Bacterial type VI secretion system augments resistance against biotic and abiotic stress

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Abstract In their natural environment, bacteria employ various mechanisms to survive and protect themselves from neighboring microorganisms. One of the most noteworthy features found in a minority approximately 25% of Gram-negative species, predominantly those inhabiting complex, competitive environment is the type VI secretion system (T6SS). T6SS gene clusters are exclusive to Gram-negative bacteria, with the number of clusters varying from one to six per organism. Bacteria utilize the T6SS to deliver effectors responsible for eliminating their competitors, using a lancet-like apparatus to transport materials outside the cells, ensuring survival in a stressful environment, and subverting host cells. Since its discovery in 2006, research on the T6SS — encompassing biochemical, structural, and molecular studies — has led to the identification and characterization of its assembly, loading, firing, and disassembly processes. Ongoing research, coupled with bioinformatics approaches, is expanding our knowledge of new T6SS effectors and their diverse functionality, shedding light on previously unexplored aspects of prokaryotic and eukaryotic organism biology. The present review focuses on recent discoveries related to T6SS effectors, their mechanisms, and regulation, enhancing our understanding of this combative system.

Keywords: Effectors, Regulation, Secretion, Signals, T6SS

1. Introduction

The T6SS serves as a molecular weapon employed by Gram-negative bacteria and various other microbial strains inhabiting both marine and terrestrial habitats. Presence of T6SS in the bacteria equips them to translocate effector proteins to the extracellular environment through a contact-dependent manner [1,2]. The T6SS-mediated delivery of effector proteins often leads to cellular damage within polymicrobial environments. Such intercellular interactions can facilitate ecological cooperation and competition, subsequently influencing the local composition of bacterial populations and consortia [3]. The remarkable diversity of bacterial species in these environments underscores the intricacy of the regulatory signals which govern the expression and activation of their T6SS. Additionally, within such diverse environments, bacteria have the capability to switch between planktonic and biofilm states, adding a diverse system to control gene expression [4]. T6SS resembles a molecular nanomachine, sharing structural and mechanistic similarities to T4 bacteriophage tail. It comprises several structural proteins that come together to form three primary complexes: the membrane, baseplate, and tail [5]. While bioinformatic surveys of available microbial genomic resources indicate that T6SS clusters are present in less than 25% of Gram-negative bacteria, this system is remarkably prevalent among Proteobacteria that thrive in polymicrobial consortia. The apparent discrepancy between its limited overall distribution and the vast diversity of T6SS-utilizing genera described in literature arises from the system's critical role in niche competition; it is a defining feature of highly adaptive genera such as *Vibrio, Pseudomonas,* and *Agrobacterium* [1].

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The T6SS membrane complex is constituted by several proteins including TssJ, TssL and TssM, whereas the baseplate consists of TssE, TssF, TssG and TssK [5]. The sheath complex composed of TssB and TssC is involved in the contraction [6], resulting in the ejection of an inner tube comprised of Hcp hexamers [6]. The inner tube tip is composed of a VgrG-trimer which is covered by a PAAR domain. TssA serves as a cap protein responsible for tail assembly, whereas ClpV acts as an ATPase, providing the energy required for the contraction and firing events [5,6]. The T6SS-associated effectors are usually secreted as a C-terminal domain along with the associated structural components Hcp, VgrG and PAAR, referred to as evolved or specialized effectors [7], or as a cargo effector either with or without the assistance of an adaptor protein. These effectors confer various cellular processes in their target prey cells [8] and may also encode antibacterial toxins decorated with cognate immunity proteins within the same operon. This immunity protein serves to shield the bacteria from self-intoxication and protect the sister cells [7].

The presence of T6SS contributes to bacterial fitness via (i) secretion of antibacterial toxins against their competitors, and (ii) through uptake of scarce metal ions [9,10]. In some cases, T6SS apparatuses spontaneously assemble and contract within the cell, releasing toxic effectors against neighboring cells, even in the absence of prior aggression from the surroundings [11]. However, defensive T6SS systems only release toxic effectors after recognizing a previous attack, signaled to trigger a counterattack. This type of activation is also facilitated by the activity of another T6SS, effectors with phospholipase activity, membrane damage, and chemical substances including antibiotics, DNA, and chelators [12]. Some bacteria possess multiple T6SS systems in their genomes, and some T6SS have dual functions, acting as antibacterial toxins or serving as both antibacterial and antieukaryotic systems [13].

2. T6SS Regulation via Abiotic and Biotic Signals

Many of the T6SS gene clusters are found in the vicinity of pathogenicity islands (PAIs) that are usually acquired by horizontal gene transfer (HGT) events. PAIs encode diverse virulence factors, represent distinct genetic elements in diverse bacterial pathogens, and seem to be absent from non-pathogenic bacteria. The integration of these PAIs into pre-existing regulatory networks confers additional advantages to the bacteria. Although PAIs are usually acquired by HGT, only few PAIs carry the mobilization and transfer genes facilitating their transmission by HGT. Moreover, previous studies have demonstrated that regulation of T6SSs is governed at three levels including transcriptional, post-transcriptional and post-translational [14]. At the transcriptional level, regulation is controlled by various factors including two-component signal transduction systems (TCS), alternative sigma factors, histone-like proteins, and quorum sensing (QS) systems. The presence of TCS equips the bacteria to sense and monitor their environment. The histidine kinase primarily senses environmental signals and the response regulator mediates a cellular response. Under certain environmental stimuli, the HK catalyzes an ATP-dependent autophosphorylation which facilitates the transfer of a phosphoryl group, thereby facilitating conformational dynamics and the cellular response. Overall, these processes equip the TCSs in metabolic adaptation, virulence, and response to stressors like osmolarity, pH, bile salts, and oxygen availability.

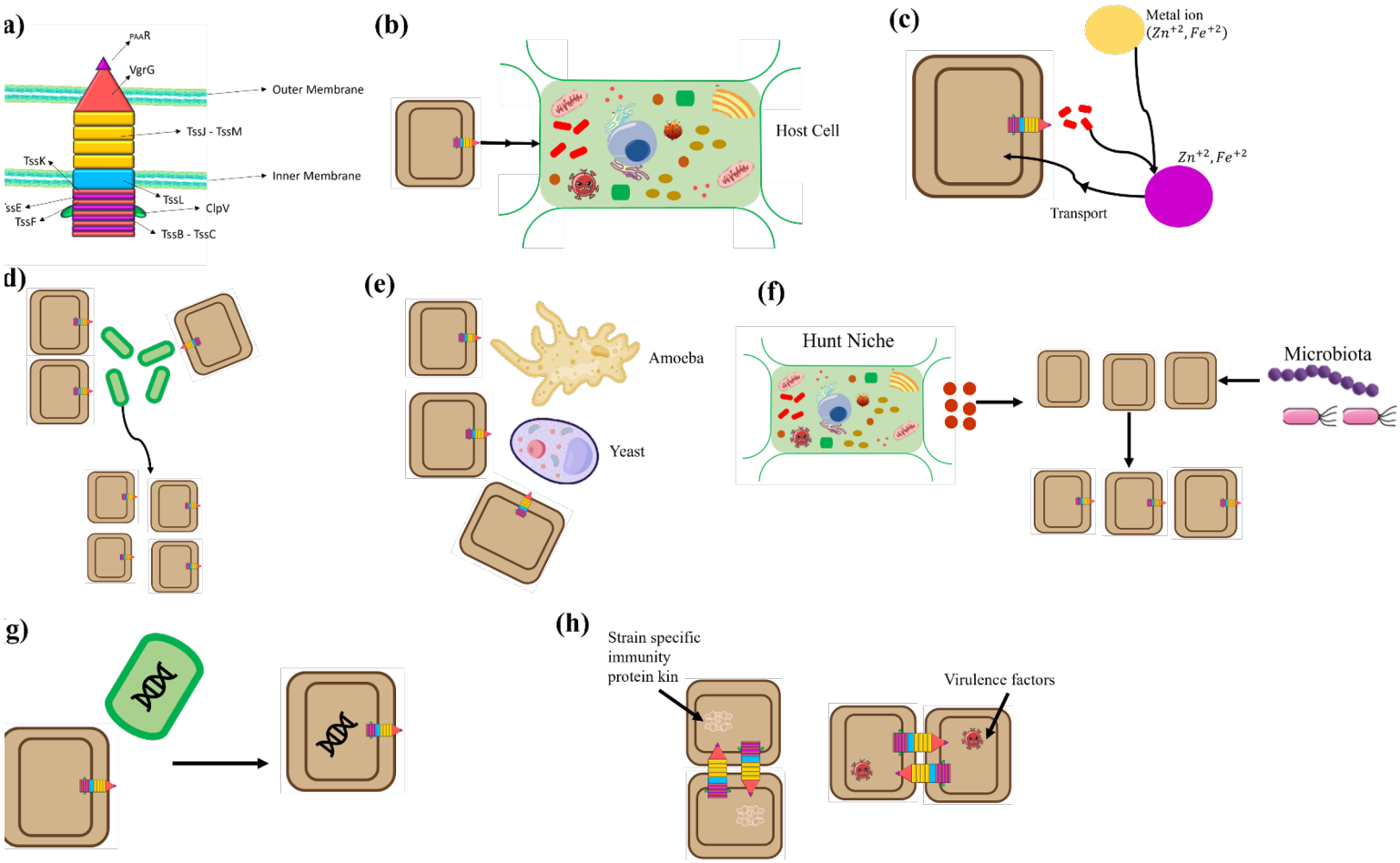
On the other hand, at the post-transcriptional level, the regulation involves RNA-binding proteins (RBPs) and small regulatory RNAs (srRNAs) which affect the processing of mRNAs and RNase-mediated degradation. However, RBPs may sometimes inhibit RNases, which leads to stabilization of mRNA resulting in increased protein production. Similarly, post-translational regulation relies on phosphorylation events which regulate the structural proteins involved in T6SS assembly. In addition to these regulatory mechanisms, T6SSs are subject to modulation by various abiotic factors including temperature, pH, oxygen levels, and heavy metals. Furthermore, biotic signals including quorum sensing molecules, antibiotics, host-derived chitin, and toxic effectors also play a role in modulating T6SS expression. Fig. 1a–h describes the activation of T6SS and its multifarious action.

T6SS gene cluster of *Vibrio cholerae* O1 was observed to be repressed at low osmolarity. However, evidence has shown that at high osmolarity the expression of T6SS was upregulated, which leads to the secretion of Hcp [15]. Considering another example, bacterium *V. cholerae* O1 did not show antibacterial activity at lower temperature of 15 °C; however, killing was observed following increase in temperature, ranging from 25 to 37 °C [16]. In the case of *V. parahaemolyticus*, T6SS1 activity was induced under warm marine-like conditions [17]. Conversely, in *V. fischeri*, T6SS2 was found to be upregulated in environments with high viscosity and acidic pH [18]. The bacterium *Klebsiella pneumoniae* upregulates its antibacterial T6SS under high salt concentrations and reduced pH [19]. Similarly, *A. tumefaciens* upregulates its T6SS in an acidic medium (pH 5.5), a condition found at plant wound sites [20].

Among various abiotic factors, the presence of oxygen and ROS synergistically generates environmental signals that trigger the regulation of the T6SS. Moreover, the activity of transcriptional regulators belonging to the Fnr family such as ‘Anr’ is directly influenced by the availability of oxygen [21]. Under oxygen-depleted conditions, Anr dimerizes and binds to the promoter regions, thereby controlling transcription events. Conversely, Anr also induces the antibacterial and exploitative H2-T6SS in *Pseudomonas aeruginosa* under anaerobic conditions. Anr is also involved in the expression of the Fnr-family regulator ‘Dnr’ which promotes H2-T6SS expression [22]. On the other hand, the response regulator ‘OxyR’ becomes oxidized in the presence of H_2O_2, leading to the activation of gene expression, which upregulates the exploitative T6SS-4 of *Burkholderia thailandensis* [10]. Additionally, in response to ROS effects, T6SS-4 is induced to secrete the Mn^{2+}-binding effector protein TseM. The secreted effector TseM is involved in the binding of extracellular Mn^{2+}, which further enhances the Mn^{2+}-specific outer membrane transporter ‘MnoT’ to facilitate the transport of Mn^{2+} back to the cell [10]. Iron transcriptional regulator ‘Fur’ binds to Fur boxes in the promoter regions to control gene transcription in the presence and absence of iron [23]. Fur was shown

to repress T6SS activity in various bacterial species, including *Salmonella typhimurium* [24], *P. aeruginosa*, enteroaggregative *E. coli*, avian pathogenic *E. coli*, and *Cupriavidus necator* [25].

Another transcriptional regulator, the copper-export regulator 'CueR', modulates the antibacterial activity by repressing the H2-T6SS of *P. aeruginosa* in the presence of copper. Under low Cu^{2+} concentrations, the upregulated H2-T6SS secretes the copper-binding effector 'Azu' extracellularly to chelate the Cu^{2+}, which is subsequently internalized via OprC [26]. On the other hand, low levels of phosphate also induce the H2- and H3-T6SS in *P. aeruginosa* [27]. In certain cases, salts and mucin secretions also regulate T6SS activity, especially in *S. typhimurium* and *V. cholerae* [28], whereas chitin is involved in the upregulation of the T6SS gene cluster of *V. cholerae* associated with aquatic animals [29]. Among other factors, QS acts as a bacterial communication mechanism to regulate a variety of behaviors through controlled gene expression. The regulation of T6SS expression via QS has been demonstrated in numerous bacteria, such as *Aeromonas hydrophila* [30], *Enterobacter*



cloacae [31], *P. aeruginosa* [32], and *Chromobacterium violaceum* [33]. The antibiotic polymyxin B induces membrane damage in *E. coli*, while on the other hand, it activates the expression of T6SS. Similarly, the aminoglycoside antibiotic kanamycin activates T6SS expression in *P. aeruginosa* [34]. Fig. 2 demonstrates a general overview of the actions of eukaryotic T6SS effectors.

Fig. 1. The schematic representation of T6SS apparatus and its multifarious action.

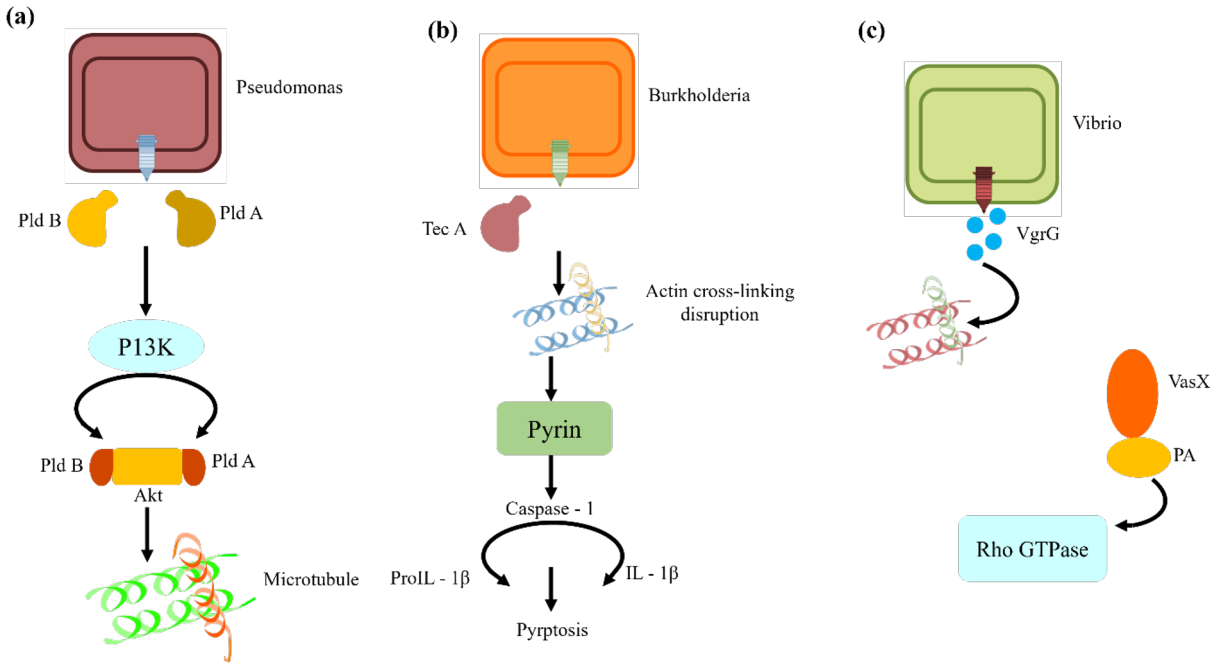


Fig. 2. A schematic demonstration of the role of some anti-eukaryotic effectors.

3. Transcriptional Regulation

At the transcriptional level, T6SS regulation is governed by the coordinated action of transcriptional complexes (TCSs) including (i) sensor histidine-kinase, (ii) a phosphorylation cascade system leading to activation of a cytoplasmic transcriptional regulator, (iii) sigma factors that govern the activity of RNA polymerases, (iv) transcriptional regulators that are controlled by specific ligands, and (v) histone-like nucleoid structuring proteins [14]. *P. aeruginosa* genome encodes three T6SS named H1, H2, and H3-T6SS that are involved in killing of competitor bacteria, ion acquisition, and virulence towards eukaryotic cells. The transcriptional regulation of *P. aeruginosa* H1, H2 and H3-T6SS as well as T6SSs of *P. fluorescens* is facilitated by the GacS/GacA system [13]. Another TCS regulator, FleS/FleR, induces the expression of the Arc-family regulator 'AmrZ' (alginate and motility regulator) [27], which directly modulates the expression of T6SS in *P. aeruginosa*.

In *P. aeruginosa* strain PA14, AmrZ induces the expression of H1- and H3-T6SS, while repressing the H2-T6SS. In contrast, in *P. aeruginosa* strain PAO1, H1-T6SS is repressed by AmrZ [27]. AmrZ-mediated repression involves c-di-GMP in association with FleQ to repress the expression of H1-T6SS. Similarly, c-di-GMP–FleQ in combination with FleN represses the K1-T6SS activity. Upon surface attachment, Wsp (wrinkly spreader phenotype) promotes c-di-GMP synthesis and T6SS repression via downstream phosphorylation of WspR. The FleQ–FleN complex also induces biofilm formation and transition between planktonic states in the presence of c-di-GMP [35].

The Enterobacteriaceae members possess an additional regulatory network known as Rcs (regulation of capsular polysaccharide synthesis). Various regulatory signals enhance the signaling network of the Rcs cascade, which modulate the expression of various genes. An outer membrane lipoprotein RcsF coordinates these signals, leading to phosphorylation of RcsC and RcsD, and the transcriptional regulator RcsB. In *Serratia marcescens*, T6SS is constitutively expressed and is used to eliminate non-aggressive competitors [36]. On the other hand, the Rcs system senses damage and responds by increasing T6SS activity. The induced gene expression facilitates the interaction of RcsB (cytoplasmic transcriptional regulator) with the RcsB-binding motif that is usually localized in the promoter region of the T6SS gene cluster [36]. Similarly, in extraintestinal pathogenic *E. coli*, antibiotic polymyxin B induces the response regulator RcsB, which controls T6SS activity [37].

In response to an environmental stimulus, high levels of c-di-GMP inhibit the T6SS activity of *A. tumefaciens*. Additionally, the transcriptional regulator ChvG/ChvI in *A. tumefaciens* facilitates the autophosphorylation of ChvG, subsequently activating the response regulator ChvI to induce T6SS expression [20]. In *K. pneumoniae*, the T6SS is regulated by PhoP/PhoQ, where PhoQ detects antimicrobial molecules, leading to the phosphorylation of the transcriptional regulator PhoP. The phosphorylated PhoP-P further activates the T6SS [19]. On the other hand, in *Burkholderia cenocepacia*, AtsR (adherence and T6SS regulator) represses its antibacterial T6SS-1 [38]. Similarly, in *V. cholerae* V52, regulator 'VxrA/VxrB' upregulates biofilm formation

and T6SS expression [39]. In the absence of chitin polymers, ChiS activates the T6SS gene expression. Moreover, in *V. cholerae* O1, regulator 'HapR' and the quorum-sensing intermediate regulator QstR also regulate T6SS expression [40]. Moreover, in the presence of chitin, TfoX upregulates the genes involved in competence in *V. cholerae*, enhancing target cell killing and DNA uptake to promote HGT events.

Many transcriptional regulators belonging to different families involved in T6SS activation remain unknown. These regulator families encode a ligand-binding domain that facilitates conformational changes and DNA binding. Such transcriptional regulators include IclR-family (isocitrate lyase regulator), Lrp-family (leucine-responsive regulatory protein), and the LysR-family. For instance, the IclR-family regulator 'OscR' represses the T6SS of *V. cholerae* O1 at low osmolarity [15]. In the case of the Lrp-family regulator 'TasR', high viscosity induces T6SS2 in *V. fischeri* [41]. Another regulator, 'TfoY', controls expression of T6SS1 under warm marine-like conditions in *V. parahaemolyticus*, whereas 'CalR' upregulates T6SS2. Additionally, the transcriptional regulator 'AggR' induces the expression of antibacterial T6SS-3 in enteroaggregative *E. coli* [41].

In some bacterial strains, T6SSs are regulated by non-chromosomal elements such as plasmids. The large conjugative plasmids (LCPs) in *Acinetobacter baumannii* encode regulators like 'TetR' (tetracycline repressor) constituting a DNA-binding domain that binds to signaling molecules to regulate T6SS expression. These TetR-like regulators suppress T6SS expression, thereby preventing bacterial killing. Other transcriptional regulators like sigma factors and enhancer-binding proteins (EBP) also control T6SS expression [14]. The EBP promotes ATP hydrolysis to induce alterations in RNA polymerase to promote transcription of T6SS genes. In *V. cholerae*, the first pair of sigma factor and EBP constitutes RpoN (σ54) and VasH [42]. VasH homologues control T6SS expression in *A. hydrophila* [41] and *V. fischeri* [43]. The EBP-interacting domain RpoN controls Hcp expression in *V. parahaemolyticus* under marine-like conditions [17]. Similarly, the EBP-interacting domain FleQ acts as a positive regulator of T6SS in *P. fluorescens*. RpoN-dependent control of T6SS expression has been demonstrated in *K. pneumoniae* [19], *Plesiomonas shigelloides*, *P. aeruginosa* [44], and *P. putida*.

Moreover, sigma factors regulate T6SS expression in *K. pneumoniae* [19], *Yersinia pseudotuberculosis* [45], and *P. plecoglossicida* [46]. Besides controlling T6SS expression, RpoN also controls motility, abiotic stress responses, and flagellar motility [47]. T6SS repression is also mediated by H-NS proteins, which control the expression of genes acquired by HGT events [48]. H-NS-mediated T6SS repression has been noted in *A. baumannii* [49], *V. parahaemolyticus* [17], and *S. typhimurium* [50]. Another study showed that the H-NS homologue MvaT represses the H2- and H3-T6SS in *P. aeruginosa*, whereas MvaT-paralogue 'TurA' represses T6SS in *P. putida* [51]. In *V. cholerae*, auxiliary T6SS clusters 1 and 2 are under the control of RpoN and VasH [47]. In *P. aeruginosa*, RpoN and Sfa2 (sigma factor activator 2) govern the upregulation of T6SS and its cognate orphan clusters. The effector TseT, secreted via the H2-T6SS of *P. aeruginosa* under high iron concentration, regulates the orphan effectors [52]. On the other hand, the secreted effector 'CccR' in *Y. pseudotuberculosis* performs as both a toxin and a transcriptional factor [53]. In some cases, CccR induces cell filamentation and growth arrest via regulating the cell division protein FtsZ, and also binds to the cccR promoter to act as a negative transcriptional regulator.

4. Post-Transcriptional Regulation

The T6SS post-transcriptional control is regulated by RBPs or srRNAs that bind to mRNAs to control their stability and translation [54]. The receptor of active C kinase 1 (RACK1) facilitates the recruitment of RBPs to the translational machinery. However, various signaling pathways involving two-component and quorum-sensing systems control these regulatory components. These systems have been well-studied in *P. aeruginosa* and *Vibrio* spp. The RBPs 'RsmA' and 'RsmN' regulate *P. aeruginosa* T6SSs [55]. The independent binding of these proteins to the 5' UTR of T6SS mRNAs blocks the ribosomal-binding sites (RBSs) and thereby prevents the translation processes [55]. Similarly, sRNAs like RsmZ and RsmY also control target mRNAs via binding to RsmA/N [116]. The regulation of RsmY/Z is governed by (i) GacS/GacA, (ii) RNase YbeY [56], (iii) the pyocin regulator PrtR, (iv) NrtR (Nudix-related transcriptional regulator), (v) RNA-binding protein RtcB, and (vi) AlgZ/AlgR (alginate biosynthesis). The regulation of histidine kinase GacS is governed by RetS, which alters GacS phosphorylation [57]; however, under external stimulus, LadS phosphorylates and activates GacS [57]. LadS phosphorylation also induces GacA to promote the expression of RsmY/Z, which induces the translation of T6SS mRNAs. PrtR inhibits GacS and GacA expression during the maturation stage of the biofilm. Similarly, YbeY (factor associated with ribosomal and sRNA maturation) controls the repression of RetS. The translation of T6SS transcripts is repressed by NrtR, which also regulates the T6SS gene clusters [58]. Similarly, the AlgR regulator activates the expression of RsmA through its histidine kinase AlgZ.

Following encounters with T6SS$^+$ competitors, the Gac/Rsm system enhances transcriptional and post-transcriptional activation of H1-T6SS [59]. Similarly, various genes related to biofilm formation or host infection are modulated by the Gac/Rsm pathway components. Another component, RetS–GacS–LadS, upregulates *P. aeruginosa* T6SSs. These regulatory pathways highlight the importance of T6SS in bacterial fitness. In *P. aeruginosa*, another component, PrtR responds to pathogen attack. Similarly, in *P. aeruginosa*, higher levels of c-di-GMP affect biofilm development as well as activation of the H1-T6SS [60]. However, decreased levels of c-di-GMP downregulate the T6SS.

In *V. cholerae*, LuxUO controls T6SS expression at the mRNA level by activating the regulatory sRNAs 'Qrr1–4'. The activated 'Qrr1–4' represses the expression of T6SS mRNAs. These processes also involve the master regulator 'HapR' to prevent T6SS transcription [61]. At low cell density, the phosphorylation of regulator LuxO is facilitated by kinases like LuxQ, CsqS, CqsR or VpsS, which finally phosphorylate the phosphotransfer protein 'LuxU' . However, at high cell density, kinases are not able to phosphorylate the signaling cascade, and thereby T6SS mRNAs are undisturbed. In *V. cholerae* O1, the cold-shock protein CspV maintains high levels of Hcp mRNAs and T6SS-mediated antibacterial activity; however, information about the interaction between CspV and T6SS mRNA transcripts is still lacking [16]. Csp proteins regulate bacterial attachment to zooplankton and also ensure correct transcription and translation under cold shock conditions [16]. In *V. cholerae*, c-di-GMP controls the T6SS transcriptional activator 'TfoY', which alters the mRNA secondary structure to prevent translation. However, low levels of c-di-GMP allow an increase in TfoY protein levels, which leads to T6SS expression [62].

5. Post-Translational Regulation

The T6SS regulation also relies on various post-translational events like acetylation, deamination, glycosylation, phosphorylation, carboxylation, and methylation that modulate protein levels and activity [63]. Among various processes, phosphorylation regulates the synthesis of T6SS structural components, including the membrane, baseplate, and tail [24]. In *P. aeruginosa*, threonine phosphorylation (TPP) is the key post-translational regulatory pathway, which is conserved in *S. marcescens* and *A. tumefaciens* [64]. In these bacteria, the signaling cascade is initiated by the membrane-associated kinase PpkA, which is involved in dimerization and autophosphorylation to promote T6SS assembly [24]. The control of the TPP pathway is overseen by TagQ and the periplasmic protein TagR. TagQ, in collaboration with TagR, catalyzes the phosphorylation of the ABC-transporter-like protein TagTS, ultimately resulting in the activation and autophosphorylation of PpkA. Subsequently, the activated PpkA interacts with the cytoplasmic protein Fha1, facilitating phosphorylation and triggering the assembly of the T6SS [65]. In certain bacterial species, PpkA assumes the role of phosphorylating additional T6SS structural components to regulate their expression. However, in *A. tumefaciens*, PpkA phosphorylation brings about changes in the T6SS membrane protein TssL, which plays a crucial role in the assembly of the TssM–TssL complex and its interaction with Hcp [66]. Furthermore, phosphorylated TssL also engages with Fha2 in *A. tumefaciens* to uphold T6SS activity. In another bacterium, *V. alginolyticus*, PpkA2 phosphorylates the T6SS component TssL, initiating Fha recruitment to the membrane complex, thus restoring T6SS activation. In *V. cholerae*, the Type VI accessory gene H (TagH) is essential for T6SS activity, as mutants lack the secretion of Hcp. However, it remains undetermined whether the FHA domain is phosphorylated or if it interacts with other T6SS structural components [67].

6. Conclusion

The diverse mechanisms governing T6SS regulation enable bacteria to control T6SS and associated components, allowing them to initiate either an attack or a counterattack in specific locations. While the expression of T6SS components is well regulated, their exact role in bacterial fitness remains to be fully established. It is worth noting that the controlled regulation primarily serves ecological purposes rather than being driven solely by energy costs. Furthermore, few works have been conducted in response to environmental signals, and less information is available regarding post-transcriptional and post-translational regulation of T6SSs. Additionally, the integration of inputs from multiple regulatory networks that govern T6SS poses a challenge when trying to identify the specific conditions and regulatory elements that control its expression.

Author Contributions

AS and RPS designed and wrote the manuscript. RPS supervised the whole work.

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Declarations

Conflict of Interest

The authors declare that they have no conflict of interest.

Ethical Approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Consent to Participate

None.

Consent for Publication

Not applicable.

Data Availability

Not applicable.

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