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Experimental approaches for the discovery and characterization of regulatory small RNA

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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.

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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]. Biocomputational 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.

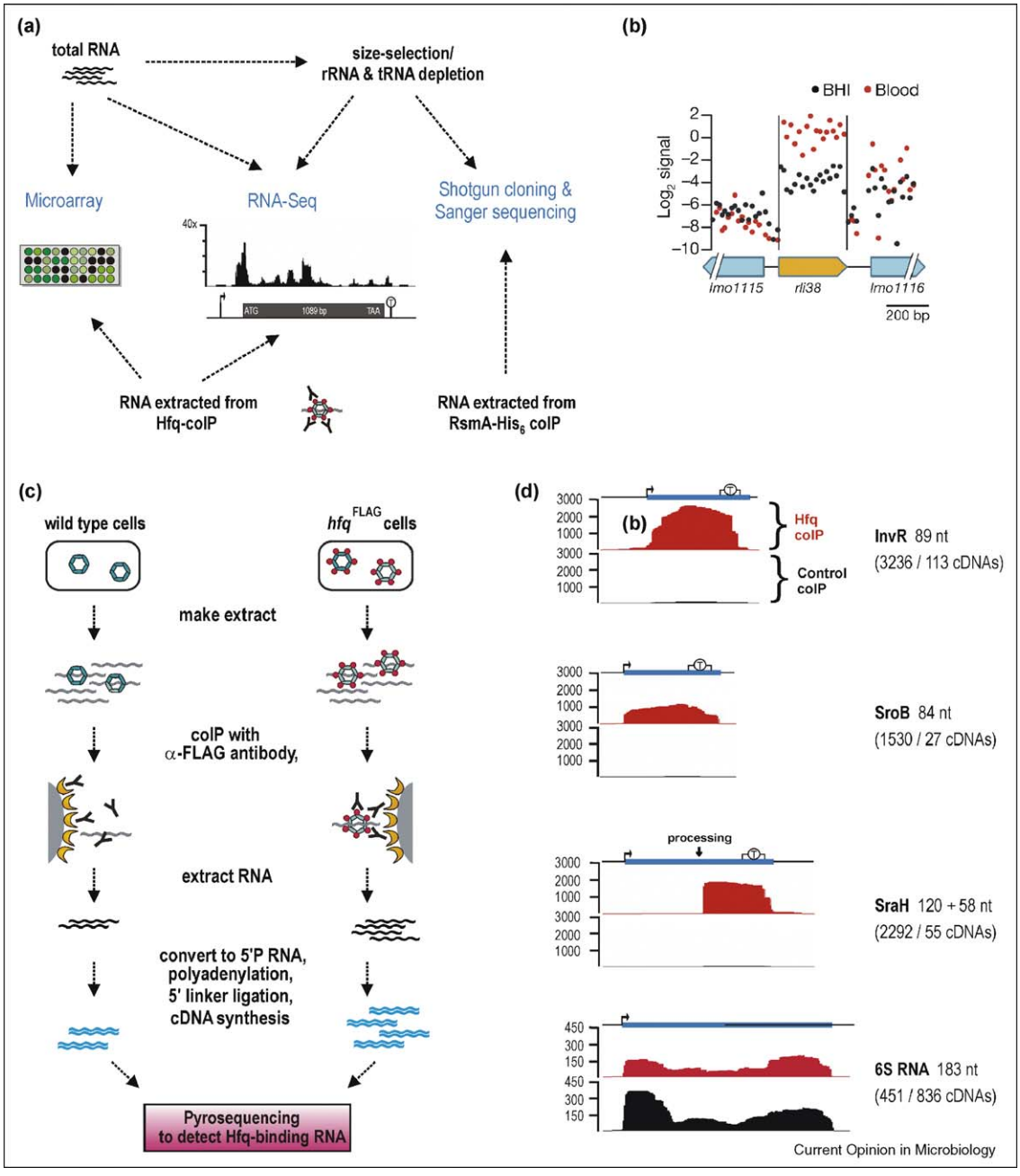
Genome-wide detection of expressed sRNAs

Notwithstanding exceptions (e.g. RNAIII, 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•].

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