* small RNA on global iron use in *Escherichia coli*.** *J Bacteriol* 2005, **187**:6962-6971.
 See annotation to Ref. [68*].
67. Tjaden B, Goodwin SS, Opdyke JA, Guillier M, Fu DX,
 • Gottesman S, Storz G: **Target prediction for small, noncoding RNAs in bacteria.** *Nucleic Acids Res* 2006, **34**:2791-2802.
 See annotation to Ref. [68*].

68. Papenfort K, Pfeiffer V, Mika F, Lucchini S, Hinton JC, Vogel J:
 • **Sigma(E)-dependent small RNAs of *Salmonella* respond to membrane stress by accelerating global omp mRNA decay.** *Mol Microbiol* 2006, **62**:1674-1688.
 Along with Refs. [66*, 67*], this study probes the concept of sRNA pulse-expression for the experimental discovery of target mRNAs.
69. Pfeiffer V, Papenfort K, Lucchini S, Hinton JC, Vogel J: **Coding sequence targeting by MicC RNA reveals bacterial mRNA silencing downstream of translational initiation.** *Nat Struct Mol Biol* 2009, **16**:840-846.
 This study provides evidence that bacterial sRNAs can target the coding sequence, with the relevant implication that reporter constructs should include a considerable number of residues downstream of the start codon of the target mRNA of interest. It is also shown that 3' RACE can be used to obtain a rough estimate of the sRNA-binding site.
70. Guillier M, Gottesman S: **Remodelling of the *Escherichia coli* outer membrane by two small regulatory RNAs.** *Mol Microbiol* 2006, **59**:231-247.
71. Rasmussen AA, Johansen J, Nielsen JS, Overgaard M, Kallipolitis B, Valentin-Hansen P: **A conserved small RNA promotes silencing of the outer membrane protein YbFM.** *Mol Microbiol* 2009.
72. Prevost K, Salvail H, Desnoyers G, Jacques JF, Phaneuf E, Masse E: **The small RNA RyhB activates the translation of shiA mRNA encoding a permease of shikimate, a compound involved in siderophore synthesis.** *Mol Microbiol* 2007, **64**:1260-1273.
73. Papenfort K, Vogel J: **Multiple target regulation by small noncoding RNAs rewires gene expression at the post-transcriptional level.** *Res Microbiol* 2009, **160**:278-287.
74. Møller T, Franch T, Udesen C, Gerdes K, Valentin-Hansen P: **Spot 42 RNA mediates discoordinate expression of the *E. coli* galactose operon.** *Genes Dev* 2002, **16**:1696-1706.
75. Vogel J, Argaman L, Wagner EG, Altuvia S: **The small RNA IstR inhibits synthesis of an SOS-induced toxic peptide.** *Curr Biol* 2004, **14**:2271-2276.
76. Bouvier M, Sharma CM, Mika F, Nierhaus KH, Vogel J: **Small RNA binding to 5' mRNA coding region inhibits translational initiation.** *Mol Cell* 2008, **32**:827-837.
77. Opdyke JA, Kang JG, Storz G: **GadY, a small-RNA regulator of acid response genes in *Escherichia coli*.** *J Bacteriol* 2004, **186**:6698-6705.
78. Kawamoto H, Koide Y, Morita T, Aiba H: **Base-pairing requirement for RNA silencing by a bacterial small RNA and acceleration of duplex formation by Hfq.** *Mol Microbiol* 2006, **61**:1013-1022.
79. Chevalier C, Geissmann T, Helfer AC, Romby P: **Probing mRNA structure and sRNA-mRNA interactions in bacteria using enzymes and lead(II).** *Methods Mol Biol* 2009, **540**:215-232.
 An excellent introduction to *in vitro* techniques for RNA structure probing and translational control.
80. Geissmann TA, Touati D: **Hfq, a new chaperoning role: binding to messenger RNA determines access for small RNA regulator.** *EMBO J* 2004, **23**:396-405.
81. Argaman L, Altuvia S: **fhIA repression by OxyS RNA: kissing complex formation at two sites results in a stable antisense-target RNA complex.** *J Mol Biol* 2000, **300**:1101-1112.
82. Afonyushkin T, Vecerek B, Moll I, Bläsi U, Kabardin VR: **Both RNase E and RNase III control the stability of sodB mRNA upon translational inhibition by the small regulatory RNA RyhB.** *Nucleic Acids Res* 2005, **33**:1678-1689.
83. Darfeuille F, Unoson C, Vogel J, Wagner EG: **An antisense RNA inhibits translation by competing with standby ribosomes.** *Mol Cell* 2007, **26**:381-392.
84. Vecerek B, Moll I, Bläsi U: **Control of Fur synthesis by the non-coding RNA RyhB and iron-responsive decoding.** *EMBO J* 2007, **26**:965-975.
85. Lease RA, Woodson SA: **Cycling of the Sm-like protein Hfq on the DsrA small regulatory RNA.** *J Mol Biol* 2004, **344**:1211-1223.
86. Maki K, Uno K, Morita T, Aiba H: **RNA, but not protein partners, is directly responsible for translational silencing by a bacterial Hfq-binding small RNA.** *Proc Natl Acad Sci U S A* 2008, **105**:10332-10337.
 Together with Ref. [46*], this study evaluates the use of an mRNA decay-free, 70S ribosome translation system to sRNA effects on mRNAs *in vitro*.
87. Morita T, Maki K, Aiba H: **RNase E-based ribonucleoprotein complexes: mechanical basis of mRNA destabilization mediated by bacterial noncoding RNAs.** *Genes Dev* 2005, **19**:2176-2186.
88. Chant EL, Summers DK: **Indole signalling contributes to the stable maintenance of *Escherichia coli* multicopy plasmids.** *Mol Microbiol* 2007, **63**:35-43.
89. Windbichler N, von Pelchrzim F, Mayer O, Csaszar E, Schroeder R: **Isolation of small RNA-binding proteins from *E. coli*: evidence for frequent interaction of RNAs with RNA polymerase.** *RNA Biol* 2008, **5**:30-40.
 Aptamer-tagging is used to identify proteins that associate with several *E. coli* sRNAs.
90. Said N, Rieder R, Hurwitz R, Deckert J, Urlaub H, Vogel J: **In vivo expression and purification of aptamer-tagged small RNA regulators.** *Nucleic Acids Research* (2009) in press.
91. Ansong C, Yoon H, Porwollik S, Mottaz-Brewer H, Petritis BO, Jaitly N, Adkins JN, McClelland M, Heffron F, Smith RD: **Global systems-level analysis of Hfq and SmpB deletion mutants in *Salmonella*: implications for virulence and global protein translation.** *PLoS ONE* 2009, **4**:e4809.
92. Brencic A, Lory S: **Determination of the regulon and identification of novel mRNA targets of *Pseudomonas aeruginosa* RsmA.** *Mol Microbiol* 2009, **72**:612-632.
93. Douchin V, Bohn C, Boulloc P: **Down-regulation of porins by a small RNA bypasses the essentiality of the regulated intramembrane proteolysis protease RseP in *Escherichia coli*.** *J Biol Chem* 2006, **281**:12253-12259.
94. Antal M, Bordeau V, Douchin V, Felden B: **A small bacterial RNA regulates a putative ABC transporter.** *J Biol Chem* 2005, **280**:7901-7908.
95. Marraffini LA, Sontheimer EJ: **Invasive DNA, chopped and in the CRISPR.** *Structure* 2009, **17**:786-788.
96. Massé E, Escorcia FE, Gottesman S: **Coupled degradation of a small regulatory RNA and its mRNA targets in *Escherichia coli*.** *Genes Dev* 2003, **17**:2374-2383.
97. Ulve VM, Sevin EW, Cheron A, Barloy-Hubler F: **Identification of chromosomal alpha-proteobacterial small RNAs by comparative genome analysis and detection in *Sinorhizobium meliloti* strain 1021.** *BMC Genomics* 2007, **8**:467.
98. Swiercz JP, Hindra, Bobek J, Bobek J, Haiser HJ, Di Berardo C, Tjaden B, Elliot MA: **Small non-coding RNAs in *Streptomyces coelicolor*.** *Nucleic Acids Res* 2008, **36**:7240-7251.