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POINT-OF-VIEW

Exploiting phage strategies to modulate bacterial transcription

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ABSTRACT
Bacteriophages employ small proteins to usurp host molecular machinery, thereby interfering with central metabolic processes in infected bacteria. Generally, phages inhibit or redirect host transcription to favor transcription of their own genomes. Mechanistic and structural studies of phage-modulated host transcription may provide inspirations for the development of novel antibacterial substances.

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Introduction

The bacterial transcription machinery is a proven target for antibiotics [1]. Besides RNA polymerase (RNAP) itself, bacteria also harbor numerous transcription regulatory factors that are essential. While sequence and structural similarities exist between bacterial and eukaryotic RNAPs, the molecular machinery comprising the transcription regulatory layers is very distinct between the different domains of life. Indeed, only the NusG/Spt5-family of transcription elongation factors appears to be universally conserved [2–4]. Thus, transcription regulatory factors or transcription complexes that are modified by specific regulatory factors may represent highly attractive targets for the development of novel antimicrobial substances. However, as most of these factors do not possess easy-to-monitor enzymatic activities, targeting this regulatory layer by traditional inhibitor screening strategies is difficult. In light of an increasing number of high-resolution 3D structures of transcription factors and their complexes becoming available, *in silico* screening methodologies may offer some remedy [5].

Bacteriophages provide large reservoirs of unique proteins that modulate diverse bacterial molecular machineries to enable successful propagation of the phages [6, 7]. Many of these phage-derived protein modulators do not exhibit any homology to known proteins from organisms in all three kingdoms of life. Often such effector molecules target host molecular machineries associated with essential metabolic pathways, eventually leading to the complete shutdown of the host metabolism and killing of the host bacteria. The essentiality of host molecular machinery is one of the major criteria to be considered when selecting a potential drug target, and identification and characterization of novel phage effector proteins could thus lead to the identification of novel, attractive drug targets in pathogens.

With an estimated number of more than a billion bacterial species on the planet [8] and more than ten phage species on average estimated to infect each microbial species [9], phages might harbor bactericidal proteins for pathogenic bacterial species in numbers that dwarf the size of chemical libraries presently in use. Phages, therefore, might offer a rich resource for discovering novel molecules that interfere with the metabolism of otherwise difficult to control human pathogens. Indeed, mycobacteriophage endolysins lyse the complex peptidoglycans of mycobacteria [10], and the gp52 protein of mycobacteriophage Fruitiloop has been shown to exert its toxicity by affecting mycobacterial cell wall biosynthesis [11]. Recently, a mycobacteriophage genomics approach

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has been initiated to identify novel mycobacteriophage factors with bactericidal properties [12].

Antimicrobial peptides (AMPs) are oligopeptides comprising around 5 to 50 amino acid residues. Due to the emergence of multi-drug resistant bacteria and due to a dearth in novel antibiotics, AMPs are presently garnering renewed attention [13, 14]. In addition to their bactericidal activities, phage-derived protein modulators are typically small (≤20kDa) [6], rendering them attractive templates for designing new bactericidal peptides or peptidomimetics. Detailed biochemical and structural analyses of their interactions with their host targets might enable the definition of small regions of the phage proteins that embody target-binding properties independent of the remaining parts of the proteins, which might be further developed into effective AMPs.

As a general strategy, phages hijack or subvert parts of the host transcription machinery [15]. In modulating host transcription, phages often target not only host RNAP but also transcription regulatory factors [16, 17]. Thus, studying the structural basis of phage-derived mechanisms to interfere with bacterial transcription may uncover hitherto unexplored transcription-modulatory strategies and, thus, may inspire the development of novel antibacterial compounds that target bacterial transcription, including the transcription regulatory layer. High-resolution macromolecular structures, as can be obtained *via* macromolecular crystallography, may serve as templates for medicinal chemists to devise small peptides, peptidomimetics or even non-protein small molecules, which could possess inhibitory prowess. Indeed, high-resolution crystal structures of a transcription factor complex have recently been used to rationally design small molecules that interfere with protein–protein interactions in this complex [18, 19]. Recent revolutionary developments in imaging of biomacromolecular complexes at the atomic level using single-particle cryo-electron microscopy (cryoEM) [20] also provide deep insights into macromolecular interactions. With additional technological and computational improvements expected in the foreseeable future, cryoEM may provide additional templates for inhibitor development, in particular, high-resolution structures of complete transcription complexes.

Phage-mediated modulation of transcription initiation

Bacteria use a conserved, multi-subunit RNAP (core subunit composition $\alpha_2\beta\beta'\omega$) to transcribe their genomes [21]. The core enzyme associates with one of the several σ-factors to initiate transcription specifically at promoter sequences [22]. After transcription initiation, RNAP forms a stable elongation complex (EC) with the DNA and nascent RNA. The EC can be modified by transcription elongation factors, such as N-utilization substance (Nus) A or G [23]. RNAP dissociates from the template only in response to certain signals, called terminators [24]. There are two major modes of transcription termination in bacteria. In intrinsic termination, a GC rich inverted repeat that forms an RNA hairpin, followed by a stretch of consecutive uridinylates induces RNAP to pause and subsequently disengage from the template [25, 26]. The predominant mode of factor-dependent termination relies on a hexameric, NTP-dependent RNA translocase/helicase, ρ [27, 28].

Traditionally, transcription has been regarded to be regulated predominantly during the initiation phase. Thus perhaps not surprisingly, several phage modulators of bacterial transcription initiation have been discovered [29]. The study of their functional mechanisms revealed an astonishing diversity of how phages can subvert the function of host σ factors. For instance, enterobacterial phage T4 proteins AsiA and MotA activate phage middle genes [30]. AsiA binds conserved region 4 of the primary *E. coli* σ-factor, σ^{70}, preventing its canonical interaction with −35 promoter elements and enabling subsequent MotA binding. Upon binding, AsiA undergoes a conformational change and engages upstream DNA [31]. MotA binds to a conserved DNA element (MotA box) that replaces the −35 element in middle promoters as well as to AsiA-remodeled σ region 4 [31]. Thus, AsiA and MotA cooperate to substitute for σ^{70} interactions with a − 35 promoter element during middle gene expression. For late gene expression, T4 proteins gp33 and gp55 form a "composite" σ factor that acts in cooperation with the T4 sliding clamp gp45 to recognize the single −10 elements of the late promoters [32]. The gp39 and gp76 proteins of *Thermus* phage P23-45 redirect host RNAP to late phage genes.

In the crystal structures of a gp39- [33] and gp39/gp76-holoenzyme complexes [34], the globular part of p39 binds to the β flap at the base of the flap tip, while a C-terminal helix interacts with σ region 4, displacing σ region 4 bound to the flap tip. As a consequence, σ region 4 can no longer interact with promoter −35 regions, leading to inhibition of transcription from the −10/-35 class of promoters, but not of transcription from an extended −10 class of phage middle/late promoters [33]. Inhibition of transcription of host genes is supported by the phage gp76 protein, which binds deep inside the active site cleft of RNAP and to a linker connecting σ regions 3 and 4, obstructing accommodation of the melted template DNA strand and thus hindering the conversion of a closed to an open transcription initiation complex. Most likely, initiation of phage genes remains efficient due to the higher affinity of RNAP to extended −10 regions, which may overcome gp76-mediated inhibition [34]. As another example, protein P7 of *Xanthomonas oryzae* phage Xp10 directly binds to the β and β' subunits of RNAP in a manner that induces σ^{70} displacement [35] and that locks the RNAP clamp in a closed conformation that inhibits loading of promoter DNA into the RNAP active site cleft [36]. Finally, enterobacterial phage T7 encodes its own RNAP and benefits from shutting off host RNAP-dependent transcription. The gp2 protein of T7 phage binds the 1.1 domain of σ^{70} and the RNAP β' subunit, thereby locking σ^{70} domain 1.1 in the RNAP active site channel [37].

However, it is now well established that the transcription elongation and termination phases also are highly regulated by both intrinsic signals on the template DNA/product RNA and by *trans*-acting, extrinsic protein transcription factors [38]. These signals and factors, among others, cause RNAP to frequently pause during elongation, offering windows of opportunity for other regulatory mechanisms to take effect [23, 38, 39], or they can modulate the strength of terminators [24, 25]. Indeed, it has been the investigation of lambdoid phages that led to the discovery of host-encoded transcription elongation factors, termination factor ρ as well as phage-derived factors that modulate the behavior of RNAP during elongation and termination [40–42].

Phage-mediated modulation of transcription elongation and termination

Lambdoid phages are known for a long time to employ strategies that modulate host RNAP pausing and termination functions during transcription of the phage genomes [40–44]. For example, most of these phages employ N and Q proteins to facilitate the switch from immediate-early to delayed-early gene expression and to support the expression of late genes, respectively, during their lytic life cycles (Figure 1). To this end, N and Q proteins interact with host RNAP, transcription factors, RNA and/or DNA, stably modifying ECs to confer pause- and termination resistance on RNAP and thus allowing it to read through intra- and intergenic terminators, even if the terminators are located far downstream of the site at which N or Q originally load onto the EC (processive anti-termination) [40–44].

Processive anti-pausing and anti-termination by n proteins

N recognizes signal sequences in untranslated regions of nascent phage RNA, so-called N-utilization (*nut*) sites (Figure 1), comprising a linear *boxA* element and a *boxB* stem-loop structure. N binds *boxB* and RNAP and recruits Nus factors A, B, E (equivalent to ribosomal protein S10) and G, building up a complex ribonucleoprotein (a "modifying" RNP) on the surface of RNAP that stays associated with RNAP during the entire transcription elongation process by an RNA looping mechanism [42, 43, 45].

Recently, the group of one of the authors (M.C.W.) and collaborators reported a crystal structure of an λN-Nus factor-*nut* RNA complex [46] and a high-resolution cryoEM structure of a complete λN-based transcription anti-termination complex (λN-TAC), comprising RNAP, template DNA, product RNA with a *nut* site, all Nus factors and the λN protein [17] (Figure 2a). N proteins are intrinsically unstructured, ~110-residue polypeptides. The structural analyses revealed that in the λN-TAC, λN only locally adopts the regular secondary structure and remains highly elongated, which enables it to contact many sites on RNAP, the Nus factors and the nascent RNA (Figure 2b). It thereby implements a multi-pronged strategy to suppress transcription pausing as well as intrinsic and factor-dependent termination.

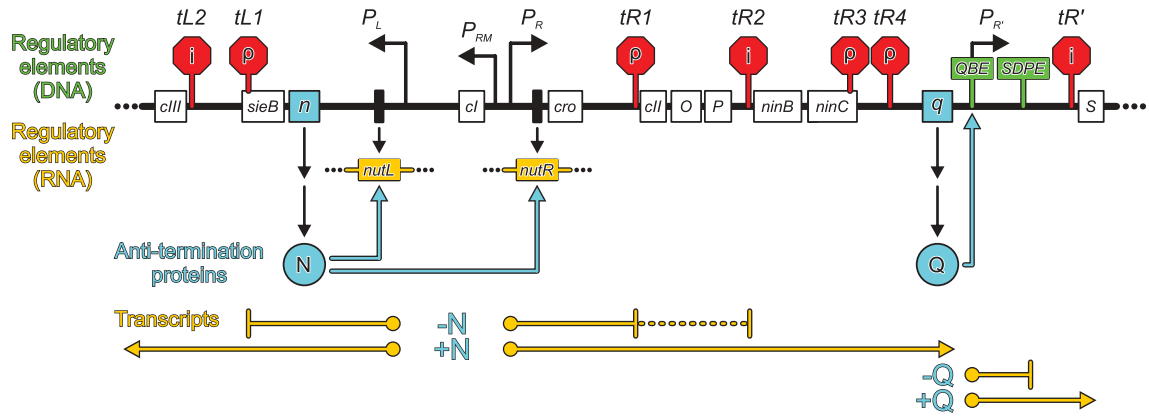

Figure 1. Biological activities of lambdoid phage N and Q proteins.

Scheme of part of the phage λ genome (thick black line) containing early and late control regions. The sizes of regions and elements, and their positions, are not drawn to scale. Open boxes with names – protein-coding regions; narrow black boxes, *nut* site DNA; black-angled arrows, promoters; red stop signs, intrinsic ("i") and ρ-dependent ("ρ") terminators; green signs, regulatory regions active as DNA (QBE and SDPE); dark yellow boxes, *nut* regulatory regions active as RNA; cyan spheres, anti-termination proteins; cyan angled arrows, sites of recruitment of anti-termination proteins to ECs; dark yellow lines, transcripts. Scheme adapted from [43] with changes.

Hairpin-stabilized pausing and intrinsic termination are disfavored by multiple strategies aimed at preventing pause-stabilizing or termination hairpins from invading the RNAP RNA exit tunnel: λN repositions NusA on RNAP, such that it can no longer stabilize exit tunnel-invading hairpins and instead may support their unfolded state. In addition, λN conformationally remodels RNAP exit tunnel elements, and its C-terminal residues line the RNA exit tunnel, constricting the tunnel and physically blocking its invasion by RNA secondary structures. Moreover, λN organizes NusA and S10/NusE regions such that they present a binding site for the C-terminal domain of NusG, which is thereby sequestered and prevented from supporting the activity of termination factor ρ. Furthermore, λN and NusG line opposite flanks of the upstream DNA duplex; λN thereby apparently supports the otherwise weak intrinsic DNA re-annealing and RNAP anti-backtracking activities of NusG. Most remarkably, the C-terminal 25 residues of λN traverse the RNAP active site cleft, stringing mobile RNAP elements, which are repositioned during pausing and presumably also during termination [47, 48], together, thus keeping RNAP in an anti-paused, processive conformation (Figure 2c).

Notably, λN employs diverse, short peptide segments to implement its many anti-pausing/anti-termination strategies, each of which may in principle lend itself to the design of novel interfering substances. The present resolution of the λN-TAC structure (3.7 Å) most likely does not suffice to serve as a reliable template for detailed modeling studies with the aim to derive new modulators. Mild cross-linking [49] and/or imaging a co-transcriptionally assembled complex may still offer room for improvement. Moreover, different lambdoid phages encode different N proteins, which may lend themselves to even higher-resolution structural analyses. Of particular interest may be a C-terminally extended N polypeptide of phage H-19B [50]. Biochemical analyses by the group of one of the authors (R.S.) have revealed that H-19B N repositions NusA and prevents ρ function [51, 52], and suggested that H-19B N may even directly interact with the RNAP active site region [50]. Thus, a detailed structural analysis may uncover yet additional strategies of RNAP modulation in the H-19B case.

RNA exit tunnel modulation as a widespread strategy to counteract pausing and termination

Structural modulation of the RNA exit tunnel and surrounding elements to prevent invasion by pause/termination-enhancing RNA hairpins, as well as prevention of NusA-mediated stabilization of such hairpins, appear to be widespread strategies employed by phages to regulate their gene expression. Again, phages have evolved surprisingly diverse molecular mechanisms to achieve these tasks. One alternative to the N-based strategy is exemplified by lambdoid phage Q proteins [44, 45]. Q recognizes a Q-binding

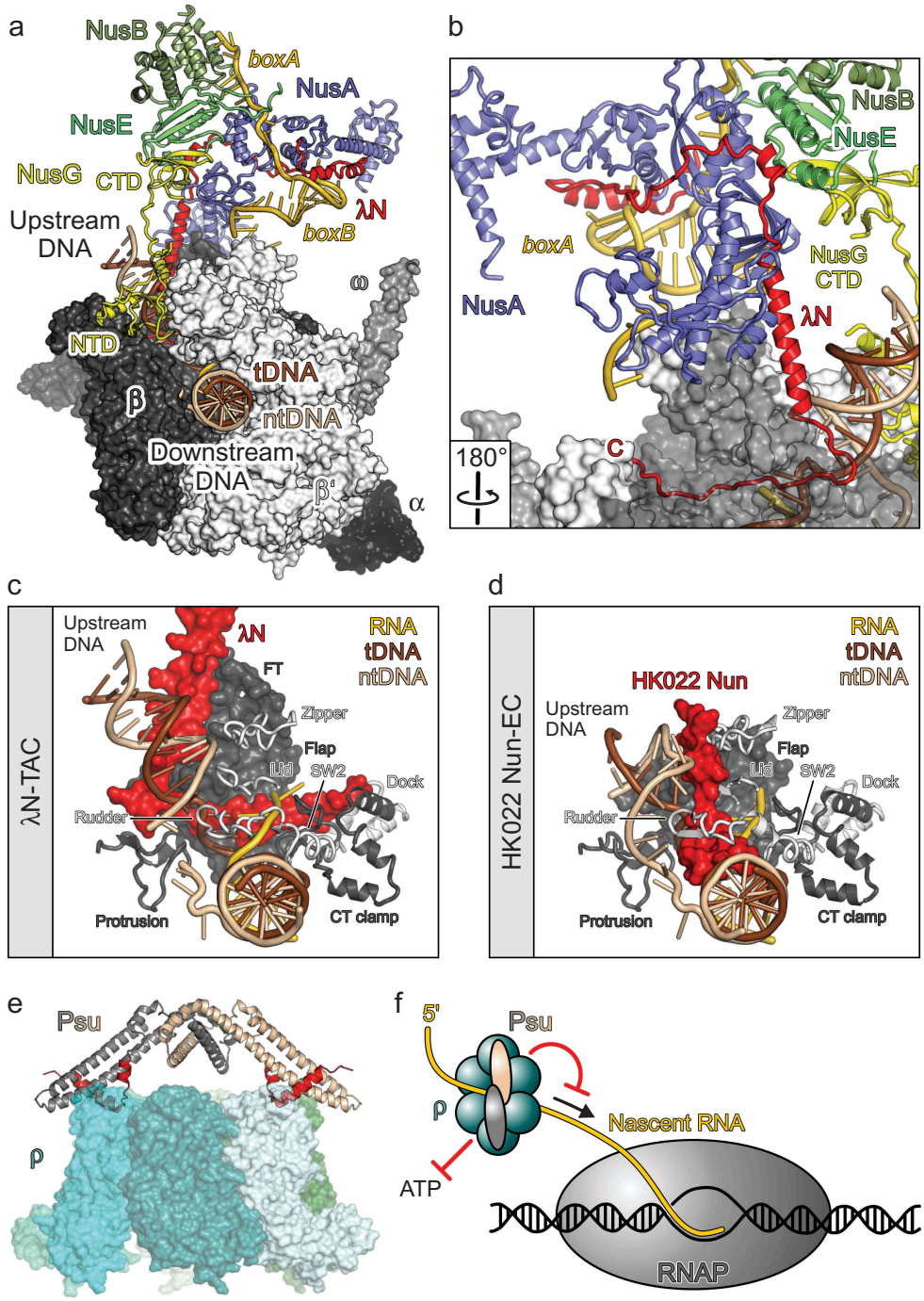

Figure 2. Molecular models of phage factors modulating host transcription elongation and termination.

(a) A Single-particle cryoEM structure of an λN-TAC [17]. RNAP subunits in surface representation. Nucleic acids, Nus factors, and λN in cartoon representation. (b) λN remains highly extended in the λN-TAC, allowing short peptides along its sequence to interact with spatially widely distributed regions on nascent RNA, Nus factors and RNAP. RNAP subunit β as semi-transparent surface. Rotation symbol – view relative to (a). (c) Interaction of the C-terminal region of λN with nucleic acids and various elements of RNAP in and around the active site cleft (β elements: flap, FT – flap tip, protrusion, CT clamp – C-terminal clamp; β' elements: zipper, lid, rudder, SW2 – switch 2, dock). View as in (a). (d) HK022 Nun interacting with nucleic acids and RNAP elements [60]. Same orientation of RNAP as in (d). (e) presumed mode of action of Psu [16]. By inhibiting ρ's ATPase, Psu will hinder the translocation of ρ along the nascent RNA toward RNAP (arrow). Red symbols – inhibition. (f) Docking model of the phage P4 Psu protein interacting with *E. coli* transcription termination factor ρ [62]. (c) and (d) adapted from [17].

DNA element (QBE) located between the −35 and −10 elements of the phage late gene promoter, which is followed by a σ-dependent pause element (SDPE) and a terminator (Figure 1). Q loads onto the σ-modified, paused EC and, upon pause escape, remains associated with the EC, persistently suppressing RNAP pausing and termination. Recent cryoEM structures of Q-loading complexes, based on the Q protein of phage 21 (Q21), revealed that two Q21 molecules recognize direct repeats of the QBE [53, 54]. In the loading complex, σ remains anchored to the paused EC *via* σ regions 2 and 3, but due to the presence of >10 nucleotides of initial RNA, the σ region 3–4 linker and σ region 4 are displaced. Besides binding the β' dock domain (occupied by σ region 4 in transcription initiation complexes) and the α_I-β interface, the Q21 protomer bound at the upstream QBE ($Q21_u$) uses a helix and neighboring linkers to form a ring-like structure (the "Q torus" [54]) around the mouth and inside of the RNA exit tunnel, which extends and constricts the RNA exit tunnel. The Q21 protomer bound at the downstream QBE ($Q21_d$) additionally contacts the β flap tip helix in a manner mutually exclusive with σ region 4-β flap tip interactions in initiation complexes. A structure of the Q21-loaded complex revealed that $Q21_u$ maintains its RNAP interactions after pause escape, while σ and $Q21_d$ are displaced [54]. This structure also showed that single-stranded RNA can be threaded through the $Q21_u$ torus, while nucleation, propagation and exit tunnel penetration of RNA hairpins are prevented [54].

P7 protein of phage Xp10 provides yet another example of exit tunnel modulation. Recent cryoEM structures of P7-modified ECs without and with NusA revealed that P7 can bind between a short N-terminal helix of β', the β' dock domain and the C-terminal region of β at the mouth of the RNA exit tunnel, thereby restricting the local diameter of the exit tunnel and preventing accommodation of an RNA hairpin [36]. Moreover, P7 in a P7/NusA-modified EC lines a concave surface of the NusA N-terminal and S1 domains [36]. Thus, P7 exploits the very surfaces of NusA that are normally used to stabilize exit tunnel-invading hairpins for its own stable binding to RNAP, essentially converting NusA from a pause/termination-supporting factor to an anti-pausing/anti-termination factor [36].

Transcription arrest by the HK002 nun proteins

Lambdoid phage HK022 resorts to a different strategy to implement transcription anti-termination for delayed-early gene expression. Here, a *cis*-acting, bi-lobed RNA structure, the polymerase-utilization (*put*) site, in the untranslated regions of the phage RNA directly binds to the β' Zinc-finger domain of RNAP and confers pause/termination resistance [55] 56]. HK022 also encodes an N-related protein, Nun [57]. Presumably due to the availability of the *put* element, Nun evolved to have a diametrically opposite function to other N proteins: It responds to the same *nut* sites as N and recruits the same set of host Nus factors to RNAP, but induces pre-mature transcription arrest [57–59], likely to prevent super-infection by other lambdoid phages. A cryoEM structure of an HK022 Nun-arrested EC has been elucidated [60]. Only the C-terminal 23 residues of Nun on RNAP could be imaged, the rest of the protein remained unresolved due to its intrinsically unstructured nature and high flexibility in the absence of the Nus factors and *nut* RNA. The structure revealed how Nun, similar to λN, inserts its C-terminal region into the interior of RNAP, but entering along a different flank of upstream DNA, where no natural crevices are available to accommodate the protein without distorting RNAP (Figure 2d). As a consequence, Nun distorts and displaces several RNAP elements and wedges into the nucleic acid network, inhibiting nucleic acid movement inside RNAP (Figure 2d). Thus, this C-terminal region of Nun provides a highly attractive template for the design of novel RNAP-inhibitory substances. It will be interesting to see in the future how other regions of Nun interact with the Nus factors and whether these interactions augment the transcription inhibitory potential of the protein.

Anti-ρ activity of the Psu protein

Another interesting phage-derived transcription modulator is the capsid protein, Psu, of enterobacterial phage P4. Psu is an antagonist of the conserved bacterial transcription termination factor, ρ [16]. The group of one of the authors (R.S.) demonstrated that Psu inhibits ρ ATPase activity but does not prevent the binding of RNA to ρ's primary and secondary RNA binding sites [16]. Thus, Psu

presumably interferes with ρ-mediated transcription termination by inhibiting 5′-to-3′ translocation of ρ on the mRNA (Figure 2e). Together with collaborators, the Sen lab also unraveled the crystal structure of Psu, showing that the protein adopts a novel fold that supports the formation of an unusual, knotted dimer [61]. Based on this structure, the known structure of *E. coli* ρ and the mapping of interacting residues on Psu and ρ, a docking model of dimeric Psu on a closed ρ hexamer was constructed (Figure 2f) [62, 63]. The biochemical data and the docking model revealed that Psu most likely uses a C-terminal α helix and neighboring residues to contact two ρ subunits on opposite sides of the ring. Importantly, the Sen group demonstrated that Psu can inhibit the ATPase and transcript release activities of ρ proteins from diverse pathogenic bacteria *in vitro* and that overproduction of Psu was bactericidal [64]. Novel AMPs could be designed based on the C-terminal helices of Psu that directly contact ρ. The rational design of Psu-derived anti-ρ peptides or peptidomimetics would strongly benefit from the elucidation of a high-resolution experimental structure of a Psu-ρ complex.

Conclusions

Ample examples have been documented for how phages employ small proteins to target all phases of transcription of their hosts. It is to be expected that with more phages being discovered and studied, more such mechanisms, as well as variations of known mechanisms, will be revealed. Notably, in many cases, not only the host RNAP but also the host transcription factors that regulate the various phases of transcription are key targets of the phage proteins. While these transcription factors are typically not conserved in eukaryotes, they are widely distributed in bacteria and in many species they are essential, rendering them "naturally selected" ("chosen" by phages) drug targets. With improved techniques for molecular docking and design, high-resolution structures of phage-derived transcription modulators in the course of their action may provide valuable assets for guiding the rational development of novel, phage-informed, transcription-targeting antibacterial substances.

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