


# The Biological and Regulatory Role of Type VI Secretion System of *Klebsiella pneumoniae*

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**Abstract:** Bacteria communicate with their surroundings through diverse secretory systems, and the recently discovered Type VI Secretion System (T6SS) has gained significant attention. *Klebsiella pneumoniae* (*K. pneumoniae*), an opportunistic pathogen known for causing severe infections in both hospital and animal settings, possesses this intriguing T6SS. This system equips *K. pneumoniae* with a formidable armory of protein-based weaponry, enabling the delivery of toxins into neighboring cells, thus granting a substantial competitive advantage. Remarkably, the T6SS has also been associated with *K. pneumoniae*'s ability to form biofilms and acquire resistance against antibiotics. However, the precise effects of the T6SS on *K. pneumoniae*'s functions remain inadequately studied, despite research efforts to understand the intricacies of these mechanisms. This comprehensive review aims to provide an overview of the current knowledge regarding the biological functions and regulatory mechanisms of the T6SS in *K. pneumoniae*.

**Keywords:** *Klebsiella pneumoniae*, T6SS, competition, drug resistance, virulence

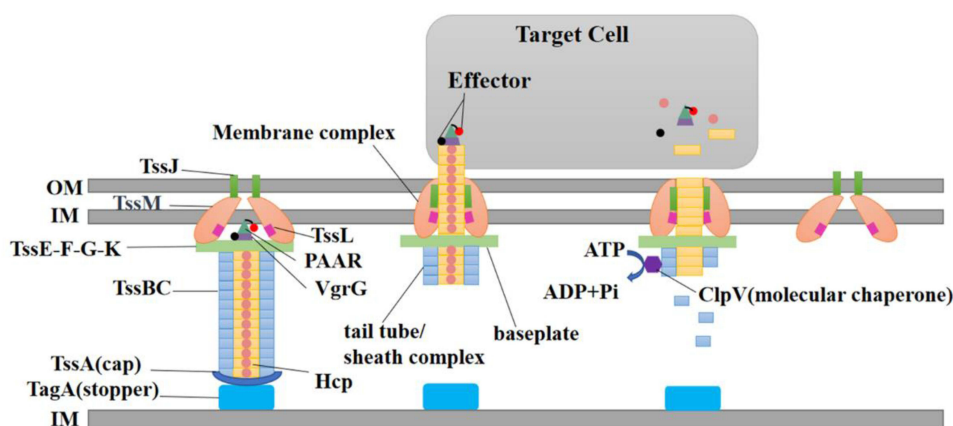
## Introduction

Bacteria inhabit densely populated communities and consistently engage in competition with other bacterial species in order to acquire essential nutrients and occupy adequate space. The bacterial secretory system is a structure present on the surface of bacterial cells that allows bacteria to exchange information with their surrounding environment to obtain nutrients and transport effector proteins. As a result, the secretory system regulates the interaction between bacteria and their hosts.<sup>1,2</sup> There are currently nine different secretion systems that have been identified in Gram-negative bacteria, ranging from type I (T1SS) to type IX (T9SS). The T7SS and T8SS predominantly play a role in the secretion of pili and fibers. The T9SS, on the other hand, is a recently discovered secretion system that has only been found in Bacteroides bacteria.<sup>3</sup> First identified in *V. cholerae* by Pukatzki et al in 2006, the Type VI Secretion System (T6SS) is involved in the secretion of hemolysin co-regulatory protein (Hcp) and valine-glycine repeat protein G (VgrG).<sup>4</sup> These secretions are associated with cytotoxic effects and require a cluster of intracellular multiplication protein F-related homologous genes.<sup>5,6</sup> The initial discovery of T6SS effectors revealed their anti eukaryotic properties, leading to the disruption of the target host cell's physiological processes.<sup>4</sup> However, significant breakthroughs and subsequent advancements in T6SS research have unveiled a predominant antibacterial activity among these effectors. Recent investigations have further expanded our understanding by revealing that certain effectors possess antifungal properties, thus broadening the traditionally defined role of T6SS from solely antibacterial to encompassing antimicrobial capabilities.<sup>7,8</sup> Beyond that, Lu et al designed antimicrobial peptides based on the bactericidal effect of T6SS effectors, which provided a new idea for the clinical treatment of multi-drug resistant bacteria.<sup>9</sup> T6SS can also facilitate symbiotic or reciprocal relationships between bacteria and hosts, and play cooperative or competitive roles in bacterial interactions.<sup>10–12</sup> Additionally, it has been demonstrated that the T6SS also serves as a general secretion system, facilitating the delivery of effector proteins into the extracellular environment to access shared resources, highlighting its versatility.<sup>13,14</sup>

*K. pneumoniae*, a Gram-negative opportunistic pathogenic bacterium belonging to the Enterobacteriaceae family, exhibits significant drug resistance. It ranks among the leading causes of nosocomial infections, encompassing conditions such as pneumonia, meningitis, liver abscesses, urinary tract infections (UTIs), wound infections, bacteremia, and sepsis.<sup>15,16</sup> Carbapenem-resistant *K. pneumoniae* (CRKP) infections pose a significant public health threat, particularly in intensive care units (ICUs), where they can lead to higher mortality rates among critically ill and debilitated patients. The financial burden associated with hospitalization and treatment for these infections is a global concern.<sup>17,18</sup> Recent studies have shown that T6SS may participate in the pathogenic process and play an important role in the virulence of *K. pneumoniae*.<sup>19–21</sup> Consequently, with the increasing pathogenicity of *K. pneumoniae*, research on *K. pneumoniae* T6SS has attracted considerable attention. This article aims to provide a comprehensive review of the fundamental genes, biological functions, and regulatory mechanisms of *K. pneumoniae* T6SS to facilitate future research on this system.

## The Typical T6SS

Current literature shows that T6SS is encoded within clusters containing at least 13 conserved core genes (*tssA-M*), which encode proteins that comprise the basic components of the secretion apparatus.<sup>22–24</sup> Based on bioinformatics, these genes were classified into three main categories.<sup>25</sup> The genes encode proteins that form the three-part architecture of T6SS, including bacteriophage-like injection apparatus, membrane complex, and molecular chaperone.<sup>26–28</sup> The most distinctive feature of T6SS is its bacteriophage-injection device-like structure, which consists of a needle structure, a sheath complex, and a baseplate (Figure 1).<sup>29</sup> The needle-like structure of the syringe includes Hcp and VgrG, which are translocator proteins secreted into the extracellular environment.<sup>30</sup> In particular, Hcp secretion is commonly considered a marker of T6SS activity.<sup>31</sup> In contrast, the VgrG homologous trimer is associated with the gp27/gp5 complex, a spike of the T4 phage.<sup>32</sup> The Hcp protein forms the tail of the inner tube with the VgrG trimer.<sup>33</sup> The length of sequences in the VgrG family proteins usually varies from approximately 750 to 1200 amino acids. The conserved domains are enclosed within the 750 amino acids. In addition, there is a variable C-terminal region in these proteins that often provides an effector function.<sup>34</sup> The spike complex commonly consists of a trimer of VgrG proteins and a spike complex protein (PAAR) containing proline-alanine-alanine-arginine repeats.<sup>27,35,36</sup> The needle structure (Hcp-VgrG-PAAR structure) is identified as the only part of T6SS that advances into the target cells. Hence, the T6SS effector is intimately linked with the needle structure.<sup>37,38</sup> The cone-shaped PAAR is thought to enhance the sharpness of the T6SS spear, enabling effective penetration. However, the reasons for the necessity of PAAR in T6SS functions among certain bacteria, while being optional in others, are still not clearly understood.<sup>39</sup> As an illustration, the effector could either be an appended structural domain fused to a needle component (eg, Hcp, VgrG, or PAAR) or a distinct protein that binds directly or indirectly to Hcp, VgrG, or PAAR.<sup>38,40</sup> The sheath complex, constructed from the TssB and TssC subunits, has the capacity to form tubular structures bearing significant resemblance to gp18.<sup>41</sup> The baseplate, composed of TssE, TssF,



**Figure 1** Schematic representation of the T6SS.

**Abbreviations:** IM, inner membrane; OM, outer membrane; Hcp, hemolysin co-regulatory protein; VgrG, valine-glycine repeat protein G; TagA, Tss-associated genes; Tss, type VI secretion system; PAAR, proline-alanine-alanine-arginine; ClpV, disassembly ATPase.

TssG, and TssK, bridges the transmembrane complex with the tail. It plays a pivotal role in triggering the polymerization of the tube/sheath complex and anchoring it to the membrane complex, thus initiating sheath contraction while keeping the entire assembled system solid during sheath contraction.<sup>42</sup> It serves as a vital link that connects the tail to the membrane complex and initiates the formation of the needle. Interestingly, these proteins exhibit significant similarity to the baseplate proteins of the T4 bacteriophage, such as gp6, gp7, and gp25.<sup>43</sup> Additionally, there is a protein called TssK, which shares certain structural characteristics with receptor-binding proteins found in siphophages. TssK interacts with TssG, and the stability of this interaction is ensured by the presence of TssF. Consequently, the TssKFG wedge complex is formed, representing an early building block of the T6SS baseplate that is analogous to the wedge complex of the T4 baseplate.<sup>44</sup> Currently, evidence has demonstrated that the fundamental components of T6SS include three proteins: TssL, TssM, and TssJ, which can collectively form a membrane complex.<sup>45</sup> TssM and TssL are located in the inner membrane, while TssJ is located in the outer membrane. TssM is capable of interacting with TssL and TssJ to connect the inner and outer membranes, thus creating a ring-like channel structure that traverses the cell membrane, allowing the VgrG spike and Hcp tube to deliver a variety of effectors to the target cells.<sup>46,47</sup> In bacterial secretion systems, ATPase or proton motive force is typically utilized to supply the energy required for the assembly of the secretion apparatus and the transfer of substrates.<sup>48</sup> ClpV, a member of the AAA+ (ATPases associated with various cellular activities) protein family, which is part of the HSP100 (Heat Shock Proteins) family, plays a crucial role in this process.<sup>36,49</sup> Upon the contraction of T6SS sheath-like structures, the exposed N-terminal structural domain of TssC is recognized by ClpV, leading to the disassembly of the structures into TssB and TssC monomers, a process that relies on ClpV-mediated ATP hydrolysis.<sup>36,50</sup> Aside from the aforementioned genes, T6SS also incorporates some non-conserved genes, such as *TssA* and *TagA*. TssA is divided into three categories, each performing similar functions in T6SS despite their structural differences.<sup>51</sup> TagA, a protein that interacts with TssA, halts the assembly of the sheath and maintains the sheath in an extended conformation.<sup>52</sup> However, TagA, the “stopper”, is not conserved within the T6SS gene cluster,<sup>29</sup> suggesting that different T6SS-positive strains may control tail-tube/sheath assembly and termination via distinct mechanisms.<sup>51–53</sup> Figure 1 provides a schematic representation of the T6SS. Table 1 lists the core genes of T6SS as well as their encoded proteins and functions.

## T6SS of *K. Pneumoniae*

In recent years, continuous research has led to the discovery of 13 conserved genes encoded in T6SS loci in various Gram-negative bacteria, including *K. pneumoniae*.<sup>61</sup> Lawlor et al have reported findings suggesting that the new virulence loci of *K. pneumoniae* appear to be homologous to the Yersinia pestis protein (YPO1467).<sup>62</sup> Further evidence supporting this notion was presented by Sarris et al, who found that the protein YPO1467 is annotated as a T6SS core

**Table 1** Genetic Organization and Coding Proteins of the Bacterial Type VI Secretion System

Gene	Encoded Protein	Function	Reference
tssA	Vas/ImpA	Tail complex	[53–55]
tssB	VipA/ImpB	Outer contractile sheath	[56]
tssC	VipB/ImpC	Outer contractile sheath	[56]
tssD	Hcp	Inner pipe, effector	[37]
tssE	HsiF	Baseplate	[57]
tssF	VasA/ImpG	Baseplate	[57]
tssG	VasB/ImpH	Baseplate	[57]
tssH	ClpV/VasG	AAA+ ATPase, sheath circulation	[57]
tssI	VgrG	Tip, effector	[47]
tssJ	VasD/Lip/SciN	Membrane complex	[57]
tssK	VasE/ImpJ	Baseplate	[58]
tssL	IcmH/DotU/VasF	Membrane complex	[59]
tssM	IcmF/VasK	Membrane complex	[57]
tagD	PAAR	A tapered structure on the tip	[60]

component and seems to be highly homologous (65.5%) with the putative *K. pneumoniae* protein. Concurrently, they detected 13 conserved genes of typical T6SS in *K. pneumoniae*. Sarris et al also proposed that a T6SS mechanism may assist the colonization and infection of the host by *Klebsiella*.<sup>63</sup> In an effort to study T6SS more comprehensively, the SecReT6 (Type VI Secretion System Database) and related reports have proposed classifying T6SS clusters into types I, II, III and IV based on gene cluster structure and core component evolutionary relationships.<sup>64–67</sup> Type I encompasses six subtypes (ie, I1, I2, I3, I4a, I4b, and I5), whereas type II is specific to the *Francisella* pathogenicity gene cluster; type III has been identified in *Bacteroides*. Type IV is newly discovered and represents a primordial system from which extracellular contractile injection systems, phages, and T6SSi–iii have evolved.<sup>68</sup> According to SecReT6 data, the complete T6SS cluster in *K. pneumoniae* belongs to type I2.<sup>20,67</sup> However, a comparative genomic analysis using *Enterobacter cloacae*—which contains a type I2 T6SS gene cluster—and *K. pneumoniae* revealed that some *K. pneumoniae* strains identified as containing a type I2 T6SS cluster lacked certain conserved T6SS genes. Notably, there are strains possessing only one unknown T6SS type, but the function of their T6SS clusters remains unclear.<sup>25</sup> T6SS core genes are tightly clustered in one or more gene clusters. It has been reported that in some *K. pneumoniae* strains, T6SS genes are primarily grouped in two loci (eg, NTUH-K2044, Kpn2146, and HS11286); in others, they are found in three loci (eg, Kp52145, MGH78578, and Kp342).<sup>20,63,69</sup> To facilitate more systematic T6SS research, Barbosa et al proposed using the TssA–M nomenclature to denote *K. pneumoniae* core T6SS components; they also reported that the majority of T6SS genes in the *K. pneumoniae* Kp52145 genome (33 genes, 87%) were clustered in three genomic loci, with only five (13%) being orphan genes. In their analysis of *K. pneumoniae* HS11286, 25 (86%) of the 29 T6SS genes were clustered in two main loci; only four (14%) were orphan genes.<sup>70</sup> However, Barbosa et al's data on *K. pneumoniae* T6SS genes were derived from computational analysis, indicating that further experimental studies are required to confirm these predictions.

In summary, the in-depth study of *K. pneumoniae* has led to the detection of T6SS core genes in its genome. More systematic genetic testing is urgently needed to enable a comprehensive investigation and confirm these computational speculations.

## Biological Function of *K. Pneumoniae's* T6SS

### T6SS Mediates Competition for *K. Pneumoniae* Participation Among Species

T6SS plays a crucial role in the interspecies competition between *K. pneumoniae* and the host microbiota.<sup>19</sup> Notably, T6SS exhibits robust antibacterial activity. Liu et al carried out competition assays to assess the antibacterial effectiveness of the T6SS in *K. pneumoniae* strain HS11286.<sup>20</sup> The findings revealed that mutant strains deficient in the T6SS apparatus or effector exhibited a diminished killing activity compared to the wild type strain. This research also established *K. pneumoniae's* significant advantage in T6SS-mediated interspecies competition with *E. coli*. Moreover, the study analyzed the T6SS gene cluster on chromosome HS11286, identifying the gene encoding for the effector protein Tle1<sup>KP</sup>. It was found that the Tli1<sup>KP</sup> immunoprotein, which is encoded within this gene cluster, could mitigate the toxicity of Tle1<sup>KP</sup>.<sup>20</sup> T6SS in *K. pneumoniae* also has a role in mediating both intraspecies and interspecies bacterial competition. Research by Storey et al demonstrated that polymyxin B and antimicrobial peptides could stimulate and enhance the expression of T6SS in *K. pneumoniae* strain Kp52145, which consequently influenced bacterial competition.<sup>71</sup> *K. pneumoniae* Kp52145 strain is particularly noteworthy as it contains highly virulent gene clones associated with human infections and encodes all virulence functions significantly linked to severe community-acquired diseases in humans.<sup>17,69</sup> In the context of competition experiments with *E. coli*, it was observed that the killing activity of the Kp52145  $\Delta$ clpV mutant strain against *E. coli*, was markedly reduced compared to the clpV complementary strain. This suggests that the eradication of *E. coli* by Kp52145 is T6SS-dependent, and the clpV gene may have a crucial role in this process. Further, the Kp52145 strain was found capable of competing with fungi in a T6SS-dependent manner. This was in stark contrast to the  $\Delta$ clpV mutant. The protein VgrG4 was identified as a critical player in this competition against fungi. The C-terminal extension of this protein, which contains the DUF2345 domain, was adequate to exhibit antibacterial and anti-eukaryotic effects.<sup>71</sup> Yet, the homologous immune protein Sel1E was found to reduce the toxin function of VgrG4.<sup>61</sup> Furthermore, the proteins VgrG1, VgrG2, and VgrG4 are essential in the T6SS-mediated killing

mechanism of *K. pneumoniae*. However, the functionality of VgrG2 and VgrG4 is primarily contingent on the bacterial growth conditions.<sup>72</sup> In a competition assay involving *K. pneumoniae* strain NTUH-K2044, Hsieh et al found that IcmF1, IcmF2, and Hcp augmented the bacterium's antimicrobial activity. Conversely, strains with *icmF1/icmF2* mutations demonstrated a decreased competitive ability in vivo.<sup>19</sup> T6SS is capable of not only delivering effectors to inhibit the growth of other bacteria, but also of injecting effectors into eukaryotic cells to promote bacterial survival by targeting the cytoskeleton. A recent investigation has demonstrated that the trans-kingdom antimicrobial T6SS effector VgrG4 from *K. pneumoniae* triggers the fragmentation of the mitochondrial network.<sup>73</sup>

In summary, *K. pneumoniae* effectively leverages T6SS to mediate both intraspecies and interspecies bacterial competition, thereby enhancing its survival. Nevertheless, the precise mechanisms underlying the competitive advantages conferred by T6SS remain elusive and warrant further investigation.

## Adhesion, Invasion, and Colonization of *K. Pneumoniae* Mediated by T6SS

The critical steps in its attack on host cells include adhesion, invasion, and colonization of *K. pneumoniae* to host cells. T6SS plays a crucial role in bacterial adhesion, invasion, and colonization, thus affecting the ability of *K. pneumoniae* to infect the host. In *K. pneumoniae* NTUH-K2044, the deletion of the T6SS gene significantly reduced bactericidal activity, expression of type-1 fimbriae, and epithelial cell adhesion and invasion.<sup>19</sup> A study using a *Galleria mellonella* infection model illustrated that the virulence of Kp52145 mediated by T6SS was orchestrated by three types of VgrG proteins.<sup>61,71</sup> The adhesion of *K. pneumoniae* to eukaryotic cells is linked to the production of type-1 fimbriae. In an experiment involving adhesion and invasion of *K. pneumoniae* in human colonic Caco-2 epithelial cells, it was discovered that after deleting the structural protein genes *icmF1* and *icmF2* of T6SS, the expression of type-1 fimbriae was down-regulated, which led to a significant reduction in adhesion and invasion to Caco-2 cells. Moreover, the bacterial load in infected mice's intestines, spleens, and livers decreased markedly.<sup>71</sup> The study conducted by Merciecca et al investigated the role of the Type VI secretion system (T6SS) in *K. pneumoniae* during long-term gastrointestinal colonization. Their findings revealed that T6SS isogenic mutants exhibited reduced efficiency in colonizing the upper gastrointestinal tract of mice compared to the parental strain over an extended period. Additionally, subsequent investigations demonstrated that T6SS had a negative impact on the richness and resilience of the gut microbiota.<sup>74</sup> This suggests that the virulence, bacterial invasion, and intestinal colonization of T6SS *icmF1* and *icmF2* mutants would be diminished.

In general, T6SS-mediated expression of type-1 fimbriae, cell adhesion, invasion, and in vivo colonization are critical for the onset of *K. pneumoniae* infection.<sup>19</sup> However, the current research on the colonization mechanism of *K. pneumoniae* T6SS is limited, and more detailed studies must be conducted.

## T6SS Affects the Acquisition of Drug-Resistant Genes in *K. Pneumoniae*

The global misuse of antibiotics has resulted in *K. pneumoniae*'s multi-drug resistance, leading to significant healthcare challenges. Under the influence of antibiotics, the secretory function of T6SS can be induced, potentially providing a novel concept for the clinical development of targeted drugs for *K. pneumoniae*. Research on the ST11 carbapenem-resistant *K. pneumoniae* strain HS11286 demonstrated that clinical abuse of antibiotics might not inhibit its proliferation, but may induce T6SS activity, making it more invasive.<sup>20</sup> PCR analysis of drug resistance genes in clinical isolates of *K. pneumoniae* by Liao et al showed that T6SS-positive strains had more drug resistance genes than T6SS-negative strains.<sup>75</sup> Liu et al reported the clinical isolation of carbapenem-producing *K. pneumoniae* strain HS11286 from sputum samples. They discovered that the T6SS gene cluster on the chromosome of *K. pneumoniae* can encode the effector protein Hcp, which possesses antibacterial properties.<sup>20</sup> Yet, when the bacteria were cultured in LB broth, there was no Hcp in the cells and supernatants. In contrast, Hcp could be detected in the cell components and supernatants of bacteria stimulated by  $\beta$ -lactam antibiotics, indicating that the T6SS secretory function occurred under the influence of antibiotics. Additionally, apramycin was used to determine that not only  $\beta$ -lactam antibiotics can induce T6SS secretion. In the competition experiment of the antibacterial activity of *K. pneumoniae* HS11286, it was found that the mortality of the non-antibiotic group was nearly two times lower than that of the meropenem or ceftazidime group, suggesting that the killing effect of T6SS activated by intercellular contact may be more potent under antibiotic stress.<sup>20</sup> It has been demonstrated that *V. cholerae* can acquire new T6SS effector genes via horizontal transfer and use them to kill

neighboring cells to promote horizontal gene transfer.<sup>76</sup> Similarly, *K. pneumoniae* can acquire new T6SS effector genes via horizontal transfer and natural transformation. They can then be transcribed into proteins and utilized to kill target cells.<sup>77</sup> Killing target cells via T6SS-mediated release DNA, which becomes accessible for uptake by naturally competent *K. pneumoniae*. Since antibiotic stimulation can activate the expression of T6SS, under antibiotic selective pressure, the coexistence of natural competence and T6SS-mediated bacterial killing may contribute to the highly efficient acquisition of drug-resistance genes in *K. pneumoniae*.<sup>77</sup> However, some studies reported opposing results. Zhang et al reported that the drug resistance rate of T6SS-positive bacteria in clinical isolates of *K. pneumoniae* blood infection was lower than that of T6SS-negative bacteria, and a lower proportion of CRKP was also found in T6SS-positive strains.<sup>21</sup> Similar findings were also reported by Liu et al in their study. They investigated a total of 169 *K. pneumoniae* strains obtained from patients with abscesses. Their results demonstrated that, with the exception of cefazolin and tegacycline, isolates positive for T6SS exhibited a reduced prevalence of antimicrobial resistance to other drugs.<sup>78</sup>

In conclusion, antibiotics induce the expression and secretion of T6SS, which may make attackers of T6SS-positive strains more aggressive in competitive growth. Simultaneously, the presence of T6SS is advantageous for strains to acquire drug resistance genes more efficiently, providing a competitive edge in biological competition. Nevertheless, some research indicates that the drug resistance rate of T6SS-positive strains is lower than that of T6SS-negative bacteria, and the internal relationship between T6SS and resistance genes in *K. pneumoniae* requires further discussion.

## T6SS Mediates the Virulence of *K. Pneumoniae*

The toxin delivery routes of T6SS can be categorized into the following two types: VgrG protein enters target cells directly as the effector protein of T6SS, and the Hcp channel transfers small molecules of virulence proteins, which are often not directly associated with T6SS-encoding gene clusters, to target cells.<sup>79</sup> Liao et al analyzed the virulence genes of T6SS positive strains in clinical *K. pneumoniae* isolates, and the results revealed that compared to T6SS-negative strains, T6SS positive strains had a higher frequency of virulence genes (*rmpA*, *fimH*, *entB*, *kfu*, *yetS*).<sup>75</sup> In a similar study, Liu et al studied the distribution of strain T6SS isolated from 169 liver abscess patients, and the study showed that the positive rate of T6SS was significantly higher in the highly virulent strains.<sup>78</sup> The study conducted by Suchanda Dey et al also demonstrated a strong correlation between the T6SS and 76 virulence factors in the highly drug-resistant and epidemic clones of ST147.<sup>80</sup> The T6SS gene frequency in clinical isolated *K. pneumoniae* was higher than that in blood and intestinal isolates and lower than that in pyogenic liver abscess (PLA) isolates.<sup>19,81</sup> The virulence of *hcp*-positive strains may be more potent than that of T6SS-negative strains, and the invasive infection rate of *hcp*-positive strains is higher than that of T6SS-negative strains.<sup>75</sup> Zhang et al investigated isolates obtained from patients with *K. pneumoniae*-induced blood infections and discovered that T6SS-positive strains were prevalent in patients with bloodstream infections.<sup>21</sup> Concurrently, the study indicated that T6SS-positive strains had a high detection rate of virulence genes and were more likely to produce hypervirulent *K. pneumoniae* (hvKp). *K. pneumoniae* liver abscess (KPLA) with extrahepatic migratory infections is defined as invasive KPLA (IKPLA). Wang et al conducted a study on the relationship between T6SS and IKPLA. The results demonstrated a significant enrichment of T6SS-related genes in the IKPLA group. Additionally, the detection rate of T6SS-positive strains in the IKPLA group was higher compared to the KPLA group (97.1% versus 78.4%), and the expression level of *hcp* was notably increased in IKPLA isolates.<sup>82</sup>

In summary, T6SS-positive *K. pneumoniae* strains have a high frequency of virulence genes. However, the association between T6SS and the virulence genes of *K. pneumoniae* and the mechanism of this bacterium's virulence effect remain unclear. Thus, further research is needed to explore the exact pathway and mechanism.

## Regulation of T6SS in *K. Pneumoniae*

T6SS-positive bacteria can utilize T6SS for bacterial competition, cell invasion, and in vivo colonization. However, the function of T6SS requires the coordinated participation of numerous proteins for assembly and secretion. When bacteria encounter changes in external environmental conditions, they can precisely regulate the transcriptional, translational, and post-translational mechanisms of T6SS expression.<sup>83,84</sup> The expression of T6SS is not fixed and can change under certain conditions. Recent studies have shown that the regulation of T6SSs can occur at three different levels, depending on the bacterial species: transcriptional, posttranscriptional, and post translational. Transcriptional regulation involves various

factors such as quorum sensing (QS) systems, two-component systems (TCS), alternative sigma factors, and histone-like proteins. Post transcriptional regulation involves the participation of RNA-binding proteins and small regulatory RNAs that control the stability and translation of messenger RNAs (mRNAs). Lastly, post translational regulation mainly relies on the phosphorylation of T6SS structural proteins to regulate the assembly of the system. Nevertheless, the research on the regulation of T6SS in *K. pneumoniae* has primarily focused on the transcriptional level.<sup>28</sup>

## Temperature Regulation

The negative regulator histone-like nucleoid-structuring protein (H-NS) of T6SS gene transcription can directly bind to the AT-rich region of DNA, acting as a silencer.<sup>85</sup> Its activity depends on temperature and osmotic pressure. Barbosa et al hypothesized that the H-NS binding site is widespread and conserved in the T6SS promoter of *K. pneumoniae*, potentially inhibiting the expression of significant virulence factors such as type 3 fimbriae and biofilms.<sup>70,86,87</sup> However, these inferences still need experimental verification. Additionally, H-NS binding sites have been detected upstream of the T6SS gene in three *K. pneumoniae* species, leading to speculation that H-NS binds to and silences the *tssD* gene promoter of the T6SS component in strain NTUH-K2044.<sup>70</sup> Furthermore, deletion of *h-ns* increases the transcription of the *hcp* gene.<sup>19</sup> A macrophage infection assay showed that *K. pneumoniae* H-NS and T6SS expression decreased in the early stage of infection.<sup>88</sup>

## Osmotic Pressure Regulation

pH, osmolarity, and NaCl can regulate the expression of T6SS encoded by hypervirulent *K. pneumoniae*. The PhoQ periplasmic domain senses acidic pH, divalent cations, and antimicrobial peptides.<sup>71</sup> The acidic residues in the periplasmic domain of PhoQ have been confirmed to activate T6SS, increasing the T6SS activity of Kp52145 in co-culture experiments with a competitive strain having active T6SS.<sup>71</sup> OmpR, the response regulator of the two-component system (EnvZ-OmpR) with the sensor kinase, has been previously reported to bind to the promoter region of a *Yersinia pseudotuberculosis* T6SS, participating in bacterial survival under high osmolarity conditions, resistance to deoxycholate, and pH homeostasis.<sup>89,90</sup> *K. pneumoniae* has been shown to use OmpR to respond to osmotic stress by regulating the cdi-GMP signaling pathway, expressing type 3 bacterial fimbriae, and forming biofilms.<sup>91</sup>

## Oxidative Stress Regulation

*K. pneumoniae* T6SS may also be activated under oxidative stress conditions, aiding the bacteria in processing reactive oxygen species (ROS) and thereby reducing the damage these species can cause to bacterial cells.<sup>70,92</sup> OxyR is a conserved oxidative stress response transcriptional regulator, and bacteria detoxify ROS by producing ROS-detoxifying enzymes, repairing DNA, sequestering metal ions, and employing mechanisms involving catalases.<sup>70</sup> *K. pneumoniae* OxyR has been shown to regulate biofilm formation, fimbrial genes, antibiotic resistance, and adhesion to epithelial cells.<sup>93,94</sup> Barbosa and Lery et al predicted that most *K. pneumoniae* T6SS promoter regions have conserved OxyR binding sites.<sup>70</sup>

Overall, *K. pneumoniae* T6SS may be activated under oxidative stress conditions, contributing to the bacterial processing of reactive oxygen species. The study also found that OxyR might regulate each gene encoding tubes, sheaths, and ATPase components. It is suggested that at least two signals are required to express *K. pneumoniae* T6SS. However, due to experimental limitations, many predictions remain unconfirmed.

## Other Regulatory Factors

Barbosa and Lery et al also predicted that RcsAB, GcvA, and Fis are regulators of *K. pneumoniae* T6SS.<sup>70</sup> RcsAB is a unique regulatory system that binds an rcsAB box and regulates the *K. pneumoniae galF* gene, affecting capsule expression and virulence.<sup>95,96</sup> GcvA, a transcriptional regulator of the glycine cleavage system, is involved in amino acid metabolism and is required for *F. tularensis* fitness and full virulence.<sup>97</sup> However, GcvA has not been studied in *K. pneumoniae*. Fis is a transcriptional regulator that responds to changes in the nutritional environment of intestinal bacteria.<sup>98</sup> Zhang et al identified BolA, an important virulence regulator in *K. pneumoniae*, and found that the expression of T6SS related proteins was also down-regulated after BolA deletion. Furthermore, Li reported that the regulation of

T6SS involves the negative regulators RcsB and MgrB, as well as the positive regulator PhoPQ. The upregulation of PhoPQ controlled the augmented activity of T6SS in mutants lacking mgrB and rcsB.<sup>99</sup> However, it is not clear whether this regulator directly regulates the expression of T6SS related genes.<sup>100</sup> Some antibiotics and antimicrobial peptides can also activate and enhance the expression and activity of different *K. pneumoniae* strains of T6SS.<sup>20,71</sup> It has been found that sub-inhibitory concentrations of  $\beta$ -lactam antibiotics (Meropenem 4mg/L or Ceftazidime 32mg/L) induced the expression of T6SS in *K. pneumoniae* HS11286, while T6SS was inactive in LB medium or M9 medium without antibiotics. In addition, similar results were obtained using apramycin, indicating that not only  $\beta$ -lactam antibiotics can induce T6SS secretion.<sup>20</sup>

However, the expression of *K. pneumoniae* T6SS appears to be associated with various environmental regulators. Whether these regulatory mechanisms are conserved in T6SS-positive strains of *K. pneumoniae* remains to be determined. At the same time, antibiotic misuse makes combating T6SS-positive *K. pneumoniae* more challenging. Consequently, there is an urgent need to explore the functions and mechanisms of *K. pneumoniae* T6SS to provide new targets for studying targeted drugs for clinical treatment.

## Conclusion

In recent years, the emergence of multi-drug resistance and hvKp has posed a significant challenge to clinical management and treatment. The rapid spread and increasing prevalence of drug-resistant and virulence genes have created an urgent need for novel therapeutic options. As research on *K. pneumoniae* T6SS has progressed, it has been discovered that this secretion system may be associated with *K. pneumoniae*'s virulence effects and the propagation of its drug-resistance genes. However, compared with other pathogens, T6SS has not been studied deeply in *K. pneumoniae*. In this review, we summarize the main role and research progress of T6SS in virulence and resistance and regulation of *K. pneumoniae*. Current studies have primarily focused on analyzing T6SS gene clusters and their functions in whole-genome sequencing, numerous questions remain unanswered. For instance, what are the molecular mechanisms by which *K. pneumoniae* T6SS recognizes target cells? As *K. pneumoniae* T6SS secretes multiple effectors, are they all secreted simultaneously, or are there selective regulatory mechanisms? Are there any new effectors to be discovered? Can *K. pneumoniae* T6SS benefit neighboring bacteria without being competitive? What is the molecular mechanism by which T6SS mediates *K. pneumoniae*'s competitive action? Moreover, T6SS mediates multiple biological effects of *K. pneumoniae*, especially the virulence effects of the bacterium, which may be closely linked to this system. This system's critical role in bacterial pathogenesis and bacterial-host interactions offers promising prospects. For example, T6SS may become a new target for the treatment of *K. pneumoniae* infection, leading to the development of new targeted drugs for effective treatment in the face of emerging multi-drug resistant *K. pneumoniae* due to antibiotic misuse.

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## Disclosure

The authors report no conflicts of interest in this work.

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