Available online at www.sciencedirect.com

ScienceDirect

Current Opinion in Microbiology

Exploring the transcriptional landscape of phage–host interactions using novel high-throughput approaches☆

Leena Putzeys[1,*], Laura Wicke[1,2,*], Ana Brandão[3,*], Maarten Boon[1], Diana P Pires[3], Joana Azeredo[3], Jörg Vogel[2,4], Rob Lavigne[1] and Milan Gerovac[2,4]

In the last decade, powerful high-throughput sequencing approaches have emerged to analyse microbial transcriptomes at a global scale. However, to date, applications of these approaches to microbial viruses such as phages remain scarce. Tailoring these techniques to virus-infected bacteria promises to obtain a detailed picture of the underexplored RNA biology and molecular processes during infection. In addition, transcriptome study of stress and perturbations induced by phages in their infected bacterial hosts is likely to reveal new fundamental mechanisms of bacterial metabolism and gene regulation. Here, we provide references and blueprints to implement emerging transcriptomic approaches towards addressing transcriptome architecture, RNA–RNA and RNA–protein interactions, RNA modifications, structures and heterogeneity of transcription profiles in infected cells that will provide guides for future directions in phage-centric therapeutic applications and microbial synthetic biology.

Addresses
[1] Department of Biosystems, Laboratory of Gene Technology, KU Leuven, Leuven, Belgium
[2] Institute for Molecular Infection Biology (IMIB), Medical Faculty, University of Würzburg, Würzburg, Germany
[3] Centre of Biological Engineering, University of Minho, Braga, Portugal
[4] Helmholtz Institute for RNA-based Infection Research (HIRI), Helmholtz Centre for Infection Research (HZI), Würzburg, Germany

Corresponding author: Gerovac, Milan (milan.gerovac@uni-wuerzburg.de)
[*] These authors contributed equally.

Current Opinion in Microbiology 2024, **77**:102419

This review comes from a themed issue on **Host-Microbe Interactions: viruses**

Edited by **Rob Lavigne** and **Julia Frunzke**

For complete overview of the section, please refer to the article collection, "Host-Microbe Interactions: Viruses 2024"

Available online 24 January 2024

https://doi.org/10.1016/j.mib.2023.102419

Introduction

Since the discovery of viruses that infect bacteria (bacteriophages, short: phages), the scientific community has a significant interest to use them for modern biotechnological applications, study their impact on global ecology and bacterial pathogenicity as well as look into them as a potential source of new antibacterials [1,2]. Advances in phage (meta)genomics have allowed for the discovery of an unprecedented catalogue of phage genomes that revealed an immense phage diversity and impact on microbial communities [3]. Concomitantly, phage genomics revealed a large knowledge gap of functionally uncharacterised and hypothetical proteins being likely connected to novel regulatory mechanisms involved in phage–host interactions. The application of state-of-the-art integrative transcriptomics, proteomics and metabolomics captures different complex layers of phage infection and sheds light on the 'viral dark matter' [4]. These integrated approaches have already proven their value in bacteria [5], yet surprisingly, the immense potential on the side of their viral predators remains underexplored. Implementation of these -omics approaches in phage research is required to fully grasp phage–host interactions and translate this knowledge into biotechnological and medical applications. For this, comprehensive high-resolution maps of transcriptomes are a fundamental requisite. Still, technical challenges to study the transcriptome of phage-infected cells remain. For example, the rapid phage replication cycle and needed synchronised infection of host cells by high multiplicity of infection [6] limit the study of phages that cannot be prepared to high titres, or in cases where the propagation host has not been identified or is not culturable. Moreover, infected bacterial cells can aggregate (e.g. [7]) or partly lyse, which makes it challenging to isolate RNA from the cells, including separation into single cells that would enable sorting. Such optimisations are essential for growth and infection conditions, together with labelling of infected cells for sorting.

In the last decade, phage transcriptomic approaches much relied upon total RNA sequencing (RNA-seq) in

☆ Given the role as Guest Editor, Rob Lavigne had no involvement in the peer review of the article and has no access to information regarding its peer-review. Full responsibility for the editorial process of this article was delegated to Julia Frunzke.

bulk to obtain gene expression levels that give insights into phage activity and host responses (Supporting Table 1) [8-11]. Sequencing of phage transcripts shortly after infection revealed anti-phage-defence and host-takeover factors [12-15]. However, total RNA-seq generally does not capture the details, diversity and interactions of transcripts such as primary transcriptional features, RNA structures and modifications that underlie regulatory mechanisms. This is especially true for the densely packed genomes of phages, with many transcriptional features in close proximity. As a consequence, the RNA biology of phages remains largely uncharted, highlighting the need for advanced transcriptional approaches to resolve the intricate transcriptional blueprints of microbial viruses. Here, we hope to inspire phage researchers to take a step further and adopt these emerging techniques to tackle new research questions in phage biology.

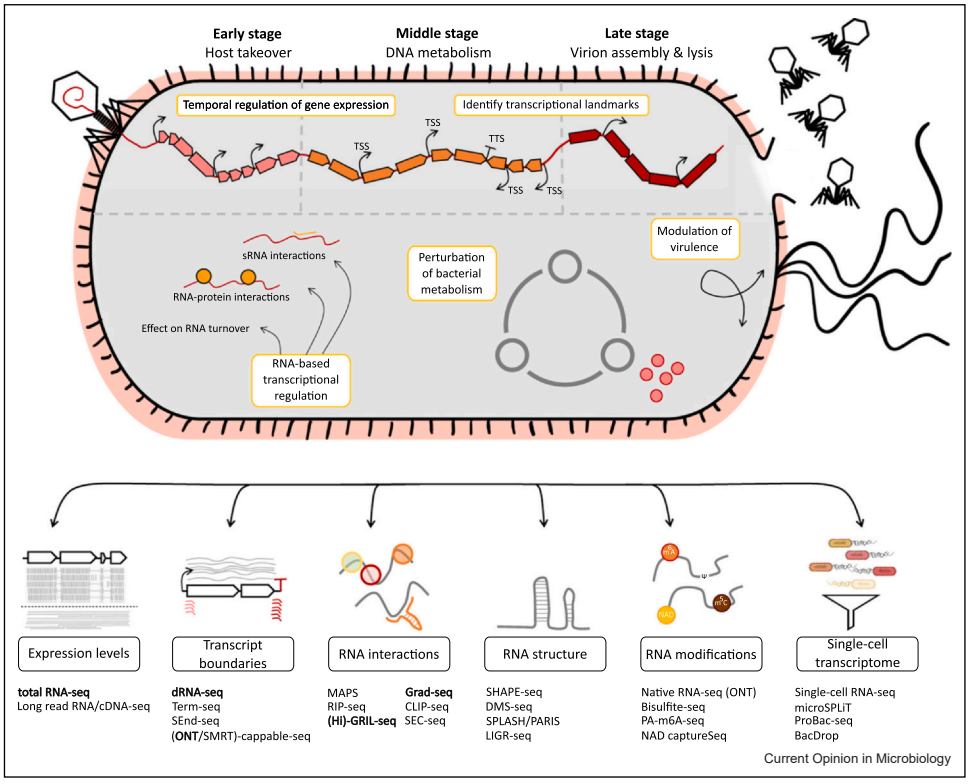
Emerging transcriptomic techniques and their untapped potential for phage biology

The prokaryotic transcriptomic toolbox has expanded continuously, giving rise to numerous new methods to study microbial RNA biology in unprecedented detail. This new generation of RNA-seq-based approaches provides the means to reveal a plethora of RNA species (Figure 1, Supporting Table 1). Phage gene expression is generally organised in the early (anti-defence and host-takeover), middle (replication) and late (virion morphogenesis and lysis) replication stages. The temporal regulation of phage gene expression is polymerase-specific and can be classified based on the utilised promoters for transcription initiation [16]. For example, phages T4 and λ rely fully on the host transcription machinery and can modulate the host RNA polymerase (RNAP) initiation via phage-encoded σ factors or covalent modifications of RNAP proteins [17]. Other phages such as T7 encode a single-subunit RNAP [18] expressed by the host RNAP in early infection, subsequently initiating transcription after the recognition of phage-specific promoters without the need of a host-encoded σ factor. Moreover, jumbo phages harbour multi-subunit RNAPs that have similar structures such as the host RNAP [19]. Another strategy is the co-injection of virion-associated RNAPs together with the phage genome for immediate transcription, enabling full independence of the host transcription machinery at the onset of infection [20,21]. Currently, knowledge on phage-regulatory mechanisms remains scarce and potentially many additional mechanisms have yet to be discovered. For example, genome compartmentalisation influences transcription, e.g. phage T5 utilises a two-step genome injection [22] or *Chimalliviridae* like ΦKZ transcribe their genome in a phage nucleus [23].

Architecture of transcripts

The delineation of transcriptional profiles of phage genes is dependent on genome-wide identification of promoters and terminators, which is crucial to fully comprehend the dependency towards specific RNAPs. Promoters can be mapped by identification of transcription start sites (TSSs) and terminators occurring at transcription termination sites. To predict promoters and terminators *in silico*, there is only a limited number of pipelines available tailored for phage genomes [24-26]. As a consequence, bacterial prediction tools are often used for this purpose, but these usually neglect non-canonical and viral-derived regulatory sequences, resulting in an incomplete prediction of the phage transcriptional programme (e.g. [27]). In the past, time-dependent transcriptional profiles of phage genes were clustered to reveal common promoter motifs for which the transcript ends were determined individually such as for the T7-like cyanophage P-SSP7 [28]. Recently, experimental transcriptomic approaches were developed and enabled a global mapping of key regulatory elements, including transcription initiation and termination events by enrichment of 5′- and 3′- transcript ends, which are generally confounded by their processed counterparts [29,30]. For example, enrichment for primary transcripts followed by sequencing reveals TSSs and their associated promoters as done by terminator exonuclease treatment that degrades processed transcripts in differential RNA-seq (dRNA-seq, [31]) and 5′-end capping of primary transcripts followed by enrichment (Cappable-seq, [32]). High-throughput discovery of transcription termination events can be achieved by specifically sequencing exposed 3′- transcript termini (Term-seq, [33]). Alternatively, both 3′- and 5′-transcriptional boundaries can be identified through sequencing of a circularised transcript product (SEnd-seq, [34]) or by sequencing the bacterial transcriptome in full-length using long-read sequencing technologies including Oxford Nanopore Technology (ONT) [35,36] and PacBio long-read sequencing [37]. These long-read methodologies greatly facilitate gene annotation and help gain insights into the complexity of fundamental regulatory signals, operon structures and gene regulation. Recently, dRNA-seq and ONT-cappable-seq were used to map the transcriptome architecture of jumbo phage ΦKZ [38], N4-like phage LUZ7 [39] and T7-like phage LUZ100 [40], all infecting *Pseudomonas aeruginosa*. These studies resulted in the first global maps of transcriptional regulatory elements in a dual fashion on the viral and the host transcriptome in a single experiment. Collectively, the data uncovered distinct promoter motifs, phage transcription unit architectures and putative phage-encoded small regulatory RNAs [38,39]. A wider adoption of these novel transcriptomic approaches in phage research is paramount to obtaining high-resolution phage transcriptomes to develop custom models [41] and

Figure 1



Overview of advanced transcriptomic approaches to illuminate phage RNA biology in a global manner. Techniques previously applied on phage-infected bacterial cells are indicated in bold, highlighting the plethora of untapped technologies in this field.

significantly improve the prediction of phage-specific regulatory elements in a high-throughput manner.

Transcripts are regulatory players in phage biology

Non-coding RNAs (ncRNA) engage in regulatory pathways through interaction with other transcripts and are often found to be associated with RNA-binding proteins (RBPs) in large regulatory networks [30,42,43]. Recently, ncRNAs appeared to add an additional layer in the transcriptional regulation of lysogens [29,44,45]. The functional role of these transcripts in lytic phages remains yet to be elucidated. Only a few examples are characterised to date, for example, prophage-encoded ncRNA anti-CRISPRs (clustered regularly interspaced short palindromic repeats) called Racrs [46] or CBASS (cyclic oligonucleotide-based anti-phage signaling system)-activating bacteriophage RNA [47] that inhibit or activate phage defence systems, respectively. NcRNAs can be primary transcripts, derived from coding sequences [48,49], or be processed from untranslated regions. In all three cases, a correct mapping of the primary transcriptome is required. In addition, high-resolution transcriptomics enable correct annotation of transcript boundaries independently of open reading frames and hence, can validate the existence of understudied viral non-coding transcripts. The intricate transcript interactome in phage-infected cells can be revealed, for example, by ligation of interacting RNAs followed by sequencing and identification of RNA–RNA chimeras (high-throughput global sRNA target identification by ligation and sequencing, Hi-GRIL-seq, [50] or RNA interaction by ligation and sequencing, RIL-seq, [51]) or MS2-tagging of RNAs for pull-down [52] coupled to sequencing of interaction partners (MS2 affinity purification sequencing, MAPS, [53]). For example, RNA–RNA-interaction studies in *Vibrio cholerae* revealed that the ncRNA SviR, encoded in a phage-inducible element, targets invading ICP1 phage transcripts to regulate their expression [44]. Notably, the detection of chimeric reads is challenging, but the high yield of phage transcripts in infected cells allows for deep detection of interactions.

Cellular complexes between ncRNAs and RBPs can be discovered by correlated sedimentation or elution profiles in gradient fractionation or size-exclusion chromatography (SEC), respectively, that analyse complexes by size (e.g. Grad-seq, [54]; SEC-seq, [55]). The cellular RNA complexome after jumbo phage ΦKZ infection was probed by gradient fractionation and uncovered phage-derived ncRNA species and shifts in sedimentation profiles of regulatory host ncRNAs [56]. The RNA targetome and interaction sites of RBP candidates can be

subsequently elucidated by RNA immunoprecipitation sequencing (RIP-seq), [57]. Optionally, UV cross-linking can be utilised to narrow down interaction sites after trimming and identification of UV-induced mutations at stringent denaturing conditions (cross-linking and immunoprecipitation sequencing, CLIP-seq), [45,58]. Application of these techniques to RBPs in a broader spectrum of infected cells and microbial viruses (Supporting Table 1), will unveil fundamental insights into intrinsic post-transcriptional regulatory networks during infection [29]. Since phage–host systems are tightly intertwined, these insights will help to answer evolutionary questions on base-pairing mechanisms between RNAs and their targets and the dependency and recognition of ncRNAs by RBPs.

RNA modifications add yet another layer to transcriptional regulation, affecting RNA function, fate and structure in various ways [59]. However, given that phage DNA genomes are collectively endowed with the largest reservoir of modified nucleobases [60], their transcripts are likely decorated with diverse modifications as well. In the continuous tugs-of-wars between phages and their hosts, phages often modify their DNA to protect themselves against bacterial defence systems, such as restriction–modification enzymes and CRISPR associated proteins (Cas) [61]. In light of the more recently discovered RNA-targeting CRISPR-Cas type-III and -VI systems, it is plausible that phages also modify their RNA to escape host immunity [62]. Internal transcript modifications and 5′-terminal structures can be captured by coupling specific chemical treatments or immunoprecipitation approaches with high-throughput RNA-sequencing (e.g. bisulfite-, m^6A-seq and nicotinamide adenine dinucleotide (NAD) captureSeq). In addition, T4 phage factor ModB RNAylates ribosomal proteins via adenosine diphosphate-ribosylation using specific 5′-NAD-capped RNAs as substrate that is important for efficient T4 phage replication [63]. Moreover, Nanopore sequencing technology allows for a global detection of RNA base modifications by direct sequencing of native RNA at the single-molecule level without the need for cDNA conversion and PCR amplification [64]. In contrast to the advances in bacterial epitranscriptomics [59], phage RNA modifications remain understudied to date.

Knowledge of secondary structures is integral to fully understand the pivotal and diverse functions of transcripts in RNA-based regulation of microbial gene expression [65,66]. In bacteria, the transcriptome-wide study of dynamic RNA structures is mainly performed by footprinting-based methods (e.g. dimethly sulfate sequencing (DMS-seq) and selective 2′-hydroxyl acylation analyzed by primer extension sequencing (SHAPE-seq)) that reveal the base-pairing tendency of nucleotides and proximity ligation-based methods that cover RNA–RNA interactions (e.g. sequencing of psoralen crosslinked, ligated, and selected hybrids (SPLASH), psoralen analysis of RNA interactions and structures sequencing (PARIS) and ligation of interacting RNA sequencing (LIGR-seq)) [66]. Moreover, RBPs can also alter the fold of the targeted RNAs [30,42]. We hypothesise that the application of these powerful RNA structurome techniques to microbial virus-infected cells would reveal novel RNA structures that impact gene expression, which, to our knowledge, has not been explored thus far. Furthermore, as bacterial riboswitches and RNA thermometers are structurally affected by environmental changes [67], one may speculate that viral RNA molecules can also alter their secondary structure throughout infection depending on dynamic host conditions to adapt their gene expression accordingly.

Recently, bacterial transcriptomics moved beyond bulk RNA-seq to single-cell RNA sequencing (scRNA-seq) that can reveal diverse transcriptional states within cell populations in microbial communities [68-75]. Strikingly, probing different growth states of *B. subtilis* or the application of DNA-damaging antibiotics revealed rare subpopulations in which PBSX or SPβ prophage genes were induced [72,75]. In addition, *E. coli* cells showed in scRNA-seq only a limited host response to λ phage infection [75]. In scRNA-seq, the detection limit of individual transcripts is pivotal to obtain a detailed picture. Of note, in phage-infected cells, the abundance per transcript is strongly elevated for phage transcripts that would allow for a detailed heterogeneity analysis on the side of the phage in scRNA-seq. The various scRNA-seq approaches differ strongly in the number of detected single cells from a couple of hundred to ten thousand cells per condition in droplet-based indexing. The challenges in scRNA-seq of phage-infected cells are to reach sufficiently high ratios of infected cells for a diverse canvas of states, and to separate infected cells into single cells. This separation is especially difficult at later time points after phage infection when the stressed cells tend to aggregate. In summary, scRNA-seq offers the possibility to study phage transcriptomes in unparalleled detail and highlight transcriptional heterogeneity between individual host cell responses, or even point out phage-specific transcription responses in different metabolic host states. In the long run, transcriptional profiles of phage-infected single cells from habitats could help to uncover specific mechanisms that phages utilise in their native niche, uncovering long-term and phage–host connections. A sneak peek is given here by metatranscriptomics in the natural context such as soil samples that can discriminate between actively transcribed phage genomes against all detected phage genomes in metagenomics [76-78].

Building on these technological advances in microbial transcriptomics and the gradual improvements in sequencing capacity, cross-kingdom interaction studies [79] are within reach. For example, phage-infected *P. aeruginosa* cells have a specific transcriptional response in the presence of lung epithelial cells [80]. In another example, prophage Φ10403S enters an active lysogeny state during

macrophage infection by *Listeria monocytogenes*, as revealed by transcriptomics [81]. Capturing the complex infection dynamics between mammalian cells, bacterial pathogens and their microbial viruses through RNA-seq, will allow deriving new RNA-based mechanisms that are important for close-to-native settings, which is imperative to ameliorate phage biology in microbiota and future phage therapy applications.

Future impact of RNA sequencing in phage biology

In the future, state-of-the-art transcriptomics beyond *classical* RNA-seq will lead to a comprehensive view of the diverse transcriptional landscape in phages, that will expand beyond model phages, shedding light on mechanisms that have been overlooked in the past, in turn giving rise to new research questions in phage and microbiology.

In an age where antibiotic resistance is a global health threat, the in-depth study of phage infection courses in different and more complex infection settings, such as biofilms and cross-kingdom communities, can support the development of successful phage therapy applications. Detailed transcriptional maps of microbial viruses are essential for engineering and *de novo* design of synthetic phages with improved antimicrobial and therapeutic potential through, for example, extended host range after initial infection, anti-phage-defence mechanisms and enforced lysis cycles to avoid (pseudo-)lysogenic pathways [82]. In addition, high-resolution transcriptome data during early infection can facilitate the discovery and functional elucidation of novel phage genes involved in bacterial reprogramming, which can serve as an inspiration for innovative phage-based antimicrobials.

Besides their biomedical potential, microbial viruses are endowed with a largely untapped reservoir of host-specific building blocks and genetic circuitry that could radically expand the potential of microbial synthetic biology. Indeed, detailed expression maps of lysogens or lytic phages throughout infection can be mined to source phage-derived regulatory elements such as promoters, transcription terminators and other RNA-based regulators that can be exploited to tune expression levels in synthetic biology circuits [2,83,84].

Data Availability

No data were used for the research described in the article.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

L.W. holds a predoctoral scholarship from the Research Foundation – Flanders (FWO, Belgium). The paper was supported by funding from the European Research Council (ERC, EU) under the European Union's Horizon 2020 research and innovation program (grant agreement No. 819800) awarded to R.L. The work was funded by Deutsche Forschungsgemeinschaft (DFG, Germany) project 465133664 in the Priority Programme 'New Concepts in Prokaryotic Virus-host Interactions – From Single Cells to Microbial Communities' (SPP 2330) awarded to J.V.

Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.mib.2023.102419.

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. Dion MB, Oechslin F, Moineau S: **Phage diversity, genomics and phylogeny**. *Nat Rev Microbiol* 2020, **18**:125-138.
2. Lammens E-M, Nikel PI, Lavigne R: **Exploring the synthetic biology potential of bacteriophages for engineering non-model bacteria**. *Nat Commun* 2020, **11**:5294.
3. Edgar RC, Taylor J, Lin V, Altman T, Barbera P, Meleshko D, Lohr D, Novakovsky G, Buchfink B, Al-Shayeb B, *et al.*: **Petabase-scale sequence alignment catalyses viral discovery**. *Nature* 2022, **602**:142-147.
4. Parmar KM, Gaikwad S, Dhakephalkar PK, Kothari R, Sing RP: **Intriguing interaction of bacteriophage-host association: an understanding in the era of omics**. *Front Microbiol* 2017, **8**:559.
5. Hör J, Gorski SA, Vogel J: **Bacterial RNA biology on a genome scale**. *Mol Cell* 2018, **70**:785-799.
6. Blasdel B, Ceyssens PJ, Lavigne R: **Preparing cDNA libraries from lytic phage-infected cells for whole transcriptome analysis by RNA-seq**. *Methods Mol Biol* 2018, **1681**:185-194.
7. Tan D, Dahl A, Middelboe M: **Vibriophages differentially influence biofilm formation by *Vibrio anguillarum* strains**. *Appl Environ Microbiol* 2015, **81**:4489-4497.
8. Brandão A, Pires DP, Coppens L, Voet M, Lavigne R, Azeredo J: **Differential transcription profiling of the phage LUZ19 infection process in different growth media**. *RNA Biol* 2021, **18**:1778-1790.
9. Doron S, Fedida A, Hernández-Prieto MA, Sabehi G, Karunker I, Stazic D, Feingersch R, Steglich C, Futschik M, Lindell D, *et al.*: **Transcriptome dynamics of a broad host-range cyanophage and its hosts**. *ISME J* 2016, **10**:1437-1455.
10. Kornienko M, Fisunov G, Bespiatykh D, Kuptsov N, Gorodnichev R, Klimina K, Kulikov E, Ilina E, Letarov A, Shitikov E: **Transcriptional landscape of *Staphylococcus aureus* Kayvirus bacteriophage vB_SauM-515A1**. *Viruses* 2020, **12**:1320.
11. Wright BW, Logel DY, Mirzai M, Pascovici D, Molloy MP, Jaschke PR: **Proteomic and transcriptomic analysis of *Microviridae* φX174 infection reveals broad upregulation of host *Escherichia coli* membrane damage and heat shock responses**. *mSystems* 2021, **6**:e00046-21.
12. Chevallereau A, Blasdel BG, De Smet J, Monot M, Zimmermann M, Kogadeeva M, Sauer U, Jorth P, Whiteley M, Debarbieux L, Lavigne R: **Next-Generation "-omics" approaches reveal a massive alteration of host RNA metabolism during bacteriophage infection of *Pseudomonas aeruginosa***. *PLoS Genet* 2016, **12**:e1006134.

13. Drobysheva A, Panafidina S, Kolesnik M, Klimuk E, Minakhin L, Yakunina MV, Borukhov S, Nilsson E, Holmfeldt K, Yutin N, *et al.*: **Structure and function of virion RNA polymerase of a crAss-like phage**. *Nature* 2021, **589**:306-309.

14. Finstrlová A, Mašlaňova I, Blasdel Reuter BG, Doškař J, Götz F, Pantůček R: **Global transcriptomic analysis of bacteriophage-host interactions between a Kayvirus therapeutic phage and *Staphylococcus aureus***. *Microbiol Spectr* 2022, **10**:e0012322.

15. Wolfram-Schauerte M, Pozhydaieva N, Viering M, Glatter T, Höfer K: **Integrated omics reveal time-resolved insights into T4 phage infection of *E. coli* on proteome and transcriptome levels**. *Viruses* 2022, **14**:2502.

16. Yang H, Ma Y, Wang Y, Yang H, Shen W, Chen X: **Transcription regulation mechanisms of bacteriophages: recent advances and future prospects**. *Bioengineered* 2014, **5**:300-304.

17. Tabib-Salazar A, Mulvenna N, Severinov K, Matthews SJ, Wigneshweraraj S: **Xenogeneic regulation of the bacterial transcription machinery**. *J Mol Biol* 2019, **431**:4078-4092.

18. McAllister WT, Raskin CA: **The phage RNA polymerases are related to DNA polymerases and reverse transcriptases**. *Mol Microbiol* 1993, **10**:1-6.

19. Sokolova ML, Misovetc I, Severinov K: **Multisubunit RNA polymerases of jumbo bacteriophages**. *Viruses* 2020, **12**:1064.

20. Ceyssens P-J, Minakhin L, Van den Bossche A, Yakunina M, Klimuk E, Blasdel B, De Smet J, Noben J-P, Bläsi U, Severinov K, Lavigne R: **Development of giant bacteriophage ϕKZ is independent of the host transcription apparatus**. *J Virol* 2014, **88**:10501-10510.

21. Lenneman B, Rothman-Denes LB: **Structural and biochemical investigation of bacteriophage N4-encoded RNA polymerases**. *Biomolecules* 2015, **5**:647-667.

22. Davison J: **Pre-early functions of bacteriophage T5 and its relatives**. *Bacteriophage* 2015, **5**:e1086500.

23. Chaikeeratisak V, Nguyen K, Khanna K, Brilot AF, Erb ML, Coker JKC, Vavilina A, Newton GL, Buschauer R, Pogliano K, Villa E, Agard DA, Pogliano J: **Assembly of a nucleus-like structure during viral replication in bacteria**. *Science* 2017, **355**:194-197.

24. Andrade-Martínez JS, Camelo Valera LC, Chica Cárdenas LA, Forero-Junco L, López-Leal G, Moreno-Gallego JL, Rangel-Pineros G, Reyes A: **Computational tools for the analysis of uncultivated phage genomes**. *Microbiol Mol Biol Rev* 2022, **86**:e0000421.

25. Sampaio M, Rocha M, Oliveira H, Blas O: **Predicting promoters in phage genomes using PhagePromoter**. *Bioinformatics* 2019, **35**:5301-5302.

26. Shujaat M, Jin JS, Tayara H, Chong KT: **iProm-phage: a two-layer model to identify phage promoters and their types using a convolutional neural network**. *Front Microbiol* 2022, **13**:1061122.

27. Coppens L, Lavigne R: **SAPPHIRE: a neural network based classifier for σ70 promoter prediction in *Pseudomonas***. *BMC Bioinforma* 2020, **21**:415.

28. Lindell D, Jaffe JD, Coleman ML, Futschik ME, Axmann IM, Rector T, Kettler G, Sullivan MB, Steen R, Hess WR, Church GM, Chisholm SW: **Genome-wide expression dynamics of a marine virus and host reveal features of co-evolution**. *Nature* 2007, **449**:83-86.

29. Altuvia S, Storz G, Papenfort K: **Cross-regulation between bacteria and phages at a posttranscriptional level**. *Microbiol Spectr* 2018, **6**:1128.

30. Hör J, Matera G, Vogel J, Gottesman S, Storz G: **Trans-acting small RNAs and their effects on gene expression in *Escherichia coli* and *Salmonella enterica***. *EcoSal* 2020, **9**:1128.

31. Sharma CM, Hoffmann S, Darfeuille F, Reignier J, Findeiss S, Sittka A, Chabas S, Reiche K, Hackermüller J, Reinhardt R, *et al.*: **The primary transcriptome of the major human pathogen *Helicobacter pylori***. *Nature* 2010, **464**:250-255.

32. Ettwiller L, Buswell J, Yigi E, Schildkraut I: **A novel enrichment strategy reveals unprecedented number of novel transcription start sites at single base resolution in a model prokaryote and the gut microbiome**. *BMC Genom* 2016, **17**:199.

33. Dar D, Shamir M, Mellin JR, Koutero M, Stern-Ginossar N, Cossart P, Sorek R: **Term-seq reveals abundant ribo-regulation of antibiotics resistance in bacteria**. *Science* 2016, **352**:aad9822.

34. Ju X, Li D, Liu S: **Full-length RNA profiling reveals pervasive bidirectional transcription terminators in bacteria**. *Nat Microbiol* 2019, **4**:1907-1918.

35. Grünberger F, Ferreira-Cerca S, Grohmann D: **Nanopore sequencing of RNA and cDNA molecules in *Escherichia coli***. *RNA* 2022, **28**:400-417.

36. Wang Y, Zhao Y, Bollas A, Wang Y, Fai Au K: **Nanopore sequencing technology, bioinformatics and applications**. *Nat Biotechnol* 2021, **39**:1348-1365.

37. Yan B, Boitano M, Clark TA, Ettwiller L: **SMRT-Cappable-seq reveals complex operon variants in bacteria**. *Nat Commun* 2018, **9**:3676.

38. •• Wicke L, Ponath F, Coppens L, Gerovac M, Lavigne R, Vogel J: **Introducing differential RNA-seq mapping to track the early infection phase for *Pseudomonas* phage ϕKZ**. *RNA Biol* 2021, **18**:1099-1110.
Simultaneous mapping of TSSs of a phage and its host by enrichment of primary transcripts followed by high-throughput sequencing and further detection of differentially expressed genes in the early state of infection.

39. •• Putzeys L, Boon M, Lammens E-M, Kuznedelov K, Severinov K, Lavigne R: **Development of ONT-cappable-seq to unravel the transcriptional landscape of *Pseudomonas* phages**. *Comput Struct Biotechnol J* 2022, **20**:2624-2638.
First study to globally map phage TSSs, termination sites and operon structures in a single experiment by applying a specialised full-length cDNA sequencing method.

40. Putzeys L, Poppeliers J, Boon M, Lood C, Vallino M, Lavigne R: **Transcriptomics-driven characterization of LUZ100, a T7-like *Pseudomonas* phage with temperate features**. *mSystems* 2023, **8**:e0118922.

41. • Coppens L, Wicke L, Lavigne R: **SAPPHIRE.CNN: implementation of dRNA-seq-driven, species-specific promoter prediction using convolutional neural networks**. *Comput Struct Biotechnol J* 2022, **20**:4969-4974.
Development of species-specific promoter prediction tools by leveraging genome-wide TSS datasets obtained by dRNA-seq.

42. Holmqvist E, Vogel J: **RNA-binding proteins in bacteria**. *Nat Rev Microbiol* 2018, **10**:601-615.

43. Papenfort K, Melamed S: **Small RNAs, large networks: posttranscriptional regulons in Gram-negative bacteria**. *Annu Rev Microbiol* 2023, **77**:23-43 041320-025836.

44. •• Dunham DT, Angermeyer A, Seed KD: **The RNA-RNA interactome between a phage and its satellite virus reveals a small RNA that differentially regulates gene expression across both genomes**. *Mol Microbiol* 2023, **119**:515-533.
First global mapping of RNA–RNA interactions between transcripts of a satellite phage and an invading phage that revealed a crucial role of a ncRNA in phage biology.

45. Tree JJ, Granneman S, McAteer SP, Tollervey D, Gally DL: **Identification of bacteriophage-encoded anti-sRNAs in pathogenic *Escherichia coli***. *Mol Cell* 2014, **55**:199-213.

46. • Camara-Wilpert S, Mayo-Muñoz D, Russel J, Fagerlund RD, Madsen JS, Fineran PC, Sørensen SJ, Pinilla-Redondo R: **Bacteriophages suppress CRISPR–Cas immunity using RNA-based anti-CRISPRs**. *Nature* 2023, **623**:601-607.
Discovery of ncRNAs that inactivate CRISPR-Cas immunity.

47. • Banh DV, Roberts CG, Morales-Amador A, Berryhill BA, Chaudhry W, Levin BR, Brady SF, Marraffini LA: **Bacterial cGAS senses a viral RNA to initiate immunity**. *Nature* 2023, **623**:1001-1008.

Discovery of an ncRNA that triggers a phage defence response.

48. Adams PP, Baniulyte G, Esnault C, Chegireddy K, Singh N, Monge M, Dale RK, Storz G, Wade JT: **Regulatory roles of *Escherichia coli* 5′ UTR and ORF-internal RNAs detected by 3′ end mapping**. *eLife* 2021, **10**:e62438.

49. Dar D, Sorek R: **Bacterial noncoding RNAs excised from within protein-coding transcripts**. *mBio* 2018, **9**:e01730-18.

50. Zhang Y, Han K, Chandler CE, Tjaden B, Ernst RK, Lory S: **Probing the sRNA regulatory landscape of *P. aeruginosa*: post-transcriptional control of determinants of pathogenicity and antibiotic susceptibility**. *Mol Microbiol* 2017, **106**:919-937.

51. Melamed S, Peer A, Faigenbaum-Romm R, Gatt YE, Reiss N, Bar A, Altuvia Y, Argaman L, Margalit H: **Global mapping of small RNA-target interactions in bacteria**. *Mol Cell* 2016, **63**:884-897.

52. 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 Res* 2009, **37**:e133.

53. Lalaouna D, Carrier M-C, Semsey S, Brouard J-S, Wang J, Wade JT, Massé E: **A 3′ external transcribed spacer in a tRNA transcript acts as a sponge for small RNAs to prevent transcriptional noise**. *Mol Cell* 2015, **58**:393-405.

54. Smirnov A, Förstner KU, Holmqvist E, Otto A, Günster R, Becher D, Reinhardt R, Vogel J: **Grad-seq guides the discovery of ProQ as a major small RNA-binding protein**. *Proc Natl Acad Sci USA* 2016, **113**:11591-11596.

55. Chihara K, Gerovac M, Hör J, Vogel J: **Global profiling of the RNA and protein complexes of *Escherichia coli* by size exclusion chromatography followed by RNA sequencing and mass spectrometry (SEC-seq)**. *RNA* 2022, **29**:123-139.

56. • Gerovac M, Wicke L, Chihara K, Schneider C, Lavigne R, Vogel J: **A Grad-seq view of RNA and protein complexes in *Pseudomonas aeruginosa* under standard and bacteriophage predation conditions**. *mBio* 2021, **12**:e03454-20.
Global mapping of RNA complexes by gradient sedimentation in phage-infected cells that showed shifts in host ncRNAs upon phage infection and predicted phage-derived ncRNAs by sedimentation profiles.

57. Sittka A, Lucchini S, Papenfort K, Sharma CM, Rolle K, Binnewies TT, Hinton JCD, Vogel J: **Deep sequencing analysis of small noncoding RNA and mRNA targets of the global post-transcriptional regulator, Hfq**. *PLoS Genet* 2008, **4**:e1000163.

58. Holmqvist E, Wright P, Li L, Bischler T, Barquist L, Reinhard R, Backofen R, Vogel J: **Global RNA recognition patterns of post-transcriptional regulators Hfq and CsrA revealed by UV crosslinking *in vivo***. *EMBO J* 2016, **35**:991-1011.

59. Schauerte M, Pozhydaieva N, Höfer K: **Shaping the bacterial epitranscriptome-5′-terminal and internal RNA modifications**. *Adv Biol* 2021, **5**:e2100834.

60. Weigele P, Raleigh EA: **Biosynthesis and function of modified bases in bacteria and their viruses**. *Chem Rev* 2016, **116**:12655-12687.

61. Mayo-Muñoz D, Pinilla-Redondo R, Birkholz N, Fineran PC: **A host of armor: Prokaryotic immune strategies against mobile genetic elements**. *Cell Rep* 2023, **42**:112672.

62. van Beljouw SBP, Sanders J, Rodríguez-Molina A, Brouns SJJ: **RNA-targeting CRISPR–Cas systems**. *Nat Rev Microbiol* 2023, **21**:21-34.

63. • Wolfram-Schauerte M, Pozhydaieva N, Grawenhoff J, Welp LM, Silbern I, Wulf A, Billau FA, Glatter T, Urlaub H, Jäschke A, *et al.*: **A viral ADP-ribosyltransferase attaches RNA chains to host proteins**. *Nature* 2023, **620**:1054-1062.
Host ribosomal proteins are ADP-ribosylated with 5′-NAD-capped RNAs by T4 phage protein ModB (RNAylation) that impacts phage replication.

64. Nielsen TK, Forero-Junco LM, Kot W, Moineau S, Hestbjerg Hansen L, Riber L: **Detection of nucleotide modifications in bacteria and bacteriophages: Strengths and limitations of current technologies and software**. *Mol Ecol* 2023, **32**:1236-1247.

65. Vicens Q, Kieft JS: **Thoughts on how to think (and talk) about RNA structure**. *Proc Natl Acad Sci USA* 2022, **119**:e2112677119.

66. Zhang J, Fei Y, Sun L, Zhang QC: **Advances and opportunities in RNA structure experimental determination and computational modelling**. *Nat Methods* 2022, **19**:1193-1207.

67. Roßmanith J, Narberhaus F: **Modular arrangement of regulatory RNA elements**. *RNA Biol* 2017, **14**:287-292.

68. Blattman SB, Jiang W, Oikonomou P, Tavazoie S: **Prokaryotic single-cell RNA sequencing by *in situ* combinatorial indexing**. *Nat Microbiol* 2020, **5**:1192-1201.

69. Homberger C, Barquist L, Vogel J: **Ushering in a new era of single-cell transcriptomics in bacteria**. *Microlife* 2022, **3**:uqac020.

70. Homberger C, Hayward RJ, Barquist L, Vogel J: **Improved bacterial single-cell RNA-seq through automated MATQ-Seq and Cas9-based removal of rRNA reads**. *mBio* 2023, **14**:e0355722.

71. Imdahl F, Vafadarnejad E, Homberger C, Saliba A-E, Vogel J: **Single-cell RNA-sequencing reports growth-condition-specific global transcriptomes of individual bacteria**. *Nat Microbiol* 2020, **5**:1202-1206.

72. •• Kuchina A, Brettner L, Paleologu L, Roco CM, Rosenberg AB, Carignano A, Kibler R, Hirano M, DePaolo RW, Seelig G: **Microbial single-cell RNA sequencing by split-pool barcoding**. *Science* 2021, **371**:eaba5257.
ScRNA-seq of cultured *B. subtilis* retrieved a cluster of cells uniquely expressing genes from the defective PBSX prophage.

73. Ma P, Amemiya HM, He LL, Gandhi SJ, Nicol R, Bhattacharyya RP, Smillie CS, Hung DT: **Bacterial droplet-based single-cell RNA-seq reveals antibiotic-associated heterogeneous cellular states**. *Cell* 2023, **186**:877-891.

74. McNulty R, Sritharan D, Pahng SH, Meisch JP, Liu S, Brennan MA, Saxer G, Hormoz S, Rosenthal A: **Probe-based bacterial single-cell RNA sequencing predicts toxin regulation**. *Nat Microbiol* 2023, **8**:934-945.

75. •• Wang B, Lin AE, Yuan J, Novak KE, Koch MD, Wingreen NS, Adamson B, Gitai Z: **Single-cell massively-parallel multiplexed microbial sequencing (M3-seq) identifies rare bacterial populations and profiles phage infection**. *Nat Microbiol* 2023, **8**:1846-1862.
ScRNA-seq of cell populations in *B. subtilis* that show induced prophages, and phage λ-infected *E. coli* cells.

76. •• Coclet C, Sorensen PO, Karaoz U, Wang S, Brodie EL, Eloe-Fadrosh EA, Roux S: **Virus diversity and activity is driven by snowmelt and host dynamics in a high-altitude watershed soil ecosystem**. *Microbiome* 2023, **11**:237.
Metatranscriptomic study that revealed actively expressed genes from DNA and RNA phages in environmental samples.

77. Chen L-X, Jaffe AL, Borges AL, Penev PI, Colenbrander Nelson T, Warren LA, Banfield JF: **Phage-encoded ribosomal protein S21 expression is linked to late-stage phage replication**. *ISME Commun* 2022, **2**:31.

78. Merges D, Schmidt A, Schmitt I, Neuschulz EL, Dal Grande F, Bálint M: **Metatranscriptomics reveals contrasting effects of elevation on the activity of bacteria and bacterial viruses in soil**. *Mol Ecol* (32) 2022,6552-6563, https://doi.org/10.1111/mec.16756

79. Westermann AJ, Vogel J: **Cross-species RNA-seq for deciphering host-microbe interactions**. *Nat Rev Genet* 2021, **22**:361-378.

80. •• Brandão A, Putzeys L, Pires D, Voet M, Paeshuyse J, Azeredo J, Lavigne R: **Impact of phage predation on *P. aeruginosa* adhered to human airway epithelium: major transcriptomic changes in metabolism and virulence-associated genes**. *RNA Biol* 2023, **20**:235-247.

RNA-seq of phage-infected bacteria in the presence of human airway epithelial cells as a gateway to cross-kingdom transcriptomics studies that mimic physiological conditions.

81. •• Pasechnek A, Rabinovich L, Stadnyuk O, Azulay G, Mioduser J, Argov T, Borovok I, Sigal N, Herskovits AA: **Active lysogeny in *Listeria monocytogenes* is a bacteria-phage adaptive response in the mammalian environment**. *Cell Rep* 2020, **32**:107956.
Discovery of active lysogeny in *L. monocytogenes* upon infection of macrophages.

82. Pires DP, Monteiro R, Mil-Homens D, Fialho A, Lu TK, Azeredo J: **Designing *P. aeruginosa* synthetic phages with reduced genomes**. *Sci Rep* 2021, **11**:2164.

83. Lammens E-M, Putzeys L, Boon M, Lavigne R: **Sourcing phage-encoded terminators using ONT-cappable-seq for synbio applications in *Pseudomonas***. *ACS Synth Biol* 2023, **12**:1415-1423.

84. Owen SV, Canals R, Wenner N, Hammerlöf DL, Kröger C, Hinton JCD: **A window into lysogeny: revealing temperate phage biology with transcriptomics**. *Micro Genom* 2020, **6**:e000330.