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Review

How to shut down transcription in Archaea during virus infection

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Abstract: Multisubunit RNA polymerases (RNAP) carry out transcription in all domains of life; during virus infection, RNAPs are targeted by transcription factors encoded by either the cell or the virus, resulting in the global repression of transcription with distinct outcomes for different host-virus combinations. These repressors serve as versatile molecular probes to study RNAP mechanisms, as well as they aid the exploration of druggable sites for the development of new antibiotics. Here, we review the mechanisms and structural basis of RNAP inhibition by the viral repressor RIP and the crenarchaeal negative regulator TFS4, which follow distinct strategies. RIP operates by occluding the DNA-binding channel and mimicking the initiation factor TFB/TFIIB. RIP binds tightly to the clamp and locks it into one fixed position, thereby preventing conformational oscillations that are critical for RNAP function as it progresses through the transcription cycle. TFS4 engages with RNAP in a similar manner to transcript cleavage factors such as TFS/TFIIS through the NTP-entry channel; TFS4 interferes with the trigger loop and bridge helix within the active site by occlusion and allosteric mechanisms, respectively. The conformational changes of RNAP described above are universally conserved and are also seen in inactive dimers of eukaryotic RNAPI and several inhibited RNAP complexes of both bacterial and eukaryotic RNA polymerases, including inactive states that precede transcription termination. A comparison of target sites and inhibitory mechanisms reveals that proteinaceous repressors and RNAP-specific antibiotics use surprisingly common ways to inhibit RNAP function.

Keywords: Archaea; transcription inhibition; RNA polymerase; viruses; evolution; antibiotics

1. Introduction

Viruses and their hosts are engaged in an endless battle whose outcome is called by the arms race between host and viral measures and countermeasures [1]. Yet, in many cases viruses thrive in a coexistence with their host (commensalism), and it is now well accepted that viruses play a key role for horizontal gene transfer and are drivers of evolution of life [2,3]. In virus-host pairs where viruses encode their own RNA polymerase, which include many bacteriophages and metazoan viruses, viral factors quickly and specifically shut down the host's gene expression to favor the viral transcription program [4,5]. This redirects host resources to produce virions and limits the host's ability to mount an effective immune response to the infection. Conversely, the host in many cases attenuates its own transcription as part of the early response to sudden unfavorable growth conditions and enters a quiescent state, which can provide the infected cell with the opportunity to mount additional antiviral responses, or alternatively as initial step leading to cell death aiming to limit virus propagation in the community [6]. The mechanisms underlying RNAP repression differ between viral and cellular transcription systems in

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Bacteria, Archaea, and Eukarya, depending on the complexity and regulatory strategies of each system.

Virus-encoded inhibitors. Viral gene expression is typically regulated temporally (early - middle - late) as a function of the infection time course, in contrast to cellular gene expression that responds to complex environmental or developmental cues. As a rule, the early genes of bacteriophages are under the control of host-like promoters and are expressed by the host RNAP [7]. Early gene products include the phage RNAP and inhibitors of the host RNAP. Once reached a critical threshold of both factors is reached, a switch from early to late transcription occurs, concomitantly with the effective repression of all host gene [5,8]. Variations to the described mechanism include phages that encode for two RNAPs that enable a more complex phage gene expression [9]. The *coliphage* T7 encodes Gp2, a small protein which binds the RNAP-σ70 holoenzyme inside the DNA-binding channel which inhibits the open complex formation [5,8,10]. Viral factors not only target the RNAP by direct physical interactions, but they can also modulate regulatory post-translational modifications of RNAP subunits. The eukaryotic *herpes simplex* virus-1 factor ICP22 (infected cell protein 22) [4] and the *Bunyamwera* virus factor NSs (non-structural protein S) interfere with the phosphorylation of the RNA polymerase II subunit RPB1 [11], while the Togaviridae factor nsP2 promotes RPB1 ubiquitination which leads to a rapid depletion of RNAPII [12]. Viruses that do not encode their own RNAP are dependent on the host RNAP, and therefore they tend to deploy regulatory factors that interfere with host promoter recognition aiming to redistribute RNAPs from host to viral promoters. The recently sequenced Italian rudiviruses encode homologues of the initiation factor TFB, but it has not been established whether the viral TFBs (vTFB) preferentially direct transcription from viral compared to cellular promoters [13].

Cellular inhibitors. Separate from the virus-host scenario, cells utilize global negative regulation of transcription via RNAP and transcription factors. Several negative regulators of bacterial RNAP have been described in literature; these are often expressed or activated during unfavorable growth conditions, such as low pH for *Thermus thermophilus* Gfh1 (Gre factor homologue 1) [14,15], or nutrient deficiency for *Escherichia coli* DksA (DnaK suppressor A) [16,17]. Likewise, eukaryotic transcription is also regulated on a global level. Naïve lymphocytes are transcriptionally silent on a genome-wide level due to severely repressed levels of the initiation factor TFIIH, a factor that catalyzes DNA strand separation and template loading into the RNAP active site during initiation [18]. Lymphocyte activation triggers TFIIH expression that in turns results in rapid global amplification of the RNAPII transcriptome. The negative elongation factor (NELF) enables promoter-proximal pausing of RNAPII, and plays an important role in viral latency during HIV (human immunodeficiency virus) infection [19,20]. Finally, MAF1 binds directly to RNAPIII and inhibits it, while also interacting with mTOR and thereby indirectly activating the host immune response [21-24] .

Archaea represent the second prokaryotic domain of life and utilize a 12-14 subunit RNAPII-like enzyme, as well as cognate initiation and elongation factors [25]. The archaeal transcription cycle is closely related to RNAPII, that in addition to the RNAPII subunits includes *bona fide* homologues of general transcription factors TBP, TFIIB, TFIIE, TFIIS, Spt4/5, Elf1 and CPSF73 (Figure 1) [25-27]. This streamlined eukaryote-like machinery transcribes a bacteria-like genome, with transcription units organized in multicistronic operons, and compacted in the so-called hyper-nucleosomes, a form of histone-based chromatin [28]. The genomes of more than 230 archaeal viruses have been sequenced, all of them are DNA-based and none of them encode genes with homology to any RNA polymerase subunit (with the exception of primase) [29]. Transcription in archaeal viruses is poorly understood, but in agreement with the absence of viral RNAPs, viral promoters include TATA and BRE host's promoter consensus elements [30]. As such, they recruit the host-encoded initiation factors TBP and TFB to facilitate transcription by the host RNAP [30], which has important implications as repressing host RNAP would also affect virus transcription. In this review we focus on two potent direct RNAP inhibitors, which play a

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key role in the virus-host conflict in archaea, the *Acidianus* two-tailed virus RNAP inhibitory protein, ATV RIP, and *Saccharolobus solfataricus* (Sso) negative regulator TFS4 [30-33].

2. RIP as a plug in the DNA-binding channel

ATV is a double-stranded DNA virus that infects hyperthermophiles of the Sulfolobaceae family including *Acidianus* and *Saccharolobus* species [34]. The ATV ORF145 gene product called RIP (RNAP inhibitory protein) is evolutionary related to the virus' capsid protein P131 (ORF131) sharing 36% sequence identity but the protein has undergone an intriguing specialization. The result is a protein that forms a high-affinity complex with RNAP, effectively inhibiting transcription initiation and elongation [30] (Figure 1).

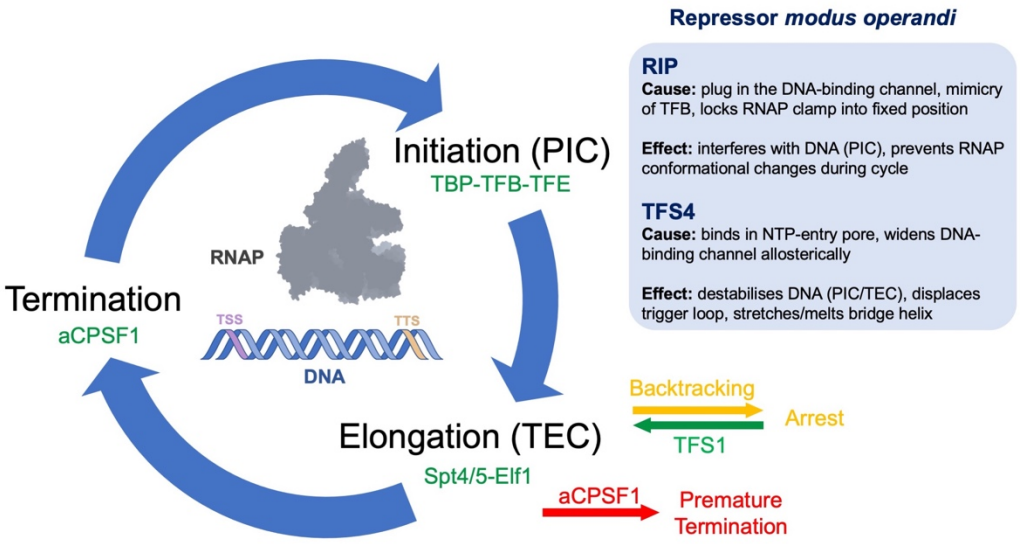

Figure 1. The archaeal transcription cycle. The basal factors TBP, TFB and TFE form together with RNAP the pre-initiation complex (PIC) on the archaeal promoter. Following promoter escape, the elongation factors Spt4/5 and Elf1 associate with the RNAP forming transcription elongation complex (TEC). When encountering roadblocks including histones or other nucleoid chromatin proteins, the TEC pauses, RNAP backtracks in a retrograde direction and extrudes the RNA 3' end through the NTP-entry pore. The transcript cleavage factor TFS1 promotes the cleavage of the 'excess' of RNA, creates a new RNA 3' end which reactivates the TEC. Transcription termination can occur through a factor-independent or -dependent mechanisms. The latter utilizes aCPSF1 (archaeal Cleavage and Polyadenylation Specificity Factor 1). aCPSF1 is also recruited proximal to the promoter where it can lead to premature termination [35]. The inhibitory regulators RIP and TFS4 interfere with RNAP in different ways as it progresses through the transcription cycle with the PIC and the TEC as listed in the light blue box and discussed in the text.

RIP is a protein with a compact six-helical bundle which is highly similar to P131, but in addition, RIP includes a long C-terminal tail which is not conserved in the capsid protein or homologues in other viruses. The high-resolution cryo-EM structure of RIP bound to the archaeal RNAP (pdb code 7oq4) reveals that the helical bundle forms a plug in the DNA-binding channel while the C-terminal tail runs along the clamp core enabling the high affinity binding to RNAP (Figure 2a) [32].

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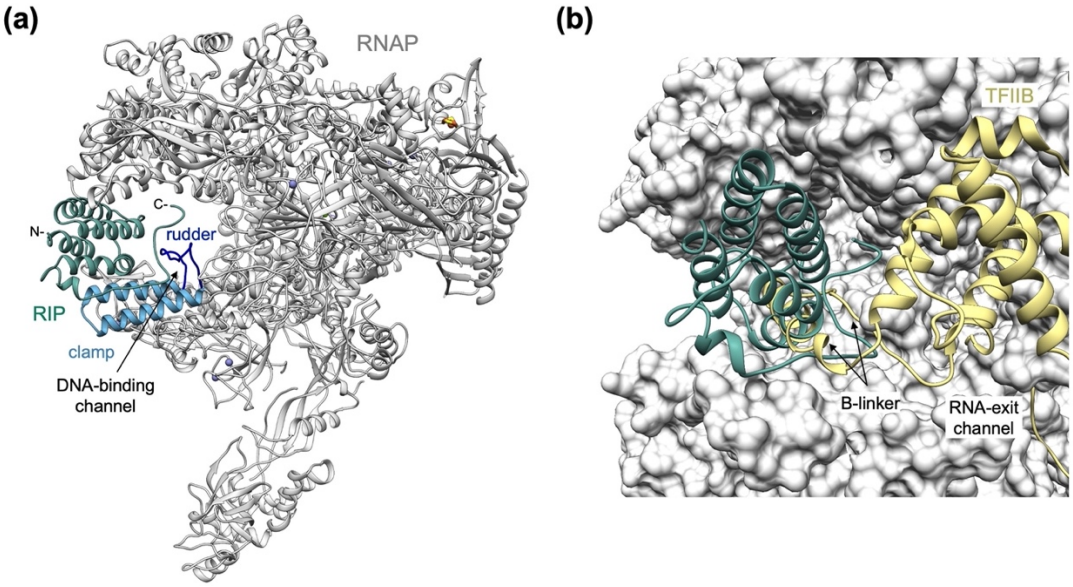

Figure 2. The archaeal RNAP-RIP complex. (**a**) The *Sulfolobus acidocaldarius* RNAP is shown in ribbon style, with ions as spheres, zinc in medium purple, magnesium in green, iron in red, and sulfur in yellow. RIP is highlighted in sea green with the N- and C-termini annotated (pdb 7oq4). (**b**) Superimposition of the RNAP-RIP complex with TFIIB of the eukaryotic PIC (pdb 6gyk [36]). The archaeal RNAP is shown as surface, while for the eukaryotic PIC complex only TFIIB has been shown (khaki).

The clamp is a flexible domain that opens and closes over the channel upon DNA binding and in response to transcription factors as RNAP progresses through the transcription cycle [37,38]. Single molecule FRET studies that monitor clamp opening and closing movements show that RIP binding locks the clamp into one fixed position [32]. As RIP seals off the DNA-binding channel, the RNAP-RIP complex cannot engage with the promoter DNA during initiation. In addition, RIP binding to the clamp sterically occludes RNAP interactions with the initiation factor TFB and the elongation factors Spt4/5, which each also compete for binding to RNAP. A deeper structural analysis and comparison with the eukaryotic PIC show that helix-4 and the C-terminal tail of RIP topologically mimic the B-linker of TFIIB, homologous of archaeal TFB (Figure 2b) [32,36]. RIP is perhaps one of the most extreme examples of evolutionary diversification where the common ancestor of P131 and RIP underwent gene duplication and speciation. Over time, one of the two paralogues evolved into the capsid component of the virus, while the other acquired a C-terminal extension that enabled RIP to bind and ultimately inhibit RNAP.

RIP expression is induced during the late stages of infection prior to cell lysis [30,32]. The strict time window of RIP expression is compatible with a role in virus particle assembly. The number of viral genomes is high during this stage, and as they are actively transcribed to produce viral proteins, most likely they are also adorned with RNAPs. The high occupancy of RNAP in turn is likely to be counterproductive during the packaging of viral DNA into the tight environment of the virus particle. RIP could be addressing this challenge by stripping RNAP off the viral genomes. In addition, the concomitant shutoff of the host transcription can activate cell death pathways leading to the lytic event and virions release.

3. TFS4 uses a 'belts and braces' approach to inhibition.

Transcript cleavage factors (TFS) are a large family of transcription regulators that interact with RNAP through its NTP-entry channel. This channel consists of a pore at the bottom of a funnel that enables the access of nucleotides to the RNAP active site, and the egress of the RNA 3'-end in the backtracked TEC [39,40]. TFS factors position two carboxylate side chains in the catalytic site of RNAP thereby triggering the cleavage of the

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backtracked RNA, allowing transcription elongation to commence [31,39,40] (Figure 1). The importance of this cleavage function is highlighted by convergent evolution; while archaeal and eukaryotic cleavage factors are homologous, the bacterial Gre factors are not sequence nor structurally related to TFS, they employ an identical mechanism of reactivating stalled TECs [41-43]. In addition, eukaryotes have stably incorporated TFIIS paralogues as RNAP subunits, RPB9 in RNAPII [40], RPA12 in RNAPI [44,45], and RPC10 in RNAPIII [46]. These RNAP subunits have acquired alternative functions that negatively influence elongation as both RPA12 and RPC10 are implicated in transcription termination. The function of RPB9 is unclear, it cannot stimulate cleavage but is essential for transcription-coupled DNA repair in yeast; curiously the rpb9 deletion can be suppressed by impairment of elongation factor Spt4/5 function through deletion of Spt4 [47].

Archaea encode only one type of RNAP, but several TFS paralogues [31], suggesting that each paralog has a distinct function. In Sulfolobales, TFS1 enhances transcript cleavage, rescuing stalled TECs and overall improving the elongation rate [31,39,48]. We have recently shown that the TFS4 paralogue, which lacks the two catalytic carboxylate residues characteristic of authentic cleavage factors, efficiently inhibits the archaeal RNAP [31,32]. The high-resolution cryo-EM map of the RNAP/TFS4 complex shows that TFS4 interacts with RNAP similarly to canonical cleavage factors, with the N-terminal zinc ribbon (ZR^N) binding between the lobe and the upper jaw, and, through the linker, positioning the C-terminal zinc ribbon (ZR^C) inside the NTP-entry channel (Figure 3a-b) [32].

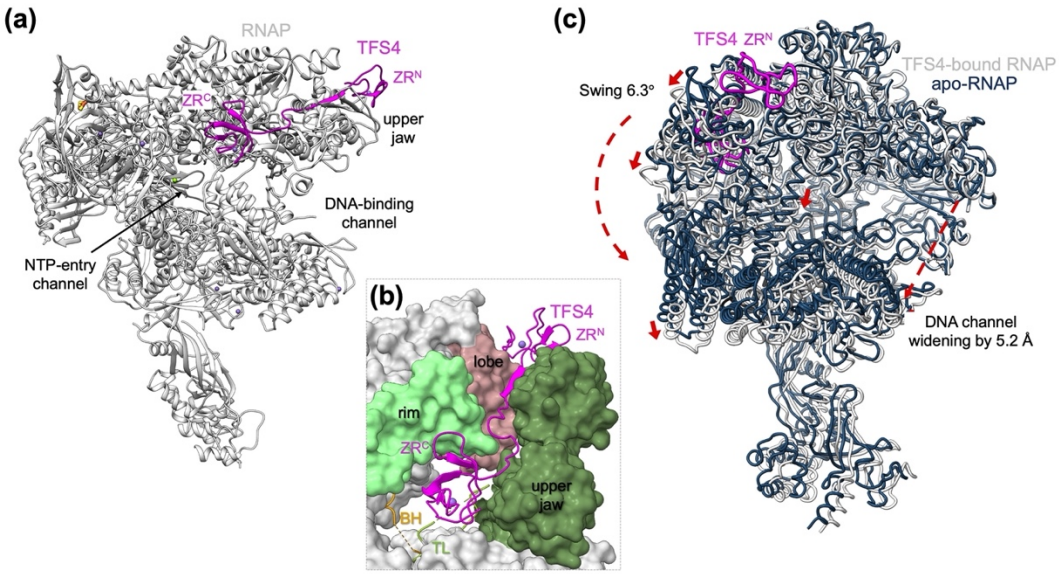

Figure 3. Molecular mechanisms of TFS4 inhibition. (**a**) Cryo-EM structure of the RNAP-TFS4 complex (pdb 7oqy) in ribbon style, with ions as spheres, zinc in medium purple, magnesium in green, iron in red, and sulfur in yellow. **(b)** Enlargement of the TFS4 binding site shown as surface, while bridge helix, trigger loop and TFS4 are in ribbon style. Zinc ions are displayed as spheres in medium purple. (**c**) Superimposition of the TFS4-RNAP complex (in grey) with the apo-RNAP (in dark blue from pdb 7ok0). The red arrows indicate the direction of the downwards swinging of the jaw and clamp, as well as the stretch of the bridge helix and the extent of the DNA-binding channel opening.

However, as the binding surface is not solvent-exposed, TFS4 displaces the jaw which causes a large conformational change with concomitant widening of the DNA-binding channel and melting of the bridge helix (Figure 3c). Finally, the ZR^C of TFS4 physically clashes with and displaces the trigger loop. As bridge helix and trigger loop are essential for the catalytic addition and translocation of NTPs during RNA synthesis, TFS4 effectively inhibits the enzymatic activity [31]. Moreover, the widening of the DNA-binding

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channel likely weakens RNAP-DNA interactions during elongation and interferes with PIC formation (Figure 1) [31].

RNAPs are large and dynamic molecular machines; the engagement with transcription factors, nucleic acids and the enzymatic reaction itself all involve conformational changes of RNAP, most of which are intrinsic to its structure and, therefore, fundamentally evolutionary conserved [25,49]. The substantial conformational changes induced by TFS4 can be easily recognized in other related inactive states, including the inhibition of bacterial RNAP by Gfh1 [15], the dimerization of inactive RNAPI [44], and transition states preceding termination of RNAPI and III that involve the TFS4 paralogues RPA12 and RPC10 [50,51]. TFS1 and TFS4 are derived from a common ancestor that, following gene duplication, have undergone speciation and evolved into a positive elongation factor and an inhibitor of transcription, respectively. This turned a positive transcription factor into a potent RNAP inhibitor. The *Saccharolobus solfataricus* TFS4 paralogue is not expressed during standard cell growth conditions, but 12 hours after infection with the *Sulfolobus* turreted icosahedral virus (STIV) the protein becomes detectable and shortly after the cell growth stagnates. The expression of a TFS4 variant from a plasmid induces growth arrest of *Sulfolobus acidocaldarius*, making it tempting to speculate that TFS4 is the causative agent of the growth phenotype caused by STIV infection. A dormant state or quiescence is often associated with a persister state in bacteria [44], allowing the infected cell to mount additional immune responses to clear the virus.

4. Similarities between antibiotics, inhibitors, TFS4 and RIP

The wide-spread emergence of multidrug resistant bacterial pathogens calls for urgent efforts to discover and develop novel classes of antibiotics [52]. RNAPs are powerful targets to fight infectious diseases by inhibiting the pathogen's RNAP, such as rifamycins, which inhibit only bacterial RNAPs [53] or nucleobase or NTP substrate analogues that are generally used to target the viral RNA-dependent RNAPs [54]. Another therapeutical application of RNAP inhibition includes anticancer treatments that either perturb the DNA template (e. g. cis-platin or actinomycin D) [55] or specifically target RNAPI [56] or RNAPII [57] or transcription factors that regulate RNAPII (e. g. p53 or myc) [58].

Antibiotics need to inhibit pathogen RNAPs with sufficient affinity and high selectivity to circumvent off-target effects such as inhibiting the host RNAP and/or other macromolecules. In addition, when considering specific RNAP motifs to be drugged, it is important to consider the likely emergence of resistance [59]. One of the most well-characterized antibiotics is Rifampicin (Rif), which is widely used as a front-line drug in the treatment of tuberculosis. Rif does not bind the RNAP active site but binds in the RNA binding pocket and blocks RNA extension. Rif resistant strains emerge readily and single amino acid substitutions in only three rpoB residues account for 88% of all clinically isolated Rif resistant tuberculosis strains [60]. For any antibiotic, the ideal target is small as well as critical for the enzyme function, as this makes it less likely that mutations arise that are both (i) deficient in antibiotic binding and (ii) remain enzymatically active. Understanding the mechanistic and structural basis of RNAP inhibition is important to rationalize the action of known drugs and to design new and improved antibiotics. As the structure and function of RNAPs is evolutionary conserved, the insights obtained from studying the inhibition of the archaeal RNAP by RIP and TFS4 can contribute to understanding the molecular basis of RNAP inhibition, the identification of novel target sites, and the search for new and improved inhibitors of bacterial and eukaryotic RNAPs. The field of RNAP-targeting antibiotics has been extensively reviewed elsewhere [59,61], below we focus on a selection of antibiotics that interfere with RNAP reminiscent of RIP or TFS4 (Figure 4).

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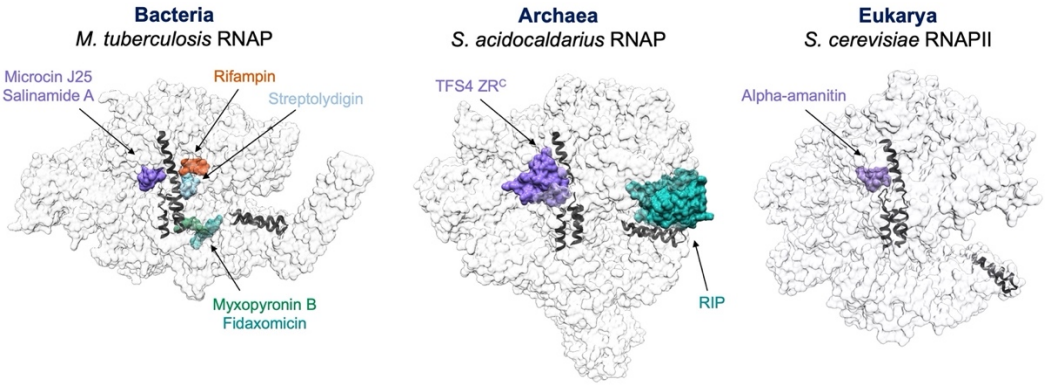

Figure 4. RNAP-specific antibiotics that are reminiscent of RIP and TFS4 action. Schematic illustration of the RNAP target sites for a selection of antibiotics and inhibitors. The RNAPs are shown as grey surfaces in transparency, the antibiotics and the inhibitors TFS4 and RIP are shown as surface representation using color coding for related binding sites. All bacterial structures were superimposed against subunit beta; pdb codes: 5uh6 (Rifampin) [62], 2a6h (Streptolydigin) [63], 4yfx (Myxopyronin B) [64], 6fbv (Fidaxomicin) [65], 6n60 (Microcin J25) [66], 4mex (Salinamide A) [67]. *S. cerevisiae* RNAPII was obtained from pdb code 3cqz [68].

The structurally mobile motifs of RNAP include the active site bridge helix and trigger loop, and the RNAP clamp that moves on so-called switches. While the oscillating movements of the bridge helix and trigger loop enable catalysis and translocation [69,70], the switch region control the opening and closing movements of the clamp, which modulates the width of the DNA-binding channel that is essential during DNA loading during initiation, and for TEC stability [49,71]. These sites are powerful[72] drug targets, e. g. the lasso peptide Microcin J25 that binds in the NTP entry channel, blocks access of NTP substrates to the active site and prevents trigger loop refolding [66], and Salinamide A that binds to the bridge helix and interferes with conformational changes necessary for nucleotide addition [67]. Streptolydigin targets the bacterial RNAP bridge helix and trigger loop from the DNA-binding channel without interfering with NTPs access [63]. In eukaryotes, the mushroom toxin alpha-amanitin traps the trigger loop and bridge helix in the active site impairing nucleotide incorporation and translocation of RNAPII [72] [73]. Examples of antibiotics that freeze clamp movements include the approved drugs Fidaxomicin and Myxopyronin which bind to the switch region [74]. The mechanisms underlying the inhibition of archaeal RNAP by RIP and TFS4 combine features of the above mechanisms. Like the switch-targeting antibiotic Myxopyronin and Fidaxomicin, RIP abrogates the mobility of the RNAP clamp. Similarly to Microcin J25, TFS4 binds in the NTP-entry channel interfering with both trigger loop and bridge helix. Furthermore, reminiscent of fidaxiomycin and alpha-amanitin, TFS4 induces a widening of the DNA-binding channel.

These compelling similarities demonstrate that while many roads lead to Rome, travelers often share the same path.

5. Discussion

RNA polymerase inhibition plays an important role for the global regulation of gene expression in response to environmental changes such as oxidative stress and nutrient deficiency, and in response to virus infection. Surprisingly, it appears that the global repression of transcription can be both of selective advantage for the host, and the virus, but for entirely different reasons. These include improved survival for the host, and aiding virus particle assembly and release for the virus. As repressors often use a range of measures to inhibit RNAPs that are reminiscent of antibiotics, characterizing the underlying inhibitory mechanisms is not only of academic interest, but has the potential to benefit the development of more potent and specific antibiotics which are less prone to resistance.

Take home messages

- RNAPs and the molecular mechanisms of RNA synthesis are universally conserved in all domains of life
- Both virus and host cell can encode repressors which tightly bind to RNAPs and efficiently inhibit their functions, leading to transcriptome repression or attenuation
- The *modus operandi* of these factors can unravel the underlying molecular mechanisms of RNAP activity and identify critical pressure points of enzyme function
- Inhibitory mechanisms include:
    - steric occlusion of DNA, NTPs and transcription factor binding sites
    - allosteric regulation by inducing conformational changes that perturb the active site (bridge helix and trigger loop) and widen of the DNA-binding channel
- Allosteric mechanisms of inhibition can reveal movements inherent to RNAP function. As these are evolutionary conserved, it is possible to draw intriguing parallels between the regulation of different transcription systems
- Antibiotics that target and inhibit RNAPs and proteinaceous repressors act via functionally closely related molecular mechanisms
- A thorough understanding of RNAP inhibition in all domains of life, including archaea, could be beneficial for the development of novel antibiotics

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