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New RNA-seq approaches for the study of bacterial pathogens

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HIGHLIGHTS (3 TO 5 - 85 CHARACTERS SPACE INCLUDED)

- A surge of new RNA-seq based methods to study bacterial pathogenesis
- RIP-seq, CLIP-seq, CLASH, RIL-seq, GRIL-seq, MAPS and Term-seq discover new RNA-based regulations
- Grad-seq guides the discovery of RNA-binding proteins
- Dual RNA-seq for simultaneous transcriptomics in host-pathogen interactions
- Single-cell RNA-seq addresses heterogeneity in infections

ABSTRACT

Understanding how bacteria cause disease requires knowledge of which genes are expressed and how they are regulated during infection. While RNA-seq is now a routine method for gene expression analysis in bacterial pathogens, the past years have also witnessed a surge of novel RNA-seq based approaches going beyond standard mRNA profiling. These include variations of the technique to capture post-transcriptional networks controlled by small RNAs and to discover associated RNA-binding proteins in the pathogen itself. Dual RNA-seq analyzing pathogen and host simultaneously has revealed roles of noncoding RNAs during infection and enabled the correlation of bacterial gene activity with specific host responses. Single-cell RNA-seq studies have addressed how heterogeneity among individual host cells may determine infection outcomes.

MAIN TEXT

The central role of RNA makes it the premier readout for a fast and comprehensive description of the physiological state of a cell. Using the so-called RNA-seq technique whereby transcripts are converted to cDNA for next-generation sequencing it is now possible to profile and quantify RNA molecules in any organism [1] as well as their interactions with RNA-binding proteins (RBPs) [2]. Unsurprisingly, RNA-seq has also become a standard method to analyse gene expression in the context of bacterial infections [3]. Following pioneering applications to profile cellular transcripts associated with the RNA chaperone Hfq in *Salmonella enterica* [4] and whole transcriptomes of *Burkholderia cenocepacia* and *Helicobacter pylori* [5,6], RNA-seq has illuminated the RNA landscapes of many diverse disease-causing bacteria [7]. These studies have provided us with a much better understanding of the transcriptional architecture of bacterial pathogens and contributed to a growing appreciation of the roles of small noncoding RNAs (sRNAs) in gene regulation [8].

While RNA-seq is now a standard method for transcriptomics of *in vitro* cultured pathogens, it has much greater potential for analyzing how bacteria succeed in infecting and subverting their eukaryotic hosts. In this short review, we aim to highlight a flurry of studies in the past few years reporting promising new RNA-seq approaches (**Table 1**) to revealing post-transcriptional networks in the pathogen itself; to obtaining the full picture of infection-relevant RNA expression changes that also integrates the host; and finally, to resolving the heterogeneous environment of bacterial pathogens within host cell populations. We have included some work on non-pathogenic bacteria where the method reported can easily (and should soon) be applied to pathogens.

Target predictions of individual sRNAs

Regulatory RNAs exert post-transcriptional control in many pathways of bacterial physiology and virulence [9]. A primary example are the sRNAs, noncoding transcripts in the 50-250 nucleotide range, the majority of which regulate *trans*-encoded mRNAs by base-pairing mechanisms [9] and in, Gram-negative pathogens, typically depend on the RNA chaperones Hfq and ProQ [10-12]. By contrast, the ubiquitous CsrB-like sRNAs control mRNAs indirectly by antagonizing CsrA-like translational repressor proteins [13]. Loss-of-function of Hfq or CsrA attenuates the virulence of diverse pathogens [14], which may be taken as evidence that the associated sRNAs have roles in bacterial pathogenesis, too. Other important riboregulators include riboswitches and RNA thermometers, which usually act in *cis* via induction of structural rearrangements in their host mRNAs [15,16].

The ability to predict their cellular targets experimentally was crucial in establishing sRNAs as a major class of post-transcriptional regulators. The method of choice has been pulse expression: an sRNA is briefly expressed from an inducible promoter, followed by microarray-based global gene expression profiling to detect mRNAs (targets) with altered abundance due to base pairing interactions [17,18]. These predictions may be made more reliable by the inclusion of sRNA variants with mutated seed regions [19]. As in standard transcriptomics, RNA-seq is increasingly substituting microarrays for target discovery by sRNA pulse expression in pathogens grown *in vitro* or even inside host cells [20]. Moreover, RNA-seq has been used to profile sRNA-induced changes in ribosome-bound transcripts, helping to distinguish between mRNAs regulated at the level of RNA stability and those regulated at the level of translation [21,22].

Whereas the above methods identify targets from induced gene expression changes, two recent approaches employed RNA-seq to identify targets of individual sRNAs

more directly. In MAPS, potential cellular RNA targets were co-purified and sequenced with *in vivo* expressed aptamer-tagged *E. coli* sRNAs [23]; besides new mRNA targets, this work discovered 3' fragments of tRNA precursors as natural buffers of sRNA activity (**Figure 1**). GRIL-seq, developed to identify targets of PrrF1 sRNA in *Pseudomonas aeruginosa*, works similarly but additionally seeks to seal the typically short sRNA-mRNA duplexes *in vivo* by expression of an RNA ligase prior to capturing candidate target mRNAs with an sRNA-specific oligonucleotide [24] (**Figure 1**). Although technically more challenging, these techniques nicely complement target predictions by mRNA abundance profiling, as judged by some *bona fide* targets of the *E. coli* RyhB sRNA which were missed by either pulse expression or MAPS [23].

RNA inventories

Target searches for individual sRNAs seek to answer specific physiological questions, but it is advisable to understand early on in what global context these sRNAs act. Which RBP does a given sRNA interact with? Are the candidate targets of an Hfq-associated sRNA also bound by Hfq?

RIP-seq offers a cost-effective approach to obtaining a quick overview of major RNA regulons in a pathogen of interest; it applies RNA-seq to analyse transcripts obtained by co-immunoprecipitation (coIP) with an RBP such as Hfq, using either a protein-specific antibody or by pulling down an epitope-tagged variant of the RBP (provided the tag maintains a functional protein) (**Figure 1**). RIP-seq was used to globally profile Hfq-bound sRNAs and potential mRNA targets in several stages of growth of *Salmonella* Typhimurium, bringing to light a new sRNA (DapZ) activated along with the invasion genes of this pathogen and a surprising number of other noncanonical sRNAs originating from the 3'

regions of mRNA genes [25]. It has determined cellular ligands of several other sRNA-related proteins [11-13,26-28], and comparison of cDNA reads from coIP samples with known *in vivo* copy numbers of sRNAs suggests that RIP-seq is semi-quantitative [25].

RIP-seq identifies major RNA ligands but gives limited positional information as to where the RBP in question binds. Therefore, methods like CLASH and RIL-seq [29,30] or CLIP-seq [31,32] include *in vivo* UV treatment to covalently cross-link RNA to proteins prior to co-purification (**Figure 1**). The crosslinking permits both more stringent denaturing conditions to reduce potential false-positive targets and on-protein trimming of bound RNAs by ribonucleases [32]. Moreover, the crosslinked position often reveals itself by a mutation in the cDNA, although this may vary from protein to protein [31]. These crosslinking-based methods have illuminated Hfq binding preferences for sRNAs and mRNAs in pathogenic *E. coli* and *Salmonella* [31,32] and improved *in silico* predictions of sRNA-mRNA pairs [31]. CLIP-seq dramatically increased the known size of the CsrA regulon, predicting that this translational repressor interacts with >500 *Salmonella* mRNAs, many from virulence regions [31]. The RNA binding preference of CsrA inferred from this global data is an AUGGA motif in apical loops, refining the previously predicted recognition motif [33].

Global sRNA interactomes in one go

The pace of discovery and vast number of sRNAs in bacterial pathogens [8] demands the development of methods that go beyond target inventories of individual sRNAs or RBPs. Two recent studies have progressed to reporting global sRNA-mRNA interactomes in one go [29,30]. Common to both studies is the use of *in vivo* UV crosslinking and ligation of sRNA-mRNA pairs during purification with central proteins, either Hfq itself [30] or

endoribonuclease RNase E [29], which degrades mRNAs upon recognition by cognate sRNAs. The first of these two techniques, RIL-seq (**Figure 1**), recovered an aggregate of ~2,800 putative RNA interactions—ten times the established sRNA interactome—from Hfq in nonpathogenic *E. coli* [30]. The recovery of known sRNA-mRNA interactions was remarkably high (56%), considering that RIL-seq sampled only three growth conditions. Further supporting the reliability of RIL-seq, mutations in the seed region of the multi-target sRNA GcvB strongly abrogated the recovery of GcvB-mRNA hybrids. The obtained *E. coli* sRNA interactome appears dynamic, with substantial re-wiring occurring upon changes in cellular conditions [30]. In addition, RIL-seq discovered a new sRNA sponge and *trans*-encoded mRNA targets of *cis*-regulatory antisense RNAs [30].

The other study [29] in enterohemorrhagic *E. coli* (EHEC) used proximity ligation by CLASH (**Figure 1**) which had previously failed to recover RNA hybrids from Hfq in this organism [32], perhaps due to more stringent purification as compared to RIL-seq [30]. However, ligation on RNase E in lieu of Hfq increased the proportion of RNA hybrids from ~0.001% [29] to ~0.8% [32]. Since these hybrids are enriched in pairs of known sRNA seed regions and co-regulated targets, they likely represent *bona fide* sRNA-mRNA interactions. This new CLASH study is particularly noteworthy for its identification of targets of sRNAs originating from horizontally acquired virulence regions [32].

Both CLASH and RIL-seq recover a comparable number of statistically significant sRNA-mRNA interactions in related conditions and similar mRNA motifs complementary to sRNA seeds, despite the use of two different bait proteins and that EHEC and *E. coli* K12 are two different strains that share only ~80% of their genomic content. They bolster a view that Hfq-dependent sRNAs primarily sequester the start codon region of mRNAs, repressing translation. Nonetheless, hundreds of sRNA hybrids with mRNA fragments outside translation initiation regions offer leads for potentially new

mechanisms of negative or positive mRNA regulation. Finally, these first global snapshots of RNA interactomes corroborate the emerging view that sRNAs acting *in trans* come from more sources than the canonical sRNA genes in intergenic regions [34].

Discovery of novel RBPs with Grad-seq

It is emerging from RNA profiling of Hfq and CsrA that these RBPs interact with but a subset of the cellular sRNAs and mRNAs. Moreover, many bacteria lack a functional Hfq protein, suggesting that other global sRNA-binding proteins must exist. Many new RBPs have been discovered in eukaryotes, but the underlying methods [2] are not transferable to bacteria whose transcripts lack a functional poly(A) tail. The new Grad-seq approach [11] (**Figure 2a**) overcomes this limitation by defining major functional RNA classes for subsequent purification of common RBPs such as ProQ, now unveiled as the second global sRNA chaperone in *Salmonella*, *E. coli* [11] and likely other bacteria [12].

In Grad-seq, a bacterial lysate is fractionated on a density gradient, sorting complexes of RNA and protein by molecular weight and shape [11]. RNA-seq of these fractions produces gradient RNA distributions which enable the co-clustering of RNAs that interact with the same protein, as shown for *Salmonella* sRNAs in **Figure 2b**. This common protein partner is then identified using aptamer-tagged variants of the sRNAs as baits [35]. Optional proteomics of the same gradient fractions identifies proteins with matching profiles, and coverage permitting, may reveal RNA-protein complexes by direct correlation [11].

In simpler terms, Grad-seq provides a generic experimental and analytical framework for RNA interactome visualization, assigning major groups of functionally related transcripts largely independently of their length or primary sequence. Grad-seq

promises to identify the outstanding general sRNA-binding protein(s) in Gram-positive bacteria [9] and to draft initial RNA landscapes for understudied microbes or even medically important microbial collectives such as the gut microbiota, the vast majority of whose species remain uncultured.

Dual RNA-seq for simultaneous analysis of pathogen and host

The lifestyle of bacterial pathogens involves interactions not only with host cells but also with other microbes. On the latter, RNA-seq is heralding a new era of microbiome research [36]. A recently developed approach called Term-seq (**Figure 3a**), which enables genome-wide mapping of transcript termination, has proven successful at discovering antibiotic-responsive riboswitches from human microbiome samples [37].

For pathogen interactions with eukaryotic cells, transcriptomic studies were long technically limited to analyzing mRNA expression changes in either bacteria or infected host cells. This has been overcome by so-called dual RNA-seq (**Figure 3b**) [3] which simultaneously captures both pathogen and host transcripts. Given that the RNA content of a mammalian host cell greatly exceeds that of an intracellular pathogen [3], the pathogen contributes only a minor fraction to dual RNA-seq reads. Nonetheless, following rRNA depletion from mixed total RNA, >25 million cDNA reads suffice to profile all major coding and noncoding RNA classes, including regulatory eukaryotic microRNAs and lincRNAs as well as bacterial sRNAs [20]. We have reviewed in depth the technical aspects of dual RNA-seq from experimental design to bioinformatic analysis elsewhere [38].

In the four years since the concept was introduced [3], dual RNA-seq has been applied to a range of infection models [38], for example, *Chlamydia trachomatis* interacting with epithelial carcinoma cells [39], *E. coli* inside macrophages [40], or *Salmonella*

replicating in 14 different cell types of human, mouse and pig origin [20]. In addition to providing information on physiological changes in pathogen and host during the course of an infection, dual RNA-seq also unveiled hidden molecular phenotypes of virulence-associated sRNAs (**Figure 3c**) [20,41]. For example, PinT sRNA was shown to be highly activated in intracellular *Salmonella*. Subsequently, a comparative dual RNA-seq time-course with a *pinT* deletion mutant unraveled the function of PinT as a post-transcriptional regulator of important *Salmonella* virulence genes inside both human and porcine cells. The activity of PinT has a dramatic effect on the host response, with ~10% of all detected human transcripts being differentially expressed between the two infections. A similar analysis revealed significant impacts for six additional *Salmonella* sRNAs on both pathogen and host RNA expression, despite a recurrent lack of obvious replication or invasion phenotypes [20]. Combined with other methods such as pulse expression, dual RNA-seq now provides a technically straightforward approach to discovering and studying sRNAs in the context of host-pathogen interactions.

Single-cell RNA-seq reveals heterogeneous host responses

Host-pathogen interactions are increasingly found to involve a high degree of cellular heterogeneity that impacts disease progression, pathogen dissemination and antimicrobial treatment [42]. New fluorescent reporter systems have revealed considerable heterogeneity in bacterial intracellular growth rate or stress response activity [43,44], pinpointing persister cells with a non/slow growing phenotype that resist antibiotic treatment [42]. Host immune cells exposed to a homogeneous stimulus exhibit largely heterogeneous gene expression programs on the single-cell level [45]. Single-cell RNA-seq, often in combination with fluorescence-activated cell sorting (FACS), is emerging

as a powerful tool to profile cell-to-cell variation [46,47] and attribute unique RNA signatures to cell identity and biological function [45,48].

Two studies have applied single-cell RNA-seq to dissect the individual responses of macrophages to *Salmonella*, obtaining single-cell profiles for ~5,000 different host mRNAs [49,50]. The first study [49] analyzed the early infection period between uptake and onset of intracellular replication of *Salmonella* (**Figure 4**). Fluorescent reporters of bacterial load and membrane integrity enabled the differentiation of three macrophage populations: uninfected, infected but cleared, and infected with intracellular bacteria. Invaded macrophages exhibited large heterogeneity in gene expression resulting from a bimodal expression of the type I interferon (IFN) response, and this could be attributed to a variable intracellular activity of *Salmonella*'s PhoP/Q system [49].

The other study [50] combined a fluorescent bacterial division reporter [44] with single-cell RNA-seq to analyze heterogeneity in the macrophage response to growing versus non-growing *Salmonella* (**Figure 4**). The results tie macrophage polarization states to the growth rate of intracellular whereby *Salmonella* either evades intracellular immunity in a quiet long-term niche, or subverts immunity and proliferates: macrophages harboring non-growing *Salmonella* displayed RNA signatures of the proinflammatory M1 polarization state, whereas those containing growing bacteria adopted an anti-inflammatory, M2-like state. With these observations in hand, single-cell RNA-seq readily lends itself to study other known cases of host cell manipulation, for example, *Salmonella* reprogramming of epithelial cells to M cells [51] or leprosy bacilli reprogramming of adult Schwann cells [52].

Current single-cell RNA-seq protocols cannot sample the bacterial transcriptome together with that of the host as they generally rely on poly(A)-dependent

reverse transcription [46]. In addition, their sensitivity is restricted to transcripts present at >10 copies per cell which is far beyond the average copy number of 0.4 in the case of bacterial mRNAs [53] (note, that sRNAs often accumulate to >100 copies/cell [25]). However, bacterial single-cell RNA-seq protocols have been described [54], hopefully paving the way for single-cell dual RNA-seq to analyze heterogeneity in RNA expression programs and its consequences in pathogen and host simultaneously. This seems particularly promising in the context of sRNA-mediated regulation which is typically characterized by fine-tuning and modulation rather than the on/off-switch functions of transcription factors.

Future perspective

The ‘-seq’ methods have become important tools in understanding “how, when, and why sRNA-mediated regulation is of such importance to bacterial lifestyles” [55] and resolving RNA expression changes important for the success of a bacterial pathogen. For space reasons, we are unable to cover several additional variations of RNA-seq measuring protein synthesis [56] or illuminating RNA processing, structure changes and modification [26,57,58]. Another largely unresolved question is ‘where’ bacterial riboregulators are acting. Prominent RBPs such as Hfq may localize near the cytoplasmic membrane [59], as do some sRNAs [60,61]. Therefore, RNA-seq combined with cellular fractionation may reveal new aspects of spatial organization, for example, different global sRNA-mRNA interactomes for the cytosol and the membrane.

The obvious task for the immediate future is to adapt these many new methods to *in vivo* models of infection. Generally, this will require improved protocols that work with significantly less input RNA, especially for single-cell analysis; new reverse

transcriptases may achieve this goal [62]. Microfluidic technology has great potential not only for the handling and manipulation of minute amounts of starting material, but also for cDNA synthesis and sequencing in small volumes and in higher throughput [63]. Beyond the study of natural infections, RNA-seq miniaturization will benefit mode-of-action studies for diverse RNA-directed antimicrobials, be these small molecules interfering with RNA-protein interactions [64], riboswitch function [65], or the multitude of antisense inhibitors that have been developed over the years [66].

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FIGURE LEGENDS

Figure 1: New RNA-seq based methods to decipher regulatory RNA networks.

MAPS (MS2-affinity purification coupled with RNA-seq): an MS2 affinity tag is fused to a sRNA of interest and co-purified with its interacting RNA and sequenced. **GRIL-seq** (Global sRNA target identification by ligation and sequencing): the method allows the identification of direct targets of sRNAs by *in vivo* proximity ligation. It is similar to MAPS, but additionally ligates short sRNA-mRNA duplexes *in vivo* by co-expressing T4 RNA ligase prior to capturing candidate target mRNAs with a sRNA-specific oligonucleotide. **RIP-seq** (native RNA immunoprecipitation followed by RNA-seq): target transcripts are immunoprecipitated with a protein of interest in cell lysates and analysed by RNA-seq. **CLIP-seq** (cross-linking immunoprecipitation-high-throughput sequencing): *in vivo* UV

treatment covalently cross-links RNA to proteins prior to co-purification and RNA-seq. RNA trimming by ribonucleases outside of the binding region enables to map the binding region at single-nucleotide resolution. **CLASH** (crosslinking, ligation, and sequencing of hybrids): RNA molecules are UV-crosslinked to a flagged RBP *in vivo*. Next, the bound RNA is trimmed using an RNase and RNA linkers are ligated to the immobilized RNA molecules in the RNA-RBP complexes. Finally, coupled RNA molecules are ligated into one single molecule of two different types, either single or chimeric fragments. **RIL-seq** (RNA interaction by ligation and sequencing): a protein-RNA complex is immunoprecipitated and bound RNAs are crosslinked to the protein. After enzymatic digestion, RNAs are ligated and subjected to RNA-seq. RNA-RNA interactions are revealed after mapping and identification of chimeric reads.

Figure 2: Grad-seq for the discovery of novel RBP complexes.

(a) In Gradient fractionation followed by RNA-seq (Grad-seq), cellular RNAs are sorted along with proteins and complexes by fractionating a bacterial lysate on a density gradient. Parallel RNA-seq and mass spectrometry (MS) analysis of every fraction produces gradient profiles for RNA and protein, respectively. **(b)** Principal component analysis (PCA) illustrating how Grad-seq assigns cellular sRNAs to major classes associated with different RBPs, here Hfq, CsrA and ProQ (adapted from [11]).

Figure 3: Term-seq and dual RNA-seq for the study of pathogens in complex environments.

(a) Term-seq workflow. A collected microbiome is exposed to some stress or compound and both mock and treated samples are subjected to Term-seq which focusses on RNA 3'

termini. Regulation by conditional termination in bacteria can be revealed, for example an antibiotic binding to the 5'UTR of an mRNA represses transcriptional termination, giving rise to the full-length mRNA (light blue: terminator sequence; light pink: anti-terminator sequence). **(b)** Dual RNA-seq workflow. Total RNA is extracted from host cells infected with a pathogen, subjected to RNA-seq, upon which the reads are mapped to their originating organism. **(c)** Application of dual RNA-seq to reveal novel sRNA function(s). Applied over the course of the infection, dual RNA-seq provides a list of the differentially expressed coding and non-coding RNAs from which candidates can be selected. Deletion of the candidate gene followed by 'comparative' dual RNA-seq and interspecies analysis can reveal the molecular functions of the perturbed gene.

Figure 4: Single-cell RNA-seq reveals heterogeneity in *Salmonella*-infected macrophages.

Macrophage (MΦ) infection by *Salmonella* leads to disparate outcomes. After 6 hours post-internalization (h.p.i.), intracellular bacteria may have been killed, grow, or remain in a non-growing state. Two studies [49,50] have combined bacterial fluorescent reporters (pHrodo and fluorescence dilution, respectively), single-cell fluorescence-activated cell sorting (FACS), and single-cell RNA-seq. This workflow has been applied across two different time points. Between 0 and 6 h.p.i. [49] (before bacterial growth), two distinct macrophage populations differing in the type I interferon (IFN I) response were linked to *Salmonella* PhoP regulation. After extended intracellular replication (20 h.p.i. [50]), a comparison of MΦs with non-growing and growing bacteria revealed differential *Salmonella*-induced polarization of MΦs towards M1 and M2 states, respectively.

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Table 1: Overview of RNA-seq methods discussed in this review

Method	Organism ¹⁾	Selected main findings	Ref.
MAPS	<i>E. coli</i>	Identifies 3' trailer of tRNA as sRNA sponge	[23]
GRIL-seq	<i>P. aeruginosa</i>	Defines direct targets of PrrF1, an iron-regulated sRNA	[24]
RIP-seq	<i>E. coli</i>	New insights into control of decay of CsrB-like sRNAs by protein CsrA	[13]
		Identifies new Hfq-associated sRNAs and targets, including many <i>cis</i> -antisense RNAs	[27]
	<i>S. Typhimurium</i>	Capture of Hfq-bound transcripts reveals many alternative sRNAs made from 3' mRNA regions which include DapZ, a GcvB-like sRNA	[25]
	<i>L. pneumophila</i>	Finds sRNA RocR which together with ProQ-like protein Lpp0148 controls natural transformation	[12]
	<i>C. jejuni</i>	Discovers flagellin mRNA (<i>flaA</i>) as the main target of CsrA in an organisms that generally lacks CsrA-antagonizing sRNAs	[26]
CLIP-seq	<i>E. coli</i>	Through profiling Hfq, reveals a bacteriophage-encoded sRNA (AgvB) which counteracts GcvB	[32]
	<i>S. Typhimurium</i>	Hfq and CsrA global pattern of motif recognition, CsrA shown to target many virulence mRNAs	[31]
CLASH	<i>E. coli</i>	Global RNA interactome via RNase E: EHEC-specific sRNA Esr41 controls iron transport and storage.	[29]
RIL-seq	<i>E. coli</i>	Global Hfq interactome, provides the first high-confidence list of sRNA-mRNA interactions and new types of sRNAs	[30]
Grad-seq	<i>S. Typhimurium</i>	Discovers ProQ as a new global RNA-binding protein and defines a new class of ProQ-associated sRNAs	[11]
Term-seq	<i>B. subtilis</i> , <i>L. monocytogenes</i> , <i>E. faecalis</i> , oral microbiota	Novel ribo-regulators that respond to antibiotics including a riboswitch controlling the transcription of a <i>L. monocytogenes</i> lincomycin resistance gene	[37]
Dual RNA-seq	<i>S. Typhimurium</i>	Discovers sRNA PinT as a post-transcriptional timer of <i>Salmonella</i> virulence gene expression, provides full pathogen and host RNA landscapes for <i>Salmonella</i> in 14 cell types from human, mouse and pig	[20]
	<i>C. trachomatis</i>	Early iron acquisition, putative immune dampening effects of chlamydial infection of epithelial carcinoma cells	[39]
	UPEC	Identifies <i>pspA</i> is being involved in intra-macrophage survival in macrophages	[40]
Single-cell RNA-seq	<i>S. Typhimurium</i>	Variable PhoP/Q activity amongst invading bacteria drives heterogeneous type I IFN response in infected macrophages	[49]
		Ties non-growing intracellular bacteria to M1 polarization state of macrophages, whereas growing bacteria are associated with macrophages in M2-like state	[50]

1) Abbreviations: *B. subtilis*: *Bacillus subtilis*; BMDMs: Bone marrow-derived macrophages; *C. jejuni*: *Campylobacter jejuni*; *E. coli*: *Escherichia coli*; *E. faecalis*: *Enterococcus faecalis*; ETS: external transcribed spacer; UPEC: Uropathogenic *E. coli*; *L. monocytogenes*: *Listeria monocytogenes*; *P. aeruginosa*: *Pseudomonas aeruginosa*; *L. pneumophila*: *Legionella pneumophila*; *S. Typhimurium*: *Salmonella enteric serovar Typhimurium*; sRNA: small RNA; asRNA: antisense RNA; SPI1: *Salmonella* pathogenicity island 1.

Figure Highlight

Figure 1

Figure 2

(a) Grad-seq

(b)

Figure 3

Figure 4