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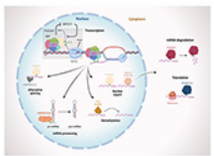

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REVIEW

Targeting bacterial transcription factors for infection control: opportunities and challenges

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ABSTRACT

The rising threat of antibiotic resistance in pathogenic bacteria emphasizes the need for new therapeutic strategies. This review focuses on bacterial transcription factors (TFs), which play crucial roles in bacterial pathogenesis. We discuss the regulatory roles of these factors through examples, and we outline potential therapeutic strategies targeting bacterial TFs. Specifically, we discuss the use of small molecules to interfere with TF function and the development of transcription factor decoys, oligonucleotides that compete with promoters for TF binding. We also cover peptides that target the interaction between the bacterial TF and other factors, such as RNA polymerase, and the targeting of sigma factors. These strategies, while promising, come with challenges, from identifying targets to designing interventions, managing side effects, and accounting for changing bacterial resistance patterns. We also delve into how Artificial Intelligence contributes to these efforts and how it may be exploited in the future, and we touch on the roles of multidisciplinary collaboration and policy to advance this research domain.

Abbreviations: AI, artificial intelligence; CNN, convolutional neural networks; DTI: drug-target interaction; HTH, helix-turn-helix; IHF, integration host factor; LTTRs, LysR-type transcriptional regulators; MarR, multiple antibiotic resistance regulator; MRSA, methicillin resistant *Staphylococcus aureus*; MSA: multiple sequence alignment; NAP, nucleoid-associated protein; PROTACs, proteolysis targeting chimeras; RNAP, RNA polymerase; TF, transcription factor; TFD, transcription factor decoying; TFTRs, TetR-family transcriptional regulators; wHTH, winged helix-turn-helix.

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Introduction

Consider a time when simple infections could be lethal, a harsh reality that troubled our ancestors before the discovery of antibiotics. Today, with persistent and escalating antibiotic resistance, this enduring nightmare remains a threat. The World Health Organization (WHO) states that drug-resistant diseases cause more than 700,000 deaths annually. This number is projected to rise 10-fold by 2050 if the issue remains unaddressed, potentially resulting in 10 million fatalities each year [1]. These staggering statistics not only highlight the human tragedy but also hint at potentially extensive economic fallout, with overburdened healthcare systems and failing treatments [2]. This situation compels us to find alternative strategies to fight bacterial infections. The rapid pace of bacterial evolution increasingly renders our traditional paths of developing efficient antibiotics inefficient [3]. Therefore, devising alternative strategies requires not just understanding, but critically analyzing the complicated processes that define bacterial physiology.

Antibiotic resistance and successful host colonization are tightly linked to the regulation of gene expression, with transcription playing a key role (Figure 1) [4]. One level of regulation is executed by sigma factors, which direct RNA polymerase (RNAP) to its promoters; while the house-keeping sigma factor is required for the bulk of transcription during balanced growth, specialized sigma factors that mediate the expression of a subset of genes are critical during host colonization or in response to specific stress conditions [5]. Moreover, factors such as the degree of DNA supercoiling and the three-dimensional organization of the genome can affect how accessible specific genes are to the transcription

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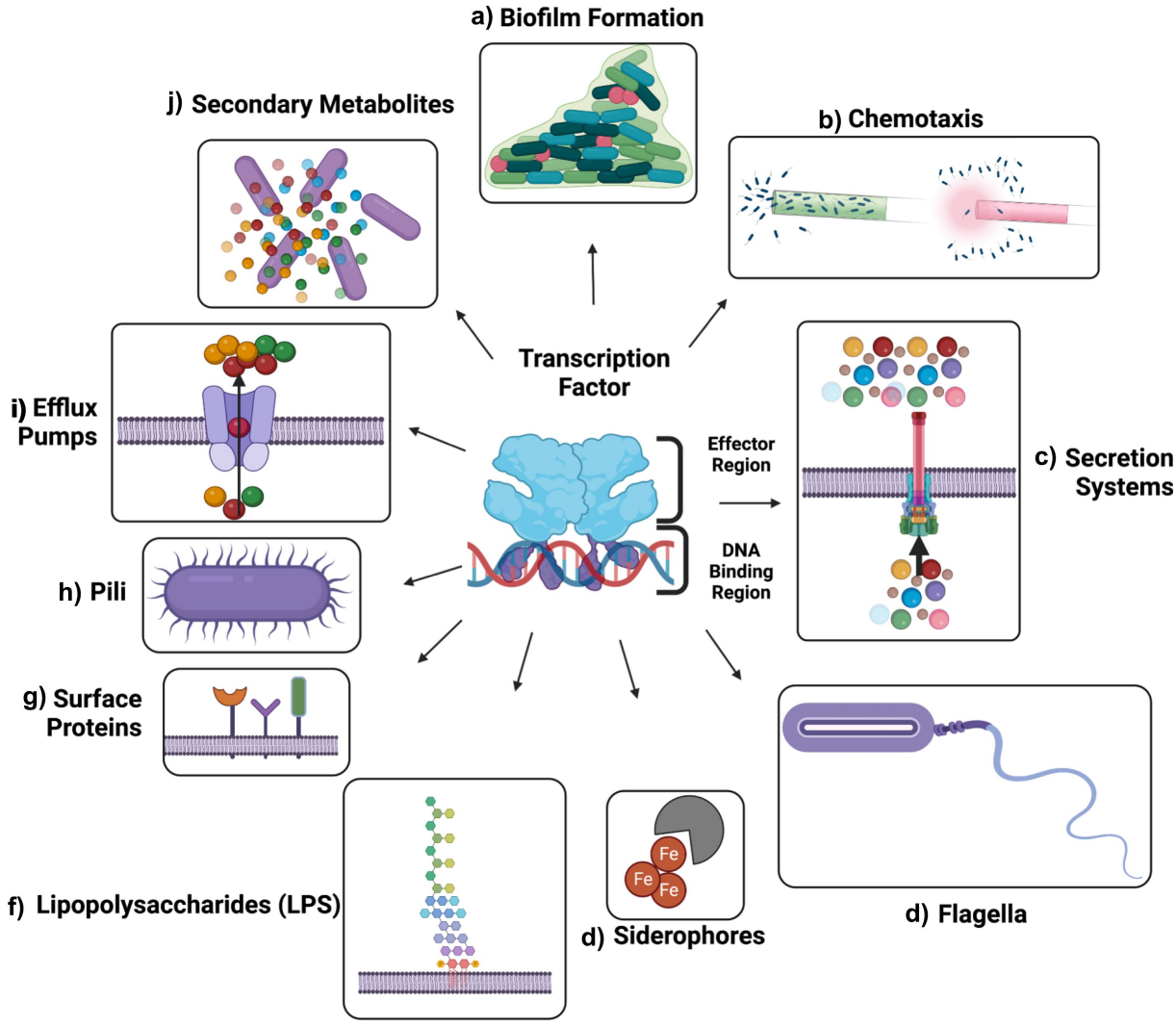

Figure 1. Central role of TFs in orchestrating key bacterial virulence mechanisms. Bacterial TF at the center with effector region in cyan and DNA-binding region in purple. Arrows point toward distinct consequences of TF activity. a) biofilm formation. These structures often confer protection against host immune responses and antimicrobial agents, aiding in persistent infections. b) chemotaxis. Bacteria may exhibit positive chemotaxis, moving toward a green chemical attractant, or they may display negative chemotaxis, retreating from a red repellant substance. c) secretion systems. Bacterial cell employing the type III secretion system to deliver effectors directly into host cells, modulating host functions to their advantage. d) flagella. Flagella facilitate bacterial movement, aiding in colonization and invasion of host tissues. e) siderophores. A bacterial siderophore binding to iron, a critical nutrient for their survival. f) lipopolysaccharides (LPS). The LPS layer, an essential component of the outer membrane of Gram-negative bacteria. LPS can act as an endotoxin, triggering strong immune responses in hosts. g) surface proteins. Proteins present on the bacterial surface may serve various functions including adhesion, invasion, or immune evasion. h) Pili. These structures aid in adhesion to host cells and surfaces and sometimes facilitate DNA transfer between bacteria. i) efflux pumps. Bacterial efflux pump actively extruding harmful metabolites or drugs. j) secondary metabolites. Production of secondary metabolites promote bacterial fitness in a competitive or hostile environment. Created with BioRender.com.

machinery, thus modulating transcription rates [6]. Environmental conditions and small molecule effectors further add layers of complexity to the regulation of bacterial gene expression. The fundamental concepts of bacterial transcription initiation and bacterial chromosome three-dimensional organization have been discussed in detail elsewhere [5–7].

Regulatory transcription factors (TFs) govern gene regulatory networks. They orchestrate bacterial adaptation, survival, and pathogenesis by binding to specific DNA sequences, thereby controlling gene expression in response to specific cues [8]. The unique functional properties of TFs arise from their bipartite structure: the DNA-binding region

and the effector-binding region. This dual design ensures specific sequence recognition while enabling the TFs to control gene expression in response to specific cues. TFs are broadly categorized into local and global types. Local TFs typically control specific pathways or individual functions by regulating a limited set of genes, whereas global TFs cast a wider net, influencing many genes across the genome, often residing at the top of a hierarchy of regulators and thereby affecting diverse biological processes [9].

As TFs play a crucial role in bacterial adaptability and pathogenicity, they have become a prime target for innovative infection control strategies [10]. Instead of conventional methods that generally aim to halt bacterial growth, targeting TFs offers a distinctive advantage, as it has the potential to disrupt bacterial virulence mechanisms selectively without compromising growth, a strategy that may lessen the selective pressure to induce resistance. This type of strategic intervention not only mitigates the harmful impact of the bacteria on the host, but it also provides the host immune system with additional time to respond, potentially reducing the severity of infections. Furthermore, by disrupting these virulence mechanisms, bacteria might become more vulnerable to traditional drugs, enhancing the overall treatment efficacy.

In recent years, significant advances have been made in targeting bacterial TFs. Generally, such approaches have entailed the delivery of small molecules that antagonize cognate TF ligands, the generation of DNA decoys, which compete with target promoters, or the design of peptides, which disrupt protein-protein interaction. As discussed below, these studies underscore the innovative and diverse approaches being adopted to counter bacterial virulence, indicating promising directions for future therapeutics.

While harnessing TFs as therapeutic targets offers promise, several challenges have become evident: a) Identifying suitable TF targets and designing drugs that are effective without disrupting host physiological processes [11]; b) Ensuring efficient drug delivery and overcoming natural barriers; c) Proactively addressing potential bacterial resistance to new therapeutic strategies [12]. Artificial Intelligence (AI) offers transformative potential to address some of these challenges. The advanced predictive modeling capabilities of AI provide a revolutionary means to identify TF targets, streamlining what traditionally has been a convoluted procedure. Moreover, AI-driven molecular simulations can refine drug design, potentially leading to compounds with increased efficacy while minimizing undesirable side effects for the host [13]. A multidisciplinary approach is important in this endeavor. By integrating insights from diverse fields, researchers can ensure a comprehensive perspective on the challenges and solutions, maximizing the potential for breakthroughs in combating bacterial virulence [14].

In this review, we discuss bacterial TFs, delineating their vital role in bacterial pathogenesis, and emphasizing their potential as therapeutic targets. We will highlight various targeting strategies, drawing insights from promising studies. Despite the potential, it is essential to acknowledge and address the associated challenges. To this end, we will shed light on the transformative potential of advanced technologies, particularly AI. Additionally, we will touch upon the crucial role of multidisciplinary collaboration and policy and funding in advancing this research. We aim to present a concise yet comprehensive overview of this complex domain, underscoring its critical importance in the ongoing battle against bacterial infections and antibiotic resistance.

Understanding bacterial TFs

Duality of bacterial TFs: activators and repressors

By definition, regulatory TFs alter transcription, either by inhibiting transcript formation or by accelerating mRNA synthesis. Activators are TFs that increase the efficiency of transcription after binding to their cognate DNA regions. The mechanism by which they increase transcription may involve a direct interaction with RNAP (Figure 2a), or it may entail a change in the conformation of promoter DNA, which in turn facilitates RNAP transcriptional activity. A classic example of the former is Catabolite Activator Protein (CAP), which when complexed with cAMP binds its cognate site to interact with the

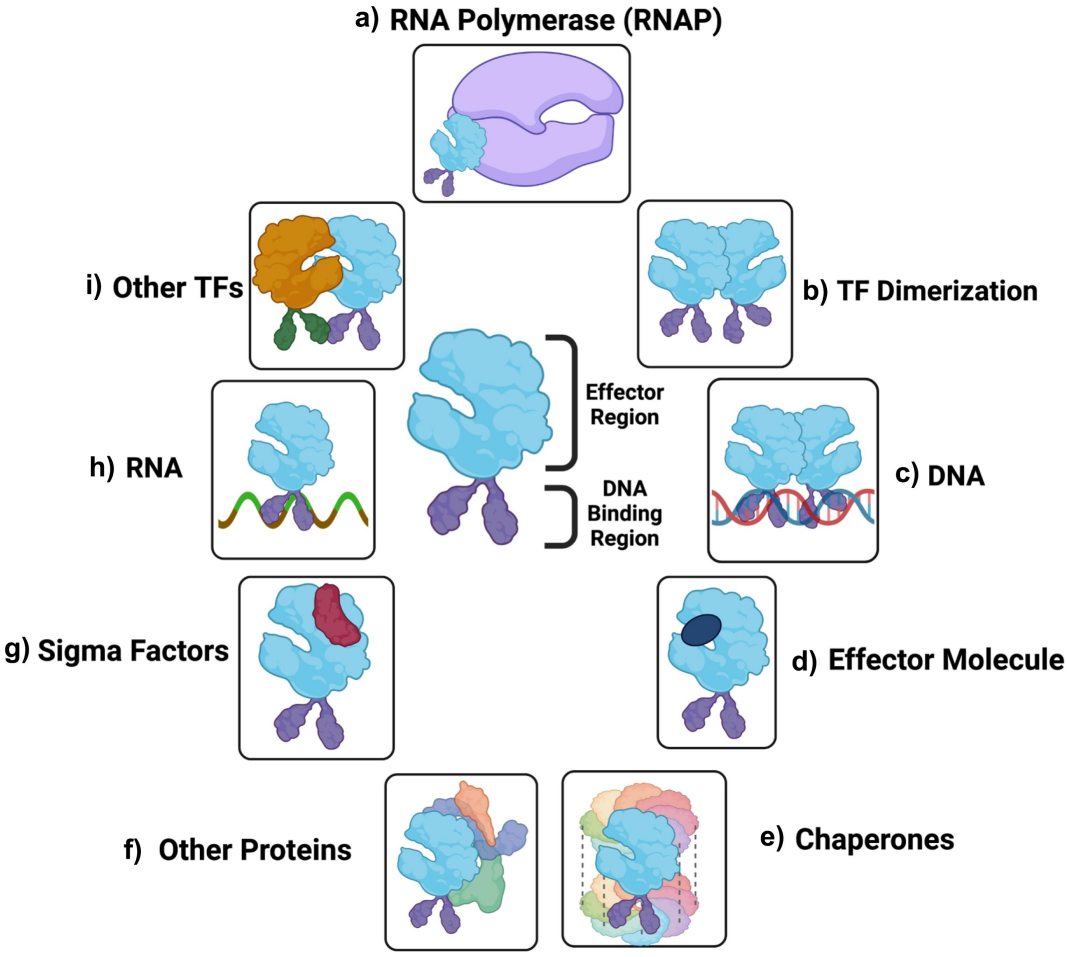

Figure 2. Examples of bacterial TF interactions. A TF in the center with its effector region in cyan and a DNA-binding region in purple. a) direct interaction with RNAP shown in lavender. b) TF dimerization. c) DNA recognition. d) effector molecule binding, with effector shown as dark oval. e) chaperone interaction. f) binding of TF to other proteins, from co-factors to those determining its post-translational state. g) interaction with sigma factor (red). h) RNA binding. i) TF cooperative binding to other TFs, forming regulatory complexes. Created with BioRender.com.

RNAP α-subunit to recruit RNAP. Since cAMP accumulates under circumstances of glucose limitation, this form of activation only occurs in this situation, thus promoting expression of genes required for metabolism of otherwise non-preferred carbon sources [15,16]. Another prototypical example is RhlR from *Pseudomonas aeruginosa*, which regulates rhamnolipid production (hence the name). It exemplifies quorum-sensing control in which the TF binds its cognate autoinducer at high cell density, and the complex in turn activates expression of genes that promote group behaviors. Notably, among the targets of RhlR are genes required for virulence and biofilm formation, which also often serves to render the bacterial communities more resistant to antibiotics and host defenses [17].

A few activators work by inducing a conformational change in promoter DNA. Well-studied examples include members of the MerR protein family, which generally bind between the −10 and −35 elements of target promoters. Since these particular promoters are characterized by having suboptimal spacing between the −10 and −35 elements, binding of RNAP does not lead to the requisite promoter unwinding or proper interaction with the sigma factor. Binding of the MerR-family protein induces a twist, which realigns the promoter elements, thus allowing transcription to initiate [18].

Conversely, repressors interfere with transcription, either by preventing RNAP binding to the promoter or by hindering elongation. For

example, LexA is part of the inducible SOS response, which senses DNA damage. A number of genes are bound by LexA, which binds to DNA sequences termed SOS boxes and blocks access of RNAP to promoter DNA [19]. However, when the bacterial DNA experiences external hazards such as UV radiation or toxic chemicals that induce DNA damage or stalled replication forks, RecA forms microfilaments. This in turn facilitates LexA auto-degradation, allowing the previously inhibited SOS genes to be expressed, thus activating DNA repair pathways, a crucial step for bacterial survival [19].

Repressive TFs have also been described, which compromise RNAP binding by altering the promoter DNA topology. For example, PecS from the plant pathogen *Pectobacterium atrosepticum* induces a distortion in promoter DNA at neutral pH. The resulting conformation of promoter DNA is incompatible with RNAP binding, the outcome of which is repression of gene expression. At alkaline pH, such distortions are attenuated, allowing RNAP to displace promoter-bound PecS and initiate gene expression. This mechanism allows differential expression of the PecS regulon in response to alkalinization of the plant apoplast following infection [20].

A few TFs bind downstream of the transcriptional start site of a gene to create a roadblock, which impedes progression of the elongating RNAP. One example is the *Escherichia coli* operons, which encode proteins required for purine biosynthesis. These operons are co-regulated by the repressor PurR (in complex with its corepressor, guanine or hypoxanthine). In one operon, the operator site for PurR is located within the protein-coding region, and binding of the repressor to this site blocks transcription elongation [21].

Organization of genomic DNA by nucleoid-associated proteins (NAPs) also has the potential to influence transcriptional efficiency. Bacterial DNA resides in a specialized region called the nucleoid, where NAPs function to shape the genome into topological domains. These proteins are not merely passive DNA-packaging elements; by actively participating in shaping the bacterial genome architecture, they become essential players in transcription regulation [7]. By binding to DNA, NAPs introduce bends, loops, or bridges, impacting the accessibility of certain genomic regions to the transcriptional machinery. This modulation can either facilitate or hinder the binding of RNAP and other TFs, thereby influencing gene expression [22]. Notably, NAPs have been associated with bacterial adaptation to stress [23]. Common NAPs include proteins such as HU (where H stands for histone-like and U refers to strain U93 of *E. coli* originally used for nucleoid isolation), Integration Host Factor (IHF), DNA-binding protein from starved cells (DPS), factor for inversion stimulation (Fis), and histone-like nucleoid-structuring protein (H-NS). These proteins have distinctive binding properties and functions. For instance, H-NS is known to silence the expression of foreign genes, playing a role in bacterial adaptation to environmental changes, whereas HU proteins have been reported to assist in forming repressive DNA loops [24,25].

Regardless of mechanism, it is essential to understand that TF binding is not a static phenomenon. It is a dynamic process, continually changing in response to environmental signals (Figure 2). For example, CAP represses target genes only under conditions of glucose limitation and the attendant cAMP accumulation, RhlR requires its cognate autoinducer for binding, and PurR requires its purine corepressor. Such adaptive versatility underscores the evolutionary ingenuity of bacteria, enabling them to thrive in diverse and ever-changing habitats.

Examples of bacterial TF families

Many families of TFs exist for which the members may be identified based on the specific DNA binding domain, usually comprising a helix-turn-helix (HTH) motif, along with distinct contiguous sequence, which may either sense a signal or facilitate interaction with RNAP. Often, the family name is based on the first characterized member. Prediction of TFs from genomes of model organisms such as the Gram-negative *E. coli* and the Gram-positive *Bacillus subtilis* indicates that LysR-type transcriptional regulators (LTTRs) are the most abundant among all model organisms tested, followed by members of the OmpR/PhoB family,

with *E. coli*, for example, encoding 35 LTTRs and 11 members of the OmpR/PhoB family [26]. In addition to these families, we highlight here a few other protein families harboring members with confirmed roles in regulation of virulence. This diversification, which has evolved to meet bacterial needs, reflects the complex design and adaptability of these cellular regulators.

LTTR

Structurally distinguished by their N-terminal HTH DNA-binding motif joined by a linker to a co-inducer binding domain, LTTRs may function either as activators or repressors. Binding of co-inducer results in a conformational change that results in differential DNA binding. Functionally, they primarily regulate genes related to metabolism, environmental stress, and virulence. Acting as homotetramers, two of these subunits bind to the DNA major grooves of palindromic DNA sequences, while the other two may establish interactions with RNAP [27]. A notable example is CsgD in *Salmonella*, which not only modulates curli fiber synthesis, essential for biofilm formation, but also regulates the expression of genes essential for virulence [28].

OmpR/PhoB

The OmpR/PhoB-type family of two-component transcriptional regulators is characterized by two distinct domains: a receiver domain, which perceives environmental signals, and a DNA-binding winged helix-turn-helix (wHTH) domain. When bacteria, such as *E. coli*, encounter varying osmotic conditions, OmpR responds by regulating the production of specific outer membrane proteins such as OmpF and OmpC. This is brought about by a sensory histidine kinase transferring a phosphoryl group to a specific aspartate in the receiver domain, a phosphorylation event that promotes homodimerization and DNA binding [29]. For instance, under high osmotic conditions, OmpR promotes the expression of *ompC* while repressing *ompF*, thereby adjusting the permeability of the bacterial outer membrane and ensuring cellular stability. On the other hand, PhoB is integral to the phosphate regulon. In environments with low phosphate concentrations, PhoB gets activated and triggers the expression of genes involved in phosphate uptake and storage, such as the *pst* operon in *E. coli*. This ensures that the bacterium acquires and retains adequate phosphate, a vital component for various metabolic processes and cellular functions.

GntR

Named after the gluconate operon repressor in *B. subtilis*, members of this protein family are ubiquitous. They feature two separate domains, a structurally conserved N-terminal wHTH DNA-binding domain and an effector-binding domain, which is also involved in protein oligomerization (usually dimerization). The effector-binding domains are heterogeneous, with the GntR protein family subdivided accordingly. Binding of ligand to the effector-binding domain is communicated to the DNA-binding domain via a flexible linker. Many GntRs repress metabolic pathways, with pathway intermediates serving as inducers. Others regulate virulence and biofilm formation [30].

TetR-family transcriptional regulators (TFTRs)

TFTRs, ubiquitous across both Gram-positive and Gram-negative bacteria, may control antibiotic resistance pathways, especially the expression of efflux pumps, but most regulate general aspects of bacterial physiology. These regulators possess a DNA-binding HTH motif at their N-terminus and a ligand-binding domain at their C-terminus. In their apo state, TetR-type regulators often act as repressors, binding to operator regions and inhibiting the transcription of their target genes [31]. However, upon binding of a specific inducer (such as tetracycline for TetR), they undergo a conformational change, reducing their DNA binding affinity and thereby initiating the transcription of the previously repressed genes. A classic example is AcrR, which controls the AcrAB-TolC efflux system in *E. coli*, a tripartite efflux pump instrumental in antibiotic resistance [32].

Multiple antibiotic resistance regulator (MarR) family

Structurally, the MarR family is distinct from the one-component regulators summarized above in that DNA- and ligand-binding regions

are not in separate protein domains. Instead, their wHTH DNA-binding motifs are directly linked to the ligand-binding pocket, which is often located in a crevice created by the wHTH motif and helices forming the dimer interface. Functionally, they play an important role in multidrug resistance, as exemplified by the eponymous *E. coli* MarR, and in sensing diverse threats, ranging from antibiotics to organic solvents and oxidants. Their mechanism of action involves sensing these environmental cues and subsequently modulating the transcription of target genes [33–35].

TFs in pathogenicity: a few examples

Bacteria utilize a range of virulence mechanisms to adeptly interact with their hosts. These mechanisms, requiring expression of specific virulence genes under control of cognate TFs, allow bacteria to adapt, invade, and flourish within the host environment. Below, we transit into specific examples to uncover how such regulators underlie bacterial virulence mechanisms.

ToxT in Vibrio cholerae

This bacterium is the causative agent of cholera, a diarrheal disease that poses significant public health challenges in many parts of the world. Functioning as a master regulator, ToxT, a member of the AraC/XylS protein family, precisely modulates the expression of a suite of genes that are pivotal for bacterial pathogenicity [36]. Among these genes are those encoding for cholera toxin (Ctx), one of the primary determinants of pathogenicity, and the toxin-coregulated pilus (Tcp). The Tcp, a type IV pilus, facilitates the initial attachment of the bacterium to the epithelial cells of the host intestine, setting the stage for colonization. Once anchored, the bacterium produces the cholera toxin, which catalyzes the active secretion of electrolytes and water into the intestinal lumen, culminating in the characteristic watery diarrhea. This mechanism not only weakens the host but also aids in the spreading of the bacterium, as the watery stools contain vast numbers of the pathogen [37].

VirF in shigella

Species of *Shigella* are the causative agents of shigellosis, a tough intestinal illness that manifests as severe dysentery, often in children. A central factor in the ability of this bacterium to invade the human colon and induce disease is the TF known as VirF, also a member of the AraC/XylS protein family. Acting as an upstream activator, VirF plays a pivotal role in the virulence cascade by regulating the expression of several genes. One of these genes encodes the virulence regulator, VirB, which further controls other genes essential for invasion. The products of these genes facilitate entry of the bacterium into intestinal epithelial cells through a Type III secretion system, a needle-like apparatus that injects bacterial effector proteins into host cells. These proteins manipulate host cell signaling pathways leading to the establishment of the infection [38].

Mga in Streptococcus pyogenes

This bacterium is behind a range of infections, from strep throat to the more severe rheumatic fever. Central to its virulent behavior is the Mga TF, a likely two-component regulator. As a master regulator, Mga controls the transcription of an array of genes that facilitate bacterial pathogenesis. Among these genes are those that code for adhesins, proteins crucial for the initial adhesion of the bacterium to host tissues. The presence of Mga ensures that, during the early stages of infection, the bacterium can effectively adhere to and colonize the host tissue. As the infection progresses, products of Mga-regulated genes mediate deeper tissue invasion and obstruct host immune responses [39].

AgrA in Staphylococcus aureus

This bacterium exhibits a broad pathogenic spectrum, ranging from benign skin infections to life-threatening conditions such as sepsis [40]. An essential regulator in this bacterium's adaptive virulence strategy is the two-component regulator AgrA. Integral to the agr quorum sensing system, AgrA modulates bacterial behavior based on population density. In the early stages of colonization, when bacterial numbers are low, AgrA-mediated gene expression prioritizes adhesion

mechanisms, facilitating colonization. However, as the bacterial population reaches a critical density, AgrA orchestrates a shift in gene expression, upregulating toxin production while downregulating adhesion genes. This dual regulatory role ensures a coordinated response: initial colonization followed by a robust pathogenic invasion once a sufficient bacterial community is established [41].

HrpL in Pseudomonas syringae

This bacterium is known for its ability to infect a variety of plant species, leading to diseases like bacterial speck or blight [42]. Central to its invasion strategy is the TF factor HrpL, which is an alternate sigma factor. Functioning as a key regulator, HrpL regulates the expression of genes that are essential for bacterial pathogenicity in plants. Among these genes, many are dedicated to forming the Type III secretion system. Once inside the host cell, these proteins manipulate plant cellular processes, weakening its defense mechanisms and facilitating bacterial colonization. Moreover, HrpL also regulates genes that produce molecules mimicking plant hormones, further destabilizing the host's internal defense signaling [43].

PecS in Dickeya dadantii

D. dadantii is a broad-host-range phytopathogen causing soft-rot diseases on both crops and ornamentals. The *D. dadantii* PecS regulon comprises more than 600 genes, with PecS at the top of a regulatory network. PecS, which is a member of the MarR protein family, controls both virulence gene expression as well as genes that are associated with enhanced bacterial fitness [44,45]. PecS-controlled genes encode, for example, pectinase and cellulase, resulting in the characteristic symptoms. PecS is conserved in other plant pathogens [46–48].

HU in Mycobacterium tuberculosis

M. tuberculosis remains a formidable pathogen, particularly with the emergence of multi-drug resistant tuberculosis. Generally, the functions of the NAP HU are manifold, ranging from regulation of DNA supercoiling to expression of virulence genes [25], yet in most bacterial species, HU is not essential. However, in *M. tuberculosis*, inactivation of the *hupB* gene encoding HU is lethal, rendering this protein an attractive target [49]. Notably, screening for compounds with the potential to disrupt DNA binding has yielded promising results, identifying the solvent-exposed DNA-binding interface to be "druggable" [50].

OrbS in Burkholderia cenocepacia

B. cenocepacia is an opportunistic pathogen, which poses a particular problem for persons living with cystic fibrosis (CF). It colonizes the CF lung, where its eradication is difficult due to inherent resistance to antibiotics. In this environment, iron is a limiting factor for the bacteria, which produce siderophores. These small molecules are secreted, upon which they chelate iron, and the iron-siderophore complexes are then taken up by dedicated bacterial transport systems. These siderophores are key virulence factors, and the expression of genes encoding biosynthetic enzymes and transporters of the main siderophore, ornibactin, is under control of a dedicated sigma factor, OrbS [51,52].

That bacterial TFs are key to pathogenicity is indubitable. But the real challenge lies ahead: How can we translate these foundational insights into actionable strategies that can enhance the way we combat bacterial infections? In the next section, we will explore possible targeted intervention strategies.

Targeting strategies for bacterial TFs

Mechanistic approach to TF targeting

Drugs target a TF to alter its activity. Such alteration may be in the form of an antagonist, which inactivates the protein function or an agonist, which induces it. In terms of identifying a druggable target, this often relies on comparisons to other members of the same protein family for which drugs have been successfully identified. The drugs in question are often small molecules, hence a druggable target should possess the ability to bind said compound with high affinity and specificity. For instance, enzymes are typically viewed as druggable targets as they inherently feature a specific substrate binding pocket. By contrast, TFs have generally been considered non-druggable, either for reasons of structural disorder

or the absence of defined binding pockets. However, many bacterial TFs do contain binding pockets for small molecule ligands in addition to the solvent-exposed protein-DNA interfaces and possibly protein-protein interfaces (Figure 2). This section explores potential strategies to target bacterial TFs, shedding light on their mechanistic foundations. Table 1 offers an overview of the advantages, drawbacks, and optimal applications for each approach.

Competitive inhibition

As previously discussed, many TFs contain specific binding pockets for small molecule ligands. Competitive inhibition, a tactic used to disrupt TF-ligand interaction, depends on the creation of antagonists that mimic the natural ligands (Figure 3a). By competing, they are meant to restrict the natural ligand's access to the site, effectively preventing the TF from responding to its natural cues and effecting differential gene expression [53]. The success of competitive inhibition relies on the antagonist associating with the binding pocket with high specificity due to its molecular shape and favorable interactions, and secondly, its effectiveness is determined by both its concentration in the system and its binding affinity for the TF, ideally exceeding that of the natural ligand.

Allosteric modulation

Allostery refers to a situation in which binding of an effector molecule alters the functionality of a protein at a distant site. By this definition, DNA binding of one-component TFs is controlled allosterically, as binding of ligand to its effector domain causes conformational changes that propagate to the DNA-binding domain. However, separate locations can be targeted by modulators, which attach to these sites and initiate conformational alterations within the TF, an approach implemented for eukaryotic TFs [54]. The results can vary, positive allosteric modulators enhance the TF activity, perhaps by increasing the receptivity of its DNA-binding domain or amplifying its affinity for the natural ligand. In contrast, negative allosteric modulators reduce TF activity, either by blocking the DNA-binding interface or diminishing its ligand affinity. What sets allosteric modulation apart is its underlying mechanism; instead of directly competing with the native ligand for the binding site, it changes the overall activity of the TFs.

Direct inhibition of DNA binding

The DNA binding region is generally surface-exposed and may be devoid of defined binding pockets, and it is therefore considered challenging to target by using inhibitors. However, examples exist in which natural ligands or designed inhibitors directly interfere with DNA binding. *S. aureus* TcaR is a member of the MarR protein family, and it regulates genes involved in teicoplanin and methicillin resistance [55]. The structure of TcaR in complex with penicillin G shows the antibiotic binding in a cleft between the two wHTH DNA binding motifs, thereby obstructing access to the DNA [56]; notably, this binding site differs from the more common ligand-binding pocket in MarR family proteins for which ligand binding induces conformational changes that propagate to the DNA recognition helices [33,35]. Similarly, the antirepressor peptide ArmR binds to *P. aeruginosa* MexR, which is also a member of the MarR protein family, and it likewise occupies a hydrophobic pocket between the DNA binding motifs [57]. These binding modes suggest that this site in MarR family proteins could be targeted by inhibitors with the aim to block DNA binding.

In AraC/XylS transcriptional activators, the non-conserved effector binding domain is connected to two C-terminal HTH DNA-binding motifs [58]. A high throughput screen was implemented to identify inhibitors of *E. coli* RhaS, which is associated with activation of an operon encoding rhamnose catabolic enzymes. In this screen, an inhibitor, subsequently named SE-1, was found to inhibit DNA binding [59]. This compound has activity against other AraC/XylS family proteins as well [60], suggesting the potential for developing more specific inhibitors.

Targeted degradation

Drug design techniques have progressed not only to inhibit but also actively degrade TFs. An example of this degradation strategy in eukaryotes is proteolysis targeting chimeras (PROTACs)

Table 1. Comparative overview of bacterial TF targeting strategies. A systematic comparison of various strategies employed in targeting bacterial TFs. Each strategy is described based on its mechanism of action, advantages, potential drawbacks, and optimal application scenarios.

Strategy	Mechanism	Advantages	Disadvantages	When to Use
Competitive Inhibition	Molecules mimic natural TF ligands, competing for binding.	1. Direct targeting allows for precise modulation. 2. Reversible, enabling dose-dependent control. 3. Well-established, with many known inhibitors.	1. Requires thorough knowledge of the binding site. 2. Continuous inhibitor presence needed to maintain effect. 3. Achieving in vivo concentration is challenging.	With detailed knowledge of the binding site and known ligands.
Allosteric Modulation	Molecules bind at sites other than DNA or ligand-binding sites, altering conformation.	1. Avoids direct competition with natural ligands. 2. Potential to modulate a range of TF functions. 3. Reduced risk of resistance via binding site mutations.	1. Requires identification of an allosteric site. 2. Less predictable due to dynamic nature of allosteric effects. 3. Potential for off-target effects.	When direct inhibition with active site is challenging or has led to resistance.
Targeted Degradation	Molecules tag specific TF for cellular degradation.	1. Ensures complete depletion of endogenous TF. 2. Less prone to resistance compared to inhibition. 3. Potential for multi-targeting with bifunctional degraders.	1. Complex synthesis and design. 2. Potential cellular toxicity. 3. Involvement of proteasome system may complicate outcomes.	When aiming to selectively deplete stubborn bacterial TFs.
TF Dimerization/ Association Modulation	Disrupts TF dimerization or association with other factors.	1. Targets the larger network of TF interactions. 2. Exploits unique interfaces, reducing off-target effects. 3. Can disrupt multiple TF functions simultaneously.	1. Potential for broad cellular impacts. 2. Requires detailed interaction maps. 3. Bacteria may evolve bypass mechanisms.	When aiming to disrupt a broader network of TF interactions.
Post-Translational Modifications (PTMs)	Modulates TF activity by altering post-translational modifications.	1. Dynamic, rapid modulation. 2. Can be transient, minimizing long-term impacts. 3. Exploits existing cellular machinery.	1. Interferes with natural cellular regulation. 2. Potential for broader cellular impacts. 3. Potential for unpredictable outcomes.	Rapid TF modulation when transient changes can have significant impact.
Decoying TFs (TFD)	Synthetic oligonucleotides mimic TF-binding genome sequences.	1. Directs TFs away from specific regulatory sites, potentially sparing other TF target genes. 2. Exploits natural TF binding preference. 3. Mimics natural DNA sequences, likely reducing host toxicity.	1. High dependency on accurate genomic data. 2. Bacteria may evolve altered TF binding preferences. 3. Risk of off-target effects on nonpathogenic strains.	TF binding sites are well-documented.

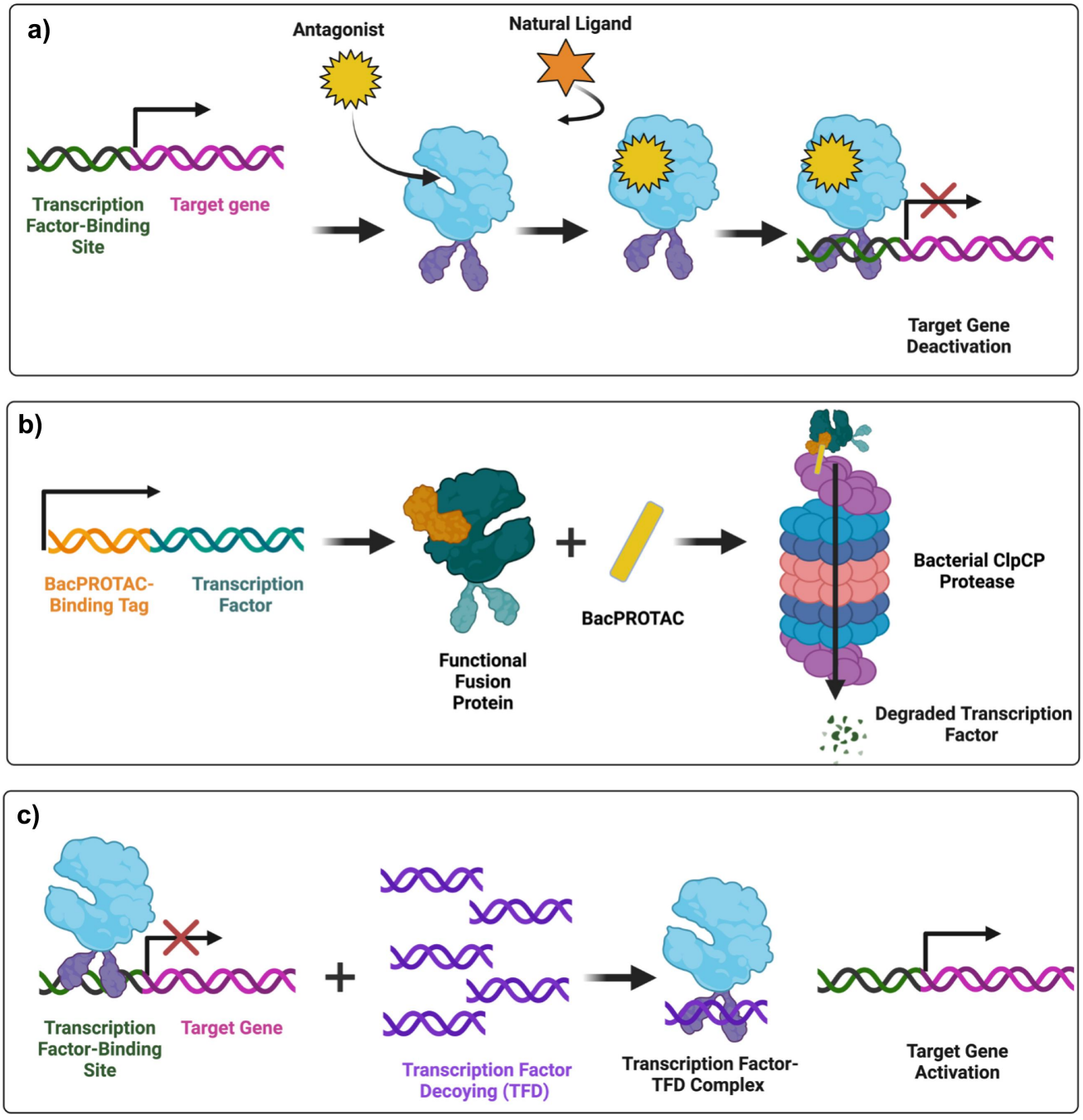

Figure 3. Strategies for therapeutic targeting of bacterial TFs. hree approaches for therapeutically targeting bacterial TFs. a) competitive inhibition. A TF bound to its natural ligand (orange star) is unable to bind its target promoter. Binding of antagonist (yellow) prevents the natural ligand from binding to the TF, resulting in DNA binding. b) BacPROTAC induced degradation. A bifunctional BacPROTAC molecule (yellow oval) links a fusion protein containing a bacterial TF and a BacPROTAC binding sequence to a ClpCP protease, initiating targeted degradation. c) transcription factor decoying (TFD). A TF binds its target promoter. Synthetic oligonucleotides designed to mimic these binding sites act as molecular decoys, diverting TFs from their native regulatory regions. Created with BioRender.com.

(Figure 3b). These bifunctional molecules link a ligand specific to the target TF with another, which recruits an E3 ubiquitin ligase [61]. By fusing these two compounds, PROTACs serve as molecular bridges. The ligand specific to the TF ensures selective binding, effectively tagging the TF. Meanwhile, the part that recruits the E3 ubiquitin ligase plays a key role in initiating the degradation process. Once the E3 ligase is brought into proximity of the TF, it facilitates the transfer

of ubiquitin molecules to the TF. This ubiquitination acts as a cellular signal for the proteasome, leading to the degradation of the tagged TF. A recent development in this domain is the use of photoPROTACs, molecules that have the capability of light-mediated activation [62]. Building on the concept of PROTACs, the recent study on BacPROTACs extends targeted protein degradation to bacterial systems. Unlike the ubiquitin-proteasome system in eukaryotes, bacteria utilize the caseinolytic protease proteolytic subunit (ClpCP) for targeted protein degradation. BacPROTACs are engineered to reprogram the bacterial ClpCP protease, enabling the targeted degradation of specific bacterial proteins. This innovative approach not only offers a new platform for antibiotic discovery but also provides a versatile tool for researching bacterial protein functions [63].

TF dimerization/association modulation

The effectiveness of TFs often depends on their ability to form homodimers or to partner with other factors. In bacterial systems, TFs can engage in a series of interactions: 1) Homodimerization: A TF might pair with an identical counterpart, forming a dimeric structure. Such dimeric assembly is often required for DNA binding by the TF, thus unlocking specific gene regulatory events. 2) Interaction with basal transcription machinery: Some TFs associate with components of the basal transcriptional machinery, the RNAP holoenzyme, to collaboratively regulate gene expression, usually by facilitating RNAP binding or promoter opening. 3) Engagement with co-factors: Such interactions may modulate TF activity, often fine-tuning its response to environmental signals and/or cellular needs. 4) Association with chaperones: Certain chaperones can support TFs, facilitating their folding or activity within the bacterial cell [64]. 5) Sequestration by cytoplasmic proteins: Some proteins can trap TFs in the cytoplasm, diverting them from their nucleoid targets. Such spatial organization can critically affect transcriptional activity [65].

Post-translational modifications (PTMs)

Like many cellular proteins, TFs are potentially subject to PTMs, events that mediate their optimal function. Beyond their primary sequence, the complex roles of TFs are profoundly shaped by modifications such as phosphorylation and acetylation. These PTMs serve as molecular switches, dynamically adjusting the activity and conformation of TFs. With advancements in molecular biology, a suite of small molecules has been identified that can modulate these PTMs, either amplifying or attenuating them. Consider, for instance, a molecule specifically designed to promote the phosphorylation of a TF. Such an intervention can induce a structural reconfiguration in the TF, potentially altering its DNA-binding proficiency [66].

Expanding our understanding of PTMs, research in actinobacteria such as *Streptomyces* and *M. tuberculosis* has revealed a fascinating addition to the repertoire of cellular modifications: pupylation. In this process, a prokaryotic ubiquitin-like protein (Pup) is covalently attached to target proteins at lysine residues through the enzymatic action of PafA ligase. Unlike ubiquitination in eukaryotes, which targets proteins for proteasomal degradation, pupylation plays a dual role. It can either target proteins for degradation in proteasomes or serve additional functions, as seen in *S. coelicolor* where mutants lacking PafA exhibit altered spore formation and antibiotic production. This mechanism is vital for optimizing protein turnover and amino acid utilization, particularly affecting proteins involved in metabolic pathways such as carbon catabolism, fatty acid metabolism, and antibiotic resistance. These discoveries not only broaden the scope of PTMs as dynamic modulators of cellular function but also hint at their potential as targets for therapeutic interventions [67].

Transcription factor decoying (TFD)

This approach aims to prevent the TF from interacting directly with its intended DNA targets (Figure 3c). TFDs are short oligonucleotides that closely resemble the DNA sequences to which a bacterial TFs typically binds. By mimicking

these sequences, TFDs operate as molecular distractions, directing TFs away from their native regulatory sites. The journey of creating a TFD starts with decoding the exact DNA sequences in the bacterial genome that are prime binding sites for TFs. With this information, researchers can craft synthetic oligonucleotides that mimic these sequences [68]. At present, this approach has been implemented only to target a repressive TF. A challenge that emerges is to ensure that these molecular decoys are both resilient against degradation and possess an attraction stronger than the authentic sites. Once designed, the focus shifts to integrating these TFDs into bacterial systems. But TFDs are more than just disruptors. Their introduction can also trigger stress responses within bacteria, making them potentially behave differently to other treatments, such as antibiotics. Looking forward, as synthetic biology and nanotechnology continue to advance at a rapid pace, we anticipate the emergence of even more refined and effective TFD delivery techniques [69].

Monoclonal antibody-mediated targeting

Monoclonal antibodies have the theoretical advantage of selective targeting. The use of monoclonal antibodies to disrupt gene regulatory circuits in human diseases such as cancer have been implemented, however, the use of such technologies to combat bacterial pathogens is lagging [70]. There is one notable exception – the NAP IHF is also an integral component of the extracellular biofilm matrix; since biofilm contributes markedly to pathogenesis, efforts to disrupt biofilm formation by targeting IHF were pursued. This approach was successfully implemented, for instance, to disrupt multicellular aggregates of *P. aeruginosa* in vivo [71].

Selected studies

The selected studies presented here excerpts some of the cutting-edge research aimed at disrupting the function bacterial transcriptional regulators. Ranging from small molecules to peptide-based strategies, these investigations illuminate the potential that lies in targeting these molecular switches. A broader survey of such studies including their key findings, strengths, and drawbacks is shown in Table 2.

Targeting the master TF PrfA of listeria monocytogenes

In their endeavor to decipher bacterial virulence mechanisms, Tran et al. focused on the master TF of *L. monocytogenes*, PrfA. This bacterium, known for its potent pathogenicity, has evolved a sophisticated technique, allowing it to escape host cell vacuoles. Such tactics facilitate its prolific intracellular replication, aiding its spread and domination within the host. PrfA activates the required virulence genes, and its affinity for cognate promoters is increased by binding of coactivator molecules. Inhibiting the PrfA coactivator site inhibits the vacuolar escape of the bacteria, which speaks to the key role of PrfA. However, it surprisingly also prompts the bacteria to multiply within expansive vacuoles. The twist? Instead of offering refuge, these vacuoles become detrimental to the bacteria, leading macrophages to systematically eliminate the entrapped *L. monocytogenes* through lysosomal degradation. The high-resolution structure of PrfA bound to an inhibitor provides crucial insights for potential therapeutic strategies. However, translating these in vitro discoveries to the complexities of in vivo settings remains a notable challenge [72].

TFDs delivered by nanocarrier to treat MRSA

Methicillin resistant *Staphylococcus aureus* (MRSA) is a Gram-positive human pathogen, which can lead to life-threatening infections. TF WalR is part of a two-component regulatory system, and it is required for viability, as it is crucial to bacterial cell wall synthesis. It is also involved in resistance to vancomycin, an inhibitor of cell wall synthesis, which remains widely used to combat MRSA infections. Hibbits et al. reported the use of TFDs designed to bind competitively to WalR and attenuate its ability to bind gene promoters. To ensure these TFDs effectively reach their target within the bacterial cell, the team introduced two distinct nanocarriers: cationic nanostructured lipid carriers (cNLCs) and chitosan-coated nanoparticles (CS-NCs). Each nanocarrier possesses unique attributes, with cNLCs being particularly notable

Table 2. Comprehensive analysis of bacterial TF targeting studies: an in-depth analysis of selected studies focusing on the targeting of bacterial TFs. Each study is dissected to illuminate the bacterial species and TF of interest, the targeting method employed, key findings, strengths, potential future directions, and the relevant reference. SM, small molecule; TFD, transcription factor decoy.

TF	Method	Key Findings	Strengths	Drawbacks/Future Directions	Reference
Listeria monocytogenes PrfA	SM	A PrfA inhibitor impedes *L. monocytogenes* from vacuolar escape in macrophages, suggesting PrfA as an antimicrobial target.	The detailed structural analysis can guide future rational drug design. The established link between PrfA inhibition and bacterial pathogenicity control is clear.	Results obtained using small molecule occupancy and genetic manipulation may not be directly comparable. Translation of in vitro findings to in vivo scenarios remains uncertain.	[72]
MRSA WalR	TFD	Co-delivery of TFD with vancomycin resulted in significant reduction in MRSA viability.	This strategy provides a novel means to inhibit microbial growth. The protective properties of TFD carriers are highlighted.	In vivo translation of in vitro findings is yet to be determined. The adaptability of MRSA to new treatments should be considered.	[73]
Mycobacterium tuberculosis CarD	Peptide	Peptides disrupt the conserved protein-protein interaction interface between RNAP and CarD.	The unique targeting site promises efficacy against multi-drug resistant bacterial strains. Broad applicability due to the conserved target.	Designing peptide-based inhibitors presents several challenges, including solubility, stability and delivery issues.	[74]
σ^{54} (RpoN)	Stapled Peptide	Peptides disrupt σ^{54}-promoter interaction.	A promising strategy to disrupt bacterial transcription. Potential applicability against multiple pathogens.	The pharmacokinetics and in vivo efficacy of the stapled peptides have not been explored.	[75]
Vibrio cholerae ToxT	SM	Virstatin was found to disrupt ToxT dimerization, which in turn reduces bacterial virulence and potential colonization.	The study provides detailed insights into virstatin's mechanism, suggesting a novel antibiotic discovery approach.	The long-term effects and potential resistance mechanisms against such treatments are not fully explored.	[76]
Vibrio cholerae/ *Salmonella* Typhi AphB/Hrg	SM	Ribavirin suppresses the functions of AphB/Hrg, leading to a reduction in bacterial pathogenesis.	The targeting is specific, reducing potential off-target effects. Ribavirin's FDA-approved status suggests potential for repurposing.	The underlying mechanism requires further exploration. Further studies needed to establish long-term efficacy and potential side effects.	[77]
Yersinia spp. LcrF	SM	N-hydroxy-benzimidazole compounds inhibit LcrF, targeting virulence without compromising growth.	Reduced resistance development potential. The potential broad applicability against *Yersinia* infections is highlighted.	The in vivo relevance of in vitro findings remains to be seen. Poor membrane permeability for some compounds.	[78]
Shigella flexneri VirF	SM	SE-1 inhibits the DNA binding activity of VirF, reducing bacterial virulence and invasion capabilities.	The novel approach offers potential cross-genera applications. Multiple validation methods ensure robust findings.	The current potency of SE-1 requires optimization. In vivo implications of in vitro findings remain uncertain.	[60]
Pseudomonas aeruginosa MvfR	SM	Benzamide-Benzimidazole (BB) inhibitors suppress both acute and persistent *P. aeruginosa* infections.	The detailed structural insights into MvfR pave the way for potential broad applications.	BB compounds might have multiple targets in the MvfR-regulated quorum sensing pathway. The full spectrum of MvfR functions and its interaction need further exploration.	[79]
Mycobacterium tuberculosis Rv0792c	SM	I-OMe-Tyrphostin inhibits Rv0792c, a TF essential for bacterial survival under oxidative stress.	Characterization of Rv0792c suggests potential broad applicability. Integration of diverse techniques offers a holistic view of the target protein.	The exact effector molecule for Rv0792c remains uncertain. Further in vivo testing is needed.	[80]

(*Continued*)

Table 2. (Continued).

TF	Method	Key Findings	Strengths	Drawbacks/Future Directions	Reference
Listeria monocytogenes σ^B	SM	Fluoro-Phenyl-Styrene-Sulfonamide (FPSS) is a potential inhibitor of σ^B, which could offer a novel antimicrobial targeting strategy.	Comprehensive screening of ~ 57,000 molecules, providing a robust foundation. FPSS specifically targeted the σ^B regulon without broad nonspecific effects. Both genetic effects and functional outcomes, such as bacterial invasion capabilities reported.	The in vivo implications of in vitro findings remain unexplored. The potential side effects and toxicities of FPSS in higher organisms, including humans, were not addressed.	[81]
Pseudomonas aeruginosa ExsA	SM	N-Hydroxy-benzimidazole inhibitors inhibit ExsA-DNA binding, reducing bacterial virulence.	The assessment of metabolic stability early in the study provides valuable information for potential drug development.	Focus remains predominantly on in vitro effects. The specificity of the inhibitors in a bacterial context remains to be determined.	[82]
Bacillus subtilis GabR	SM	(S)-4-amino-5-phenoxypentanoate selectively binds to GabR and potentially functions as a GabR agonist.	The SM showed selectivity for bacterial GabR over eukaryotic enzymes. Combines chemistry, structural biology, and molecular biology techniques. Obtained 2.75 Å resolution crystal structure of the SM bound to GabR.	While the SM binds to GabR, the exact mechanism of action is not fully elucidated. No information on potential toxicity of the synthesized SM.	[83]

for enhancing the potency of vancomycin when loaded with TFDs and effecting decreased bacterial viability. This suggests that this method can potentially revitalize older antibiotics, making them more potent against resistant strains [73].

Peptide-based strategy targeting CarD TF and RNAP β-subunit interaction in M. tuberculosis

In addressing the pressing global health issue of tuberculosis, the study by Kaur et al. offers an innovative approach toward inhibiting bacterial transcription in *M. tuberculosis*. Tuberculosis remains a significant cause of death globally, with rifampicin being a first line defense, targeting the bacterial RNAP. The global TF CarD interacts directly with the mycobacterial RNAP β-subunit to stabilize the open complex and promote transcript formation, a function that makes it an attractive drug target, particularly as this interaction confers antibiotic and oxidative stress resistance. Kaur et al. explore this interaction by designing peptide-based inhibitors that target the conserved protein-protein interaction interface between the bacterial RNAP β-subunit and CarD. This strategy is notable as targeting an interface, which is solvent-exposed and lacks a binding pocket, is particularly challenging. Using a combination of in silico protein-peptide docking and biochemical assays, the team identified peptides that are both stable and soluble in aqueous solutions. One of their promising findings is a peptide that can inhibit in vitro transcription with IC_{50} ~50 μM. Their approach of using peptide-based molecules to disrupt this interaction presents a novel angle in the fight against tuberculosis [74]. How such in vitro inhibition translates to bacterial survival remains to be determined.

Targeting σ^{54} in Gram-negative bacteria with hydrocarbon-stapled peptides

The σ^{54} subunit of bacterial RNAP is instrumental in controlling the expression of virulence genes in response to specific environmental stresses. It achieves this control through a distinctive process: upon its association with RNAP, the σ^{54} subunit facilitates promoter opening, a prerequisite for transcription. Central to this mechanism is the RpoN box, a helix located in the C-terminal region of σ^{54}. This RpoN box specifically inserts itself into the major groove of promoter DNA at the −24 position, a step crucial for initiating transcription. Recognizing the importance of this interaction, and exploiting the helical shape of RpoN, Payne et al. adopted the hydrocarbon-stapled peptide methodology. This innovative technique involves modifying peptides by stapling them with hydrocarbon chains. This stapling not only stabilizes the helical structure, but the stapled peptide more readily penetrates the cell membranes and is more resistant to degradation. Their objective was to craft stapled RpoN helices capable of penetrating Gram-negative bacteria. Once inside the bacterial cell, these peptides would bind to the σ^{54} promoter, preventing σ^{54} from interacting with its target DNA sequence. This interference would result in reduced transcription and deactivation of genes that depend on σ^{54}, ultimately decreasing bacterial virulence [75].

The implications of this study extend far beyond its immediate results. σ^{54} is linked to virulence of a multitude of pathogenic bacteria, including species such as *P. aeruginosa* and *V. cholerae*. By inhibiting the σ^{54}-DNA promoter interaction, the research team was able to suppress the activation of σ^{54}-dependent genes, presenting a novel strategy to combat bacterial pathogens and possibly sidestep the challenge of therapeutic drug resistance. Furthermore, the study underscores the therapeutic potential of hydrocarbon stapled peptides. These peptides not only breach the robust defenses of Gram-negative bacteria but also target specific intracellular interactions with precision. The research, thus, sets a guide for using such peptides to modulate bacterial TF, particularly in pathogens where σ^{54} regulates virulence properties [75].

Virstatin as a modulator of ToxT dimerization and cholera toxin expression

The *V. cholerae* transcriptional activator ToxT has been shown to be critical for production of cholera toxin [36]. The expression of ToxT mutants within the O395 strain has underscored the critical role of the N-terminal domain, particularly amino acids 6–9, in facilitating dimerization. This dimerization of ToxT is essential for the subsequent expression

of cholera toxin, implicating the N-terminal domain as a potential target for therapeutic intervention. The introduction of virstatin, which inhibits ToxT dimerization, has been shown to effectively reduce homodimer formation at concentrations starting as low as 10 μM. Notably, a point mutation (L113P) in ToxT confers resistance to virstatin, underscoring the specificity of the inhibitory interaction and suggesting a conformational basis for the activation and inhibition of ToxT [76].

Employing a bacterial two-hybrid system to assess the direct impact of virstatin on ToxT dimerization provided concrete evidence of its inhibitory action. Confirming these findings, gel filtration chromatography illustrated that ToxT is predominantly monomeric in the presence of virstatin, demonstrating the ability of virstatin to prevent the dimerization that is observed in its absence. Analysis of virstatin structural analogs illuminated the relationship between the compound inhibitory potential and its molecular structure, offering insights into the molecular mechanisms underpinning ToxT inhibition and paving the way for the development of novel antimicrobial agents.

Furthermore, the effect of virstatin on various ToxT-regulated promoters was found to be heterogeneous. Promoters such as *ctxAB* and *tcpA* were particularly sensitive to virstatin, while others showed a lesser degree of repression. This indicates a differential requirement for ToxT dimerization across its regulon, suggesting a nuanced role for ToxT in the regulation of virulence factors. The elucidation of virstatin's mechanism of action in preventing ToxT dimerization expands our understanding of pathogenic strategies, and it suggests that targeting crucial protein-protein interactions can substantially attenuate virulence. These findings suggest that the inhibition of ToxT by virstatin represents a strategic departure from traditional antimicrobial approaches, offering a promising target for novel drug discovery and a potential avenue to circumvent the issue of antibiotic resistance.

FPSS as an inhibitor of σ^B in L. monocytogenes *and* B. subtilis

In a pivotal study aimed at undermining the virulence of *L. monocytogenes*, scientists have unveiled a small molecule, FPSS (Fluoro-Phenyl-Styrene-Sulfonamide), which selectively blocks the activity of σ^B, a stress response sigma factor integral to the pathogen's ability to cause disease [81]. The discovery of FPSS emerged from an extensive high-throughput screen of approximately 57,000 small molecules. FPSS is a potent inhibitor of σ^B-mediated transcription, as evidenced by qRT-PCR and microarray analyses of gene expression. When tested against human enterocytes, FPSS significantly impeded the invasion of *L. monocytogenes* at concentrations of 8 μM and 64 μM, with reductions of 1.50 log and 1.42 log, respectively, illustrating its therapeutic potential.

The inhibitory activity of FPSS is not exclusive to *L. monocytogenes*, it also extends to *B. subtilis*, indicating its cross-genera effectiveness [84]. The mechanism by which FPSS inhibits σ^B-dependent transcription appears to be by preventing release of σ^B from its anti-sigma factor RsbW. The specificity of FPSS is particularly noteworthy, as it limits σ^B activity without disrupting the housekeeping sigma factor σ^A-dependent processes, underscoring its targeted action and implying a reduced likelihood of collateral damage in microbial communities. FPSS heralds a novel antimicrobial paradigm, selectively inhibiting σ^B-regulated virulence genes in *L. monocytogenes* and suggesting a path to novel therapies for listeriosis and other Gram-positive infections. Its specificity and potent action against key pathogenic processes holds promise for addressing the urgent need for new antimicrobial strategies with minimal impact on beneficial microbiota and low tendency for resistance development.

Inhibition of shigella flexneri *virulence by the small-molecule inhibitor SE-1*

A search for innovative antimicrobial therapies identified the small molecule inhibitor SE-1 as a potent antagonist of *S. flexneri* virulence [60]. SE-1 specifically targets VirF, a transcriptional regulator that is pivotal in the activation of a cascade of genes essential for bacterial invasion and survival within host cells. Through qRT-PCR analyses, it was established that SE-1 exerts a dose-dependent suppression on the expression of VirF-dependent virulence genes. Notably, at higher concentrations, SE-1 significantly diminished the

ability of *Shigella* to invade mouse fibroblast L-929 cells, a standard model for studying bacterial pathogenicity. This inhibition was achieved without imparting any detectable toxic effects on host cell viability or bacterial growth, suggesting a mode of action that is remarkably selective for the virulence mechanisms of the pathogen.

Surprisingly, the mechanism by which SE-1 applies its inhibitory role appears to be through direct interference with the DNA-binding activity of VirF, a hypothesis supported by thermal shift assays among other experimental approaches. This specificity not only underscores the potential of SE-1 to serve as a novel anti-virulence therapeutic agent but also suggests a lower risk for the development of drug resistance due to its targeted action. However, the efficacy of SE-1 as a therapeutic agent is dependent on further optimization to enhance its potency and selectivity; as noted above, SE-1 was originally identified as an inhibitor of a different member of the AraC/XylS family [59]. Additionally, the capacity of SE-1 to penetrate host cells is unclear, suggesting that an open question for future research includes the development of analogs, which can enter host cells. The implications of this study are significant, offering a glimpse into the future of antimicrobial drug design where mitigating virulence rather than killing the pathogen outright could reduce the pressure for the development of antibiotic resistance.

The subsequent section will highlight the challenges associated with manipulating bacterial TFs.

Challenges in targeting bacterial TFs

Identifying and validating targets

One of the primary challenges in targeting bacterial TFs lies in the precise identification and laborious validation of these proteins. Structural insights, such as those derived from X-ray crystallography, have deepened our structural knowledge of TFs. However, the dynamic nature of many TFs raises issues for drug design. The ever-changing conformations in vivo suggest that static models may be insufficient, highlighting the need for dynamic techniques to represent these shifts accurately. This is of particular concern for eukaryotic TFs, though, which are prone to harboring regions of intrinsic disorder [85]. By comparison, bacterial TFs frequently comprise a well-defined wHTH DNA-binding domain to which an equally well-defined effector domain is connected by a flexible linker. We therefore suggest that bacterial TFs can be excellent targets, even if the specifics related to ligand-induced conformational changes are incompletely understood. Once the structure is mapped, the focus moves to the optimal intervention areas. While ligand-binding pockets are the most common regions to target, not all TFs possess such well-defined pockets. This particularly pertains for two-component regulators, sigma factors, and NAPs. This variability means that in some cases, traditional binding-site-focused interventions may not be feasible. This demands the identification of alternative target regions, such as allosteric sites or protein-protein interaction interfaces [86]. The temporal activity of TFs adds another layer of complexity. Their activity is influenced by bacterial growth and environmental stimuli, which means that interventions may only be efficacious at specific points in the gene expression cycle. Factors such as slow bacterial doubling times or entry into stationary phase or dormancy may necessitate prolonged periods of treatment. Beyond theoretical interest, selected TFs must demonstrate genuine therapeutic potential. This underscores the need for rigorous validation, utilizing methods ranging from genetic manipulations to advanced proteomics.

Ensuring drug stability and effective delivery

The path to the successful employment of drugs targeting bacterial TFs extends beyond mere TF identification. It involves challenges tied to the stability, penetration, and effective delivery of these therapeutic molecules. Stability is paramount, as a perfectly designed drug might weaken if it cannot resist the diverse environments within biological systems. The pharmacokinetics of a potential therapeutic agent demands its resilience against different physiological conditions, ranging from the varied pH landscapes to enzymatic degradation mechanisms in both the bacterial and host

cells [87]. Penetration of the bacterial cell membrane is also complex due to the robust defensive structures of bacterial cells. For instance, the sophisticated cell walls of Gram-negative bacteria present difficult penetration challenges. Designing molecules capable of navigating through these barriers, while escaping mechanisms such as efflux pumps, is vital [88]. The actual delivery of the drug to the site of infection presents another layer of complexity. Molecules need to be transported to their destinations without premature metabolism or elimination, ensuring optimal bioavailability. Advanced delivery systems, such as liposomal platforms and nanoparticles, hint at the promising future of precision drug delivery [89].

Functional redundancies and overlapping roles of TFs

A notable concern stems from potential functional redundancies and overlapping roles of TFs. Bacteria are exquisitely able to adapt to changing conditions, a characteristic facilitated by the existence of redundancy. This may arise, for example, if one protein can substitute for another or if an essential metabolite can be synthesized by multiple pathways or acquired from the environment. In either scenario, elimination of one option may have little effect on bacterial fitness [90]. Hence, designing interventions requires a panoramic view of the bacterial TF landscape to anticipate and counter these built-in redundancies.

Orthologous TFs, encoded by homologous genes, which derive from a common ancestor, may exist in bacterial species that are related to the intended target bacterium. Such orthologous TFs may or may not conserve function. In addition, paralogs may exist within the target bacterial species, representing genes that arose from a duplication event. Such shared lineages pose a dual challenge: ensuring specificity in targeting while avoiding unintended collateral damage to closely related proteins [91]. Beyond redundancies and homology, the crosstalk between TFs introduces another layer of complexity. In some bacterial systems, TFs can influence the activity or expression of others, either enhancing or diminishing their effects. Anticipating these communication networks is crucial to ensure that interventions do not unintentionally amplify an undesired outcome or reduce a desired one [92].

Host-related implications

When considering the host's physiological and immunological context, a distinct and complex set of challenges arises. One is precision in targeting. While bacterial TFs are the intended target, the potential for off-target interactions with host TFs or other cellular proteins cannot be overlooked. Such unintended interactions can cause a range of side effects, emphasizing the importance of perfect specificity in drug design. The host microbiome is likewise vulnerable. This rich assembly of microorganisms, many of which are beneficial, plays pivotal roles in various physiological processes. Precise targeting becomes even more critical here, as interventions could disrupt this microbial balance. The loss or impairment of commensal bacteria could have cascading effects on vital processes like digestion and overall immune health [93]. Finally, introducing drugs targeting bacterial TFs might unintentionally trigger or modulate the host's immune response. It is essential to test how these interventions interact with the immune system, ensuring that they do not stimulate inflammatory reactions or compromise the body's natural defenses. This interplay can be complex, given that the immune system has evolved to recognize and respond to bacterial components. Any therapeutic strategy must, therefore, be designed with an understanding of these potential immune interactions in mind [94].

Bacterial resistance

Bacteria are adept at developing resistance to antibiotics or other agents. Their high mutation rates can lead to alterations in proteins targeted by specific interventions, ultimately diminishing drug efficacy. The selective advantage these mutations confer in the presence of a therapeutic agent drives such mutations. This adaptability ensures that even the most targeted interventions might only offer a temporary solution [95]. Bacteria have also perfected communal survival strategies. Horizontal gene transfer stands out as a potent

tool, allowing resistance genes to circulate within bacterial communities. This communal sharing ensures that even bacteria not initially exposed to a drug can acquire resistance, posing challenges for long-term therapeutic strategies [96]. Lastly, even if a drug effectively targets a TF, bacteria may evolve or activate alternative pathways to mitigate the impact. This versatility means we may need treatments that can address multiple possible bacterial responses at the same time [97].

Combination therapies have long been implemented in the treatment of multidrug resistant bacterial infections. An underlying concept may be for a combination of two or more antibiotics targeting different steps in a particular pathway, as exemplified by sulfamethoxazole and trimethoprim. These drugs both inhibit steps in the generation of the critical one-carbon donor methylene-tetrahydrofolate, in turn compromising nucleic acid and amino acid biosynthesis [98]. Alternatively, completely different targets may be inhibited simultaneously, or a single target may be inhibited by more than one mechanism. A different approach is to combine an antibiotic with a compound, which enhances the activity of the antibiotic – a so-called adjuvant. An example of the latter is to combine a β-lactam antibiotic with a β-lactamase inhibitor to generate the antibiotic augmentin. Compared to monotherapy, combination therapies may lower the risk of developing resistance and potentially reduce the duration of therapy. Conversely, combination therapies must be carefully evaluated to guard against drug-drug interactions [99]. The advent of Artificial Intelligence may furnish an avenue toward developing models of the infection environment and predictions for drug combinations, which may prove superior to monotherapy.

Artificial Intelligence (AI)

AI is considered a fundamental cornerstone in contemporary scientific advancements. Tracing its roots back to the mid-twentieth century, AI establishment was marked by the visionary ideas of pioneers such as Alan Turing [100]. At its essence, AI combines sophisticated computational algorithms with advanced statistical methods, aiming to imitate human cognitive functions such as learning, reasoning, and problem-solving. AI systems are characterized by their ability to adapt and learn directly from datasets, in contrast to

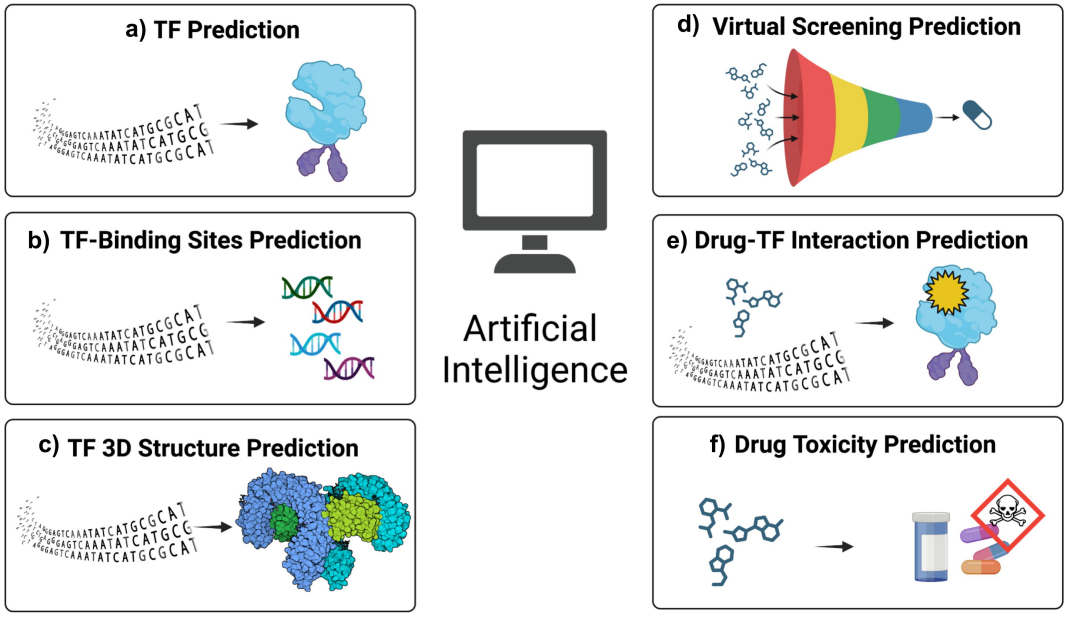

Figure 4. Applications of AI in targeting bacterial TFs. a) prediction of bacterial TFs using genome sequences. b) prediction of binding sites within bacterial genome. c) TF structure prediction using algorithms such as AlphaFold. d) virtual screening to identify possible inhibitors or activators of TFs. e) prediction of drug-TF interaction. f) drug toxicity prediction to assess the safety profile of the identified compounds. Created with BioRender.com.

traditional computational models, which are constrained by specific predetermined instructions. Through iterative refinement, AI systems adjust parameters to minimize errors, enabling them to detect complex patterns and relationships within vast datasets [101]. In the biology domain, AI has emerged as a transformative tool, reshaping how researchers approach complex biological systems and phenomena. Specifically, machine learning, a specialized branch of AI, serves as the main platform for many of these advanced applications. Recent comprehensive reviews have been published, elaborating on the extensive applications and significant impact of AI across various subdisciplines within biology, offering in-depth analyses for readers interested in the field [102–104]

Our next focus will be on examining selected research studies that demonstrate how AI technologies are effectively addressing the challenges associated with therapeutic targeting of bacterial TFs (Figure 4).

AI in target identification

ate identification of TFs and their respective binding sites. Traditional approaches to solve this problem are often labor-intensive, requiring extensive laboratory experiments that can take a considerable amount of time to yield reliable result. DeepTFactor is a pioneering deep learning-based tool for the prediction of TFs using protein sequences as input. This tool uses a CNN (convolutional neural networks) architecture to extract hidden features from protein sequences and categorize them as either TFs or non-TFs. In practical applications, DeepTFactor was used to predict 332 TFs in *E. coli* K-12 MG1655, one of the most studied bacteria, but for which approximately 35% of the genes are still poorly annotated. Of these, 80 were from a set of genes with previously unknown functions. The study went further to experimentally validate three of these predicted TFs [105]. In a separate study, DeepGRN was developed to exploit deep learning with attention mechanisms to predict TFs binding sites across cell types. These attention mechanisms allow the model to focus on specific, informative regions within the vast genomic data, similar to how human attention selectively concentrates on portions of information while filtering out the rest. The tool efficacy was tested using the ENCODE-DREAM in vivo Transcription Factor Binding Site Prediction Challenge datasets, a well-regarded benchmark in the field. Remarkably, DeepGRN outperformed the best four methods in the challenge achieving higher overall scores [106].

AI in structure prediction

Understanding the 3D structure of bacterial TFs is vital for drug design, providing crucial insights into how these proteins interact with other molecules and regulate gene expression. Addressing this demand, AlphaFold is a deep learning-based system that sets a new standard for protein structure prediction. At the core of AlphaFold is its innovative architecture, the Evoformer, designed to process both individual amino acids and their pairwise interactions in the protein sequence. This enables the model to capture complex relationships and dependencies among amino acids. AlphaFold model also incorporates Multiple Sequence Alignments (MSAs) and, where available, 3D templates, enhancing its predictive accuracy. The algorithm has proven itself in the Critical Assessment of Structure Prediction CASP14 benchmark, a highly regarded competition that evaluates the accuracy of protein structure prediction methods globally [107].

The AlphaFold model employs a unique loss function that measures the accuracy of its 3D atom position predictions against the true protein structure, thus guiding its training process. Additionally, the model uses evolutionary data in the form of MSAs to understand the constraints and contexts that shape protein structures. However, the AlphaFold performance can diminish when MSAs are scarce or when proteins have fewer intra-chain contacts. Despite these limitations, the ability of AlphaFold to make highly accurate protein structure predictions within minutes to hours marks a significant advance in the fields of structural biology and drug design [107].

AI in drug-target interaction (DTI)

Computational drug discovery was recently enhanced by DeepConv-DTI, a dual-input deep learning model specifically designed for predicting DTIs. The uniqueness of this model lies in its dual-input architecture, which accommodates both amino acid sequences of proteins and molecular fingerprints of drugs. For the protein sequences, the authors employ a CNN as a feature extraction mechanism. This choice is particularly insightful as CNNs excel in capturing localized patterns in a sequence, thereby enabling the model to identify critical amino acid residues that facilitate drug binding. What sets this study apart is the rigorous validation process in which a comprehensive training dataset was compiled by curating information from databases such as DrugBank, KEGG, and IUPHAR. To further ensure the validity of their model, external validation was conducted using different datasets sourced from MATADOR, PubChem BioAssay, and ChEMBL KinaseSARfari. Despite the heterogeneity and class imbalance inherent in these datasets, a recurring obstacle in DTI prediction, the model exhibited remarkable predictive performance. This high degree of accuracy emphasizes the model capability to generalize across varying data conditions, an attribute pivotal for its applicability in real-world drug discovery scenarios [108].

AI in toxicity prediction

In a recent study, Sharma et al. advanced multi-task deep learning frameworks to refine both the accuracy and interpretability of clinical toxicity predictions. Multi-task deep learning frameworks are designed to handle multiple related tasks simultaneously, thereby enhancing the model ability to generalize and make accurate predictions. The study tackled two crucial concerns: first, improving the reliability of toxicity predictions across various experimental settings, from lab-based to animal studies and human clinical trials. Second, they developed machine learning models that make accurate predictions and provide explanations for those predictions, an essential feature for medical professionals and researchers. This methodology not only improves the accuracy of clinical toxicity predictions, but it also addresses ethical concerns by potentially reducing the need for animal testing. Future research could focus on refining these machine learning models to predict additional types of toxicity or to incorporate more complex molecular features for even greater predictive accuracy [109].

AI limitations

One of the most significant critiques of AI, especially deep learning models, is their opaque nature. These algorithms often act as a "black box", offering high predictive power but little to no model transparency. This is particularly concerning when identifying bacterial TFs for therapeutic targeting, where the biological rationale for selecting specific targets is as critical as the prediction itself. Without this transparency, there is a potential risk of missing critical biological insights that could direct better drug design and validation [110]. AI models are intrinsically data-dependent, meaning the quality of the training data directly impacts the model performance. In the realm of bacterial TFs, the available data can be skewed or biased toward well-studied strains or specific conditions. Such biases can negatively impact the algorithm ability to accurately predict TFs in non-model bacteria that are underrepresented or not included in the training data [111].

The risk of overfitting is especially high in biological data, which is often high-dimensional. An overfitted model may achieve excellent performance on the training data but perform poorly on unseen or novel data. This is a critical issue when the goal is to identify novel bacterial TFs that could serve as effective drug targets [112]. The computational resources required for running complex AI models can be extensive. This is particularly relevant when targeting bacterial TFs, where iterative simulations are often essential for capturing their dynamic behavior. The computational cost thus becomes a significant barrier for institutions that may not have access to high-performance computing facilities. Irrespective of the level of sophistication of AI predictions, they remain hypothetical until experimentally validated [113]. Each of these limitations presents unique

challenges that researchers must consider when applying AI methods to the identification and analysis of bacterial TFs as therapeutic targets.

Interdisciplinary collaboration

Effectively targeting bacterial TFs necessitates a multidisciplinary scientific approach. No single discipline can hope to unravel the full complexity of the biological pathways, molecular mechanisms, and pharmacological variables involved. For instance, microbiologists may provide a detailed understanding of bacterial physiology and behavior, yet the full potential of the vast biological datasets may need to be unlocked by data scientists skilled in AI algorithms. Geneticists contribute by analyzing the role of specific genes and their regulatory mechanisms, but it is the systems biologists who model these interactions, providing a comprehensive understanding of their interplay. This interdisciplinary synergy is not a luxury, it is a requirement for generating insights that are both deep and broad [14]. Success in such collaborative efforts requires effective communication. Different disciplines often use the same terms differently. For example, the word "model" could mean a mathematical framework in data science, but in microbiology, it usually refers to a living organism used for experiments, such as a *E. coli*. Similarly, how research methods are chosen and valued can differ; a research physician might focus on clinical trials, while a data scientist might rely on AI to validate results. These differences in language and methods can cause confusion and slow down research. To solve this, regular meetings, a shared glossary, and agreed-upon research methods can be very helpful [114]. The journey from scientific research to real-world treatment involves more than just work in a research laboratory or on a computer server. The identification of a potential TF target and the development of a corresponding drug are milestones, not endpoints. Experts in clinical trials and regulatory affairs play a key role at this stage, guiding the complex processes of human trials and government approvals. Their work ensures that early discoveries turn into treatments that meet the highest standards for safety and effectiveness [115].

Policy and funding catalysts

The translation of research into actionable therapeutic solutions depends not only on scientific innovation but also on a robust policy and funding ecosystem. This environment serves as the scaffolding upon which interdisciplinary collaborations can flourish and ground-breaking technologies can be achieved. For instance, policy frameworks can play a pivotal role in promoting public-private partnerships, which are often essential for bringing cutting-edge technologies into practical use. Regulatory bodies, governmental agencies, and private sector organizations must work cohesively to support research, enacting policies that encourage commercial investment in long-term, high-risk research areas like bacterial TF targeting. Such partnerships can accelerate the transfer of technology from the laboratory to the marketplace, thereby accelerating the availability of new therapies [116]. Tailored research grants, particularly those focusing on interdisciplinary projects, can also serve as a significant catalyst. Funding agencies should recognize the complex nature of targeting bacterial TFs and allocate resources to encourage the integration of AI, molecular biology, pharmacology, and other related fields. Special funding categories may be created to support projects that combine these diverse areas, ensuring that the research is as comprehensive as it is innovative [115]. Lastly, as AI technologies become more integrated into the targeting of bacterial TFs, policies must also address the ethical implications, such as data privacy and model interpretability. Regulatory guidelines should be established to ensure that AI algorithms used in this domain meet strict standards of reproducibility and ethical conduct [117].

Conclusions

Antibiotics continue to be of paramount importance in the treatment of bacterial infections. However, the ability of bacteria to develop resistance, likely fueled by overuse of conventional antibiotics, threatens to undermine the efficacy of existing treatments. Further exacerbating this problem is the reality that the discovery of new antibiotics has slowed [118]. Substantial changes in

approach and policy are therefore urgently needed. TFs, in general, are not essential proteins, and they do not necessarily feature the defined small-molecule binding pockets traditionally thought to be required for drug targeting. However, we suggest that these features should be embraced as a potential advantage; targeting a bacterial TF responsible for regulating virulence genes, as opposed to a protein essential for growth, mitigates the incentive for induced mutations to develop resistance. We are also encouraged by the implementation of powerful AI technologies to assist in the design of new intervention strategies and look forward to witnessing the development of a new generation of antibiotics.

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