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Comprehensive analysis of the type VI secretion system in *Neisseria*: identification, distribution, and evolutionary insights

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Abstract

The genus *Neisseria*, a gram-negative diplococcus, includes commensal and pathogenic species that infect mucosal tissues, causing diseases such as gonorrhea and meningitis. The type VI secretion system (T6SS), a multifunctional molecular machine that facilitates the ability of gram-negative bacteria to deliver effectors for bacterial competition, virulence, and interaction with host cells, has been widely studied across various bacterial taxa. However, research on the T6SS in the genus *Neisseria* remains limited. In this study, we employed comparative genomics and pangenomics, among other bioinformatics approaches, to characterize the distribution of the T6SS and its related proteins, including effectors, immunity proteins and regulators, across different species within the genus. Through an analysis of 5,067 *Neisseria* genomes, we identified two complete T6SS loci. We found that more than half of the *Neisseria* species possess at least one complete T6SS locus. Further investigation revealed multiple T6SS-related loci. We also applied a statistics-based method for identifying T6SS-associated orthologous groups and revealed 64 new T6SS-associated proteins within the genus. Our research provides a comprehensive analysis of the T6SS in *Neisseria*, advancing the understanding of T6SS-related mechanisms.

Keywords *Neisseria*, Type VI secretion system, Effectors, Comparative genomics

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Background

Bacterial secretion systems are essential for diverse functions, ranging from nutrient acquisition to microbial competition and host–pathogen interactions [1–3]. Among the ten known secretion pathways in gram-negative bacteria, the type VI secretion system (T6SS) is among the most recently characterized, with its functional elucidation beginning with studies by Pukatzki et al. in *Vibrio cholerae* [4, 5]. The T6SS cluster encodes 13 core proteins (TssA–TssM) [6], which assemble into a syringe-like structure primarily responsible for delivering effector proteins [7]. T6SS genes are typically organized in gene clusters, but their transcriptional organization varies: some are transcribed as operons, while others are regulated by multiple promoters [8]. Effectors are bacterial virulence proteins that disrupt target cells. These proteins exhibit diverse enzymatic activities, including DNase, lipase, peptidoglycan hydrolase, and phospholipase, facilitating bacterial antagonism and playing key roles in interbacterial competition and pathogenesis [9]. To prevent self-intoxication, bacteria encode cognate immunity proteins [10]. These immunity proteins neutralize the toxic effects of effectors, thereby ensuring bacterial self-protection [4]. Furthermore, T6SS functionality is precisely regulated by a number of regulatory proteins that control effector expression, activity, and secretion, enabling dynamic system modulation [11]. These regulators operate at both transcriptional and post-translational levels in response to environmental and intracellular cues. Some directly modulate T6SS gene expression, while others interact with structural components to influence effector loading and secretion [12]. Beyond dedicated regulatory proteins, T6SS expression is governed by complex regulatory networks incorporating environmental signals (e.g., pH, temperature, nutrient availability), or quorum-sensing systems [13]. Collectively, the interplay between T6SS genetic organization, effectors, immunity proteins, and regulatory factors underscores the sophisticated control mechanisms governing this secretion system and its role in bacterial adaptation and competition [11]. Beyond interbacterial antagonism, the T6SS is implicated in additional biological functions, including nutrient acquisition and biofilm formation [14]. Furthermore, studies have shown that the T6SS is closely associated with bacterial virulence and colonization [11, 15, 16]. This system is conserved in approximately 25% of gram-negative bacteria [17], many of which are associated with human diseases, including *Enterobacter cloacae* [18], *Acinetobacter baumannii* [19], *Klebsiella pneumoniae* [20], *Vibrio cholerae* and *Neisseria cinereus* [11, 21]. In summary, the T6SS plays crucial roles in both the pathogenicity and symbiosis of bacteria, highlighting the importance of elucidating its function and identifying novel T6SS components. However, the distribution of T6SS in the *Neisseria* genus and the identification of its associated proteins remain poorly understood and warrant further investigation.

The genus *Neisseria* encompasses both pathogenic and commensal species, is widely distributed among various environmental niches and commonly colonizes the skin and respiratory tracts of humans and animals [22, 23]. While clinically prevalent *Neisseria* pathogens such as *N. meningitidis* and *N. gonorrhoeae* typically lack complete T6SS loci, these loci have been identified in various symbiotic species, including *Neisseria mucosa*, *Neisseria desiccata*, *Neisseria flava*, and *Neisseria cinereus* [21, 24]. Recent studies have advanced our understanding of the T6SS in *Neisseria* species. Research by Calder et al. has provided the first comprehensive description of T6SS subtypes across different *Neisseria* species, revealing the evolutionary adaptation and specialization of these systems in various ecological niches [5, 25]. A study published by Custodio et al. explored the T6SS in *Neisseria cinerea*, a commensal of the human respiratory tract, demonstrating that the T6SS in *N. cinerea* plays a crucial role in interbacterial competition, effectively eliminating competitors through a contact-dependent mechanism [24]. This finding underscores the importance of the T6SS in maintaining microbial balance within the respiratory microbiome. *N. subflava*, traditionally regarded as a commensal, has been implicated as a pathobiont in bronchiectasis, where it disrupts epithelial integrity and promotes inflammation. Although the role of the T6SS in this process remains uncharacterized, the presence of T6SS loci in *N. subflava* suggests potential links between T6SS-mediated interactions and its pathogenic potential under certain conditions [26].

Despite these advances, the functional diversity and broader ecological roles of the T6SS in *Neisseria* remain poorly understood. For example, only six T6SS effector proteins have been experimentally validated in *N. cinerea*. This highlights the need to characterize additional T6SS-associated genes, particularly effectors, immunity proteins, and regulators, to fully understand their ecological and evolutionary significance.

To address these gaps, we comprehensively characterized the complete T6SS genome within the genus and studied the distribution of these loci within the *Neisseria* genus. We subsequently conducted an in-depth analysis of the various components of the *Neisseria* T6SS, with a focus on the distribution and diversity of effectors, immunity proteins, and regulatory proteins, and explored VgrG and its downstream effector proteins within the genus. Finally, we identified 500 T6SS-associated orthologous groups through statistical analysis and identification, which further enriched the current understanding of the *Neisseria* T6SS. Overall, our study presents one most comprehensive characterization of the *Neisseria*

T6SS, revealing its diversity, conservation, and functional importance, highlighting its critical roles in microbial interactions and adaptations, and expanding the repertoire of T6SS-associated proteins. Through an investigation of the *Neisseria* T6SS, this research enhancegs our understanding of its diversity, conservation, and functional significance, providing a solid foundation for future studies on microbial interactions and adaptations.

Materials and methods

Genome acquisition, quality control and genome annotation

Genome sequences of members of the genus *Neisseria* (comprising 5,896 genomes) were obtained from the NCBI genome database (project genome download date: October 20, 2023). To ensure the reliability of the data, strict selection criteria were employed. CheckM with *Neisseria* lineage marker genes was utilized to assess genome completeness and contamination levels. Genomes exhibiting less than 99% completeness and more than 1% contamination were excluded. This rigorous screening process resulted in a final dataset of 5,067 genomes (Supplementary Table 1), uploaded by 196 different institutions over a 20-year period (2003 to 2023), encompassing 39 species of *Neisseria*.

Exploring the arrangement and distribution patterns of T6SS gene loci within the genus

For the genomes obtained via filtration, a homology search (hmmsearch) was conducted against the SecReT6 protein database (downloaded on 2023 November 10) [27]. T6SS gene clusters were identified on the basis of 13 core components (including TssA-TssM) and other intrinsic proteins in the database. It was required that no more than 5 non-core genes separate two T6SS genes to ensure that they were within the same gene locus and that the identified locus was within the same contig of the assembly. Instances of the same arrangement sequence occurring in more than 2% of the total genome count were classified as a T6SS locus type. On the basis of the number of core genes contained in each gene locus, the strains were defined as complete T6SS strains (T6SS + strains containing all 12 T6SS core components) or T6SS-deficient strains (T6SS- strains in which at least one T6SS core component was absent).

To explore the distribution and quality of the T6SS within *Neisseria* spp., we selected a representative genome from each species and constructed a phylogenetic tree of the *Neisseria* genus using PhyloPhlAn3 [28], with *Kingella kingae* (GenBank: GCA_900475905.1) as the outgroup. The model with the lowest Bayesian information criterion (BIC) (Q.insect + F + R3 model) was selected, and 10,000 bootstrap replicates were performed to increase the robustness of the phylogenetic construction [29]. Finally, the phylogenetic tree was visualized using iTOL (v7.1), with annotations of the genomic locus distribution.

Building on this analysis, we employed the TssB typing method to further classify T6SSs as described previously [27]. We extracted representative TssB proteins from genomes containing the T6SS in *Neisseria* and constructed a phylogenetic tree alongside TssB proteins from the SecReT6 database. First, protein alignments were performed using MAFFT (v7.520), low-quality or unreliable alignment regions were removed using the trimAl (v1.4.rev15) tool [30], and the phylogenetic tree was constructed using IQ-TREE (v2.2.6) [29].

Investigating the diversity and distribution patterns of known T6SS-related proteins in the genus *Neisseria*

We analyzed the types and quantities of T6SS effector proteins, immunity proteins, and regulatory proteins across 39 species of the genus *Neisseria* via homology searches across genomic datasets. We utilized Proteinortho software (v6.0.14) for homology determination. Comparisons were made to delineate inter- and intraspecies differences, thereby elucidating patterns of diversity and distribution. We define the T6SS immune proteins, effector proteins, and regulatory proteins present in all species as the core immune proteins, core effector proteins, and core regulatory proteins of *Neisseria*. Core effector, immune, and regulatory proteins were identified within the genus, and functional information pertaining to key protein families was analyzed. Efforts were made to ascertain the presence of effector, immune, and regulatory proteins present among *Neisseria* species. Functional domain analysis of the core protein sequences was conducted using InterProScan [31] and Pfam (2.08-3) [32]. The FGENESB program (Softberry) was used for finding operons in the genome of the type strain.

T6SS-associated orthologous group analysis in the *Neisseria* genus

To test the hypothesis that T6SS-associated orthologous groups (TAOGs) are significantly more correlated with T6SS loci, we conducted a normalization analysis of the distribution of known T6SS-related proteins in the presence (T6SS+) or absence (T6SS-) of T6SS loci, as described in the previous section. The differences in the distributions of immune proteins, effector proteins, and regulatory proteins were normalized to the number of genomes in each group, and their significance was calculated using the chi-square test.

To identify protein families significantly associated with different T6SS gene clusters, protein clustering was performed using CD-HIT (v4.8.1) [33] (with a sequence identity threshold of 0.6 and a length difference cutoff of 0.6) on 39 species of *Neisseria*. All pangenomic

homologous protein families across different species were analyzed, and the genome counts for each protein family in T6SS + and T6SS- species were normalized to the number of genomes in each group. A chi-square test was employed to perform statistical analyses on the normalized data, allowing for the identification of protein families significantly associated with different T6SS gene clusters. The screening criteria were as follows:

$$Log2\left(\frac{Normalized\ No.of\ Orthologs\ in\ T6SS+\ Genomes}{Normalized\ No.of\ Orthologs\ in\ T6SS-\ Genomes}\right) \geq 2$$

$$p - value < 0.05$$

We selected the top 500 representative clusters (T6SS+/T6SS-: log2 presence/absence value > 7,645, p value < 0.0001; top 500 clusters, Supplementary Table 2) (Supplementary Table 1). The proteins identified through clustering were subsequently annotated using the SecReT6 database, Bastion6 database [34], eggNOG-Mapper functional annotations [35], Pfam families [36], and Prokka (v1.14.6) annotations [37]. To further investigate the functions of these proteins and identify those most likely associated with the T6SS, Cytoscape [38] software was used to characterize the significantly associated proteins of the repeatedly linked T6SS, enabling the identification of potential novel T6SS-associated loci or TAOGs.

Results

Identification and distribution of two distinct T6SS loci in the genus *Neisseria*

Our study identified two distinct type VI secretion system loci within the genus *Neisseria* from the analysis of 5,067 genomes (Supplementary Table 1), designated T6SS^{nessi1} and T6SS^{nessi2}. The T6SS^{nessi2} locus is fragmented into 2 different locations in the genome and cooccurs with T6SS^{nessi1} (Chi-square test, p value ≤ 0.001) (Fig. 1a). The co-occurrence of T6SS^{nessi2} with T6SS^{nessi1} suggested that evolutionary pressures maintained functional synergy between these loci. Although intact T6SS loci accounted for only 3.39% of the total genomes analyzed (Fig. 1b), they were widely distributed across 20 species of the genus (51.3%) (Fig. 1c) with various degrees of occupancy (Fig. 1d). Complete T6SS loci were identified in nearly half of the strains of *N. mucosa*, *N. subflava*, *N. flava*, *N. cinerea*, *N. sicca* and *N. macacae* (Fig. 1d). These species are predominantly found in the human upper respiratory tract and are opportunistic pathogens. Next, on the basis of the TssB typing method of the T6SS, we classified the type VI secretory systems present in *Neisseria* into 2 different subtypes: T6SS^{nessi1} aligns with subtype i2, whereas T6SS^{nessi2} corresponds to the i3 subtype (Fig. 1e)**.** Moreover, we performed a comprehensive whole-genome analysis using representative genomic data from 39 species of *Neisseria* to construct a phylogenetic tree for this genus. The phylogenetic tree clearly reveals distinct clades formed during evolution, with each clade representing a unique haplogroup that harbors varying numbers of T6SS clusters (Fig. 1f).

Conservation, diversity, and adaptive evolution of T6SS effectors, immunity proteins, and regulators in *Neisseria*

To gain insights into the conservation, diversity, and adaptive evolution of the T6SS and related proteins, we analyzed the distributions of known effectors, immunity proteins, and regulators. A homologous protein search of the effectors across the SecReT6 database of the 5,067 *Neisseria* genomes revealed the presence of a total of 33 types of effector proteins within the genus (Fig. 2a). EFF01823, EFF01485 and EFF01477 are present across the genus (Fig. 2b). The majority (30 out of 33) of the identified effector orthologs were distributed among fewer than 30 species, indicating significant diversity in effector protein composition. The nonuniform presence of the effectors suggests that the diversity of the effectors is likely caused by environmental factors. For example, EFF0135 and EFF0188 are exclusively present in *Neisseria perflava* and *Neisseria subflava*, respectively, which are found in the upper respiratory tract of humans. The unique presence of these effectors indicates a potential adaptation to specific host-related conditions. Similarly, a total of 27 immune proteins were present in the genus (Fig. 2c), with two (IMU00306 and IMU00344) being widely distributed across the genus (Fig. 2d).

To determine the relationships between effectors and immunity proteins, we investigated immunity proteins located within three genes adjacent to known effectors (Fig. 2e). We observed instances in the genus *Neisseria* where the same immunity protein is associated with multiple different effectors and where the same effector is linked to multiple different immunities. We have provided two representative locus maps of these cases (Fig. 2f and g). For example, IMU17307 is adjacent to several effectors, whereas EFF18627 is located near IMU38226, IMU17305, and others. Furthermore, we conducted an analysis to predict whether these protein pairs form operons, with the results provided in Supplementary Table 4. Additional experimental validation (e.g., RT-PCR, RNA-seq) is required to confirm their co-expression and functional association.

We identified a total of 118 different regulator orthologs from the *Neisseria* genus (Fig. 2h), 62 of which were present across all the species within the genus (Fig. 2g). Considering that the T6SS is regulated by various factors, including bacterial internal factors and environmental conditions, this broad distribution of regulatory proteins

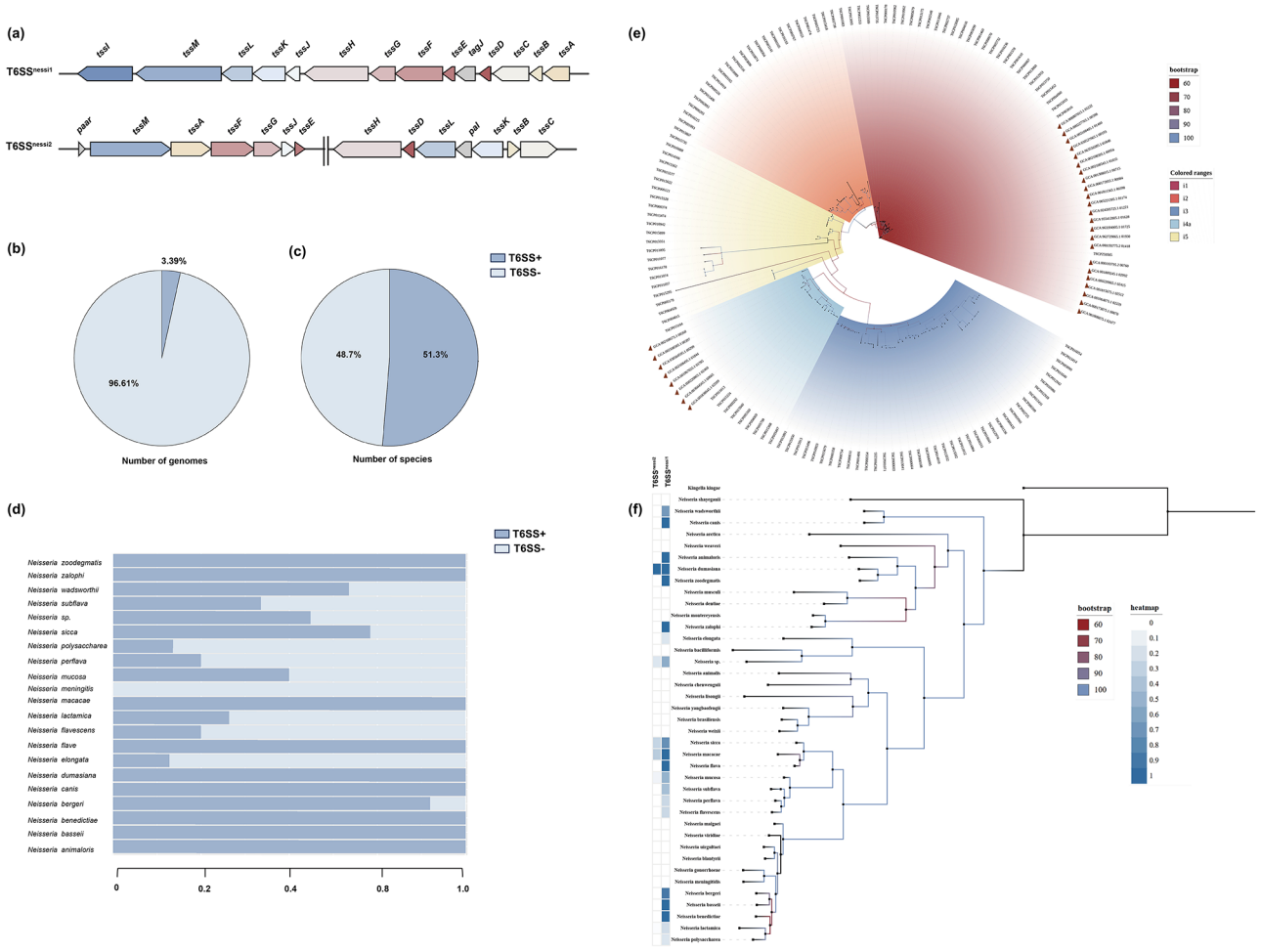

Fig. 1 Genomic Organization and Distribution of the Two Gene Clusters in *Neisseria*. (**a**) Genomic arrangement of the two identified T6SS loci within *Neisseria*. (**b**) Proportion of genomes with complete T6SS gene clusters among the 5,067 genomes analyzed. (**c**) Percentages of *Neisseria* species (out of 39) with complete T6SS gene clusters. (**d**) Distribution of genomes with complete T6SS gene loci across the various *Neisseria* species. (**e**) Phylogenetic tree of the *Neisseria* genus illustrating the distribution of the two T6SS loci across different species on the basis of TssB sequences. (**f**) Phylogenetic tree of *Neisseria* based on 158 core genes and the distribution of the two T6SS gene clusters across the genus

underscores the complex regulatory mechanisms governing the T6SS.

Characterization of VgrG-associated effector proteins in *Neisseria*

It is well known that certain effector/immunity proteins are located downstream of VgrG tip proteins, facilitating the delivery of the effectors and promoting interactions with host cells, thereby increasing bacterial infectivity and conferring a survival advantage. To identify the classical effectors downstream of VgrG, we examined them in *Neisseria* to explore the diversity and potential new effectors of their effector proteins.

We first quantified VgrG in the presence or absence of the T6SS. Our analysis revealed that the majority of the T6SS + genomes (99%) contained at least one *vgrG* gene, with the number of *vgrG* genes ranging from 0 to 14 and the majority of genomes having 0 to 2 *vgrG* genes. On the other hand, almost all the T6SS- genomes (100%) do not contain any *vgrG* genes. Overall, genomes containing the complete T6SS locus were significantly more prevalent than those lacking it (Fig. 3a and b).

Additionally, we identified effector proteins downstream of VgrG. The frequently occurring T6SS-related domains and their interconnections are illustrated in Fig. 3c. The RHS domain is the largest node in the network and has multiple connections with many other domains, such as Ntox34, HNH, Phage_GPD and TNT, indicating the crucial role of the RHS domain in carrying out toxin-related functions in collaboration with other domains. In addition, phage-related domains such as Phage_GPD and Phage_base_V suggest that the VgrG system may leverage phage-associated mechanisms to carry out its effector protein functions [39]. Domains such as Sel1, CBS, DNA_methylase, and DUF2235 have fewer connections to other domains, possibly indicating

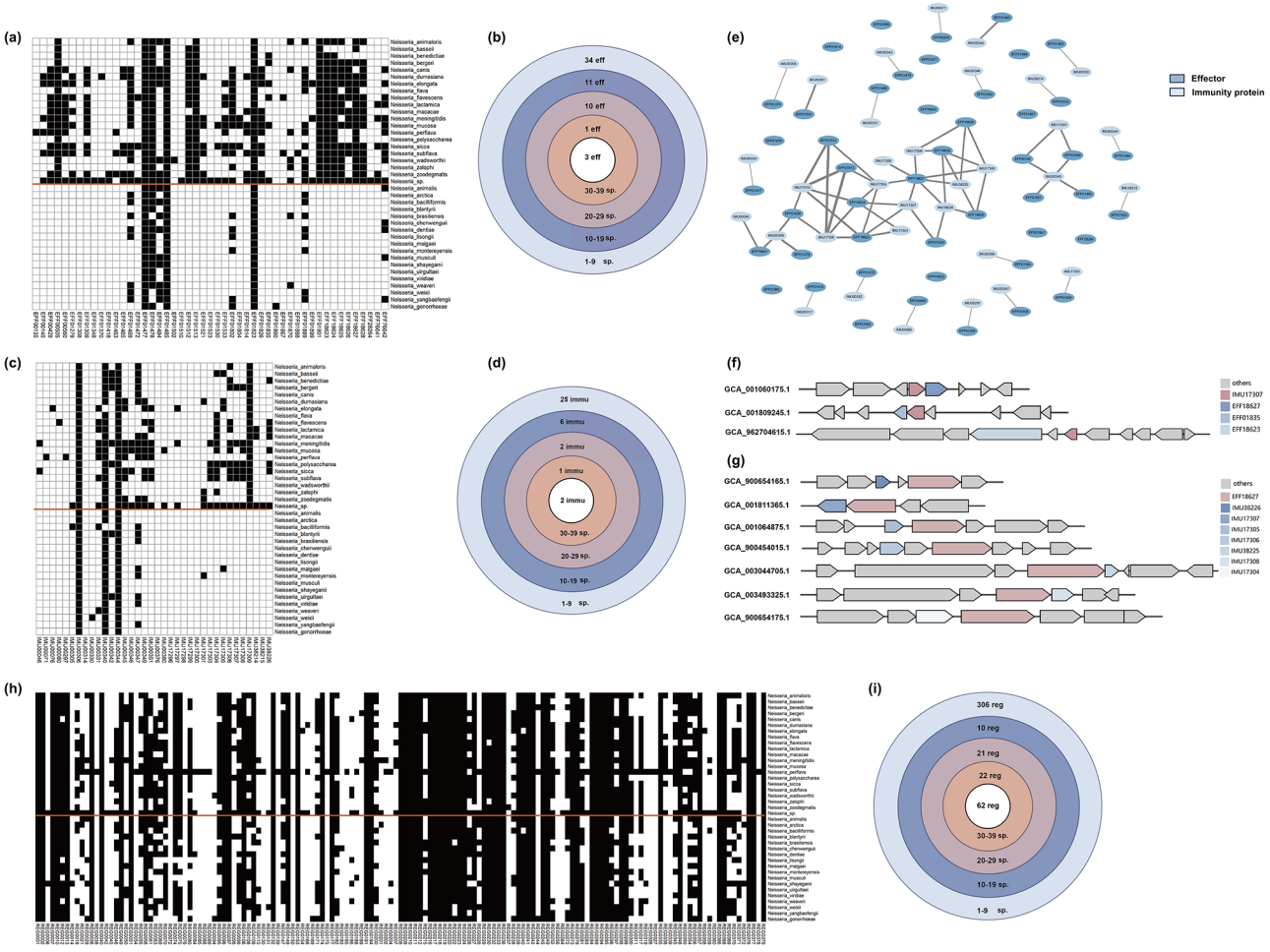

Fig. 2 Distribution Patterns of Known Effector, Immunity, and Regulatory Proteins Across *Neisseria* Species. A presence/absence heatmap showing the distributions of (**a**) effectors, (**c**) immunity proteins, and (**h**) regulatory proteins among *Neisseria* species. Shared sets of (**b**) effector proteins, (**d**) immunity proteins, and (**i**) regulatory proteins are illustrated using circular diagrams, where each circle represents the density of different *Neisseria* species. The top segment of each circle highlights the number of species in which these proteins are present, whereas the bottom segment indicates the number of proteins within each set. (**e**) Linkages between immune and effector proteins oriented in the same direction within the same genome are illustrated, with proteins separated by no more than two genes on the same contig. (**f**) Two representative locus diagrams demonstrate cases where multiple effectors are associated with a single immunity protein (**g**) and where multiple immunity proteins are linked to a single effector

that they occupy more niches or play more specific roles in the function of effector proteins.

Prevalence and enrichment of T6SS-associated genes in T6SS + *Neisseria* genomes

We hypothesis that T6SS-associated genes are more prevalent in genomes containing T6SSs than in those lacking T6SSs. To test this hypothesis, we explored known immunity proteins, effectors, and regulatory proteins in the strains containing (T6SS+) or lacking (T6SS-) the T6SS. Overall, the T6SS + strains presented greater diversity of known T6SS-associated proteins than did the T6SS- strains, as shown in the Venn diagrams (Fig. 4A, b and c). For example, although T6SS- strains contain 15 different types of immunity proteins, these represent a subset of the immunity proteins found in the T6SS + strains, which possess 33 distinct categories of immunity proteins. Next, we quantified the T6SS-associated orthologous groups in the presence or absence of the T6SS. Overall, the T6SS + genomes presented a significantly greater number of known effectors, immunity proteins and regulators, as indicated by the rightward shift of the red bars (Fig. 4d, e and f). Specifically, compared with the T6SS- strains, the T6SS + strains presented a significantly greater number of effectors per genome (Fig. 4g, T6SS+: 9.35 effectors/genome, T6SS+: 3.45 effectors/genome, p value < 0.001) and immunity proteins (Fig. 4h, T6SS+: 6.29 immunity proteins/genome, T6SS+: 3.21 immunity proteins/genome, p value < 0.001). Regulators were also more abundant in the T6SS + strains. However, the difference in regulator counts between the T6SS + and T6SS- genomes was less

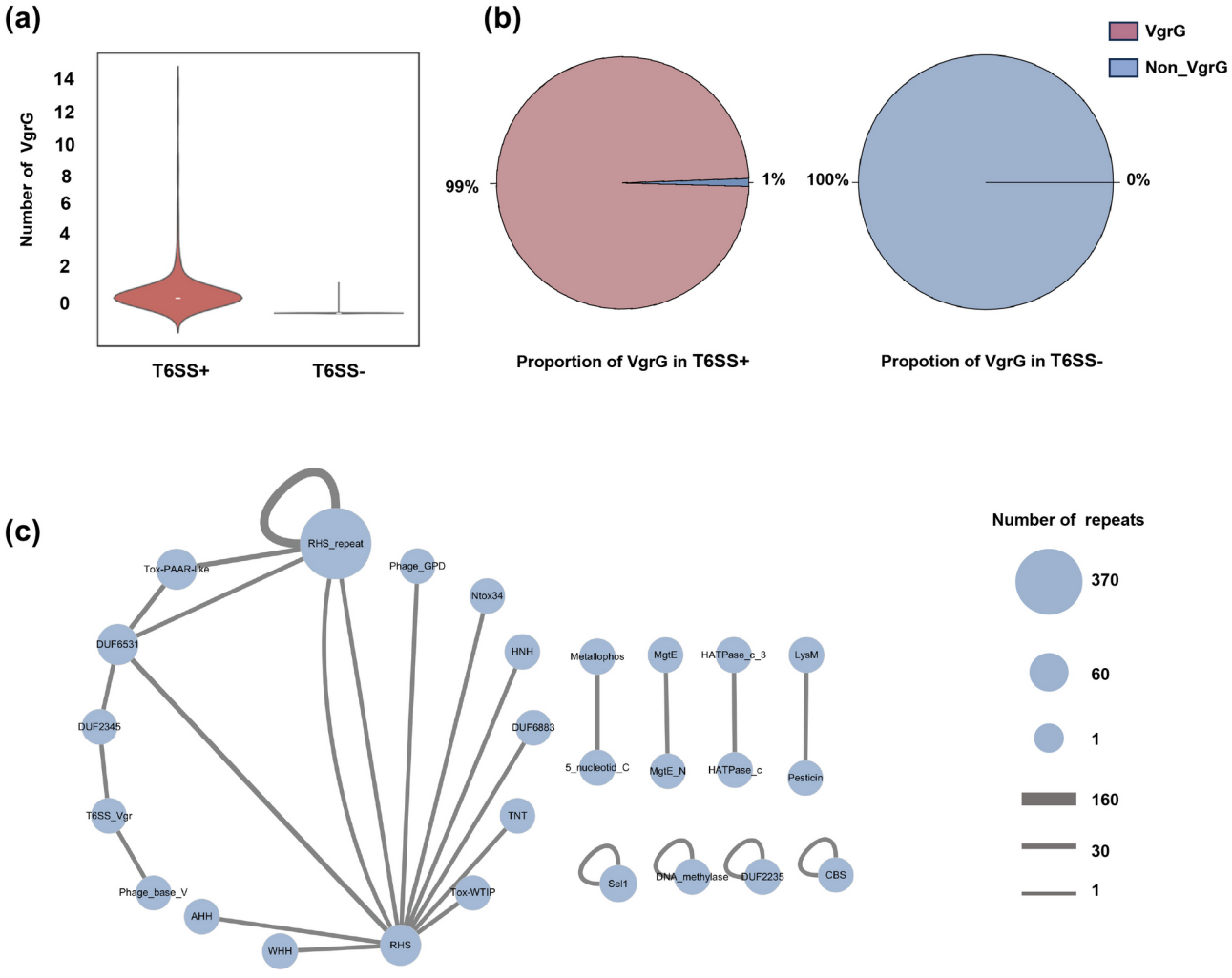

Fig. 3 Analysis of VgrG abundance and domain relationships in downstream effector proteins. (**a**) Comparison of the number of *vgrG* genes between genomes with complete T6SS (T6SS+) gene clusters and those lacking complete T6SS gene clusters (T6SS-). (**b**) Proportion of *vgrG* genes in the T6SS + and T6SS- genomes, illustrated by pie charts. (**c**) Cytoscape network analysis illustrating the relationships between domains within effector proteins downstream of VgrG. Each node represents a specific domain identified within the effector proteins, with the node size corresponding to the frequency of occurrence for that domain. Edges indicate associations between domains within the same effector protein, with edge thickness representing the frequency of these domain pairings

pronounced (Fig. 4i, T6SS+: 66.95 regulators/genome, T6SS-: 63.75 regulators/genome, p value < 0.05). This phenomenon may suggest that certain regulators are conserved beyond T6SS-dependent functions. Statistical analysis of the fold-change ratios for individual immunity proteins, effectors, and regulator proteins (Supplementary Fig. 1a, b and c) highlighted the relative abundance of each protein in the T6SS + compared with the T6SS- genomes. The results revealed that the mean fold-change values for each protein type were greater than zero, indicating a greater relative abundance of these proteins in the T6SS + genomes than in the T6SS- genomes. This analysis underscores the utility of discerning differential protein family distributions between the T6SS + and T6SS- strains as a means to discern novel protein families, at least for effectors, immune and regulatory proteins, potentially associated with the T6SS apparatus.

Identification of novel T6SS-associated protein families and functional insights

As elucidated in the previous section, known T6SS effectors and immunity proteins are significantly more abundant in genomes with a complete T6SS than in genomes without the T6SS. To explore potential novel T6SS function-related proteins, such as T6SS effectors or immunity proteins, we conducted a statistical test to explore the T6SS-associated orthologous groups across the *Neisseria* genomes. We examined the distribution of grouped orthologous groups in the presence or absence of the T6SS and performed data normalization and statistical tests. Protein families with significant distribution

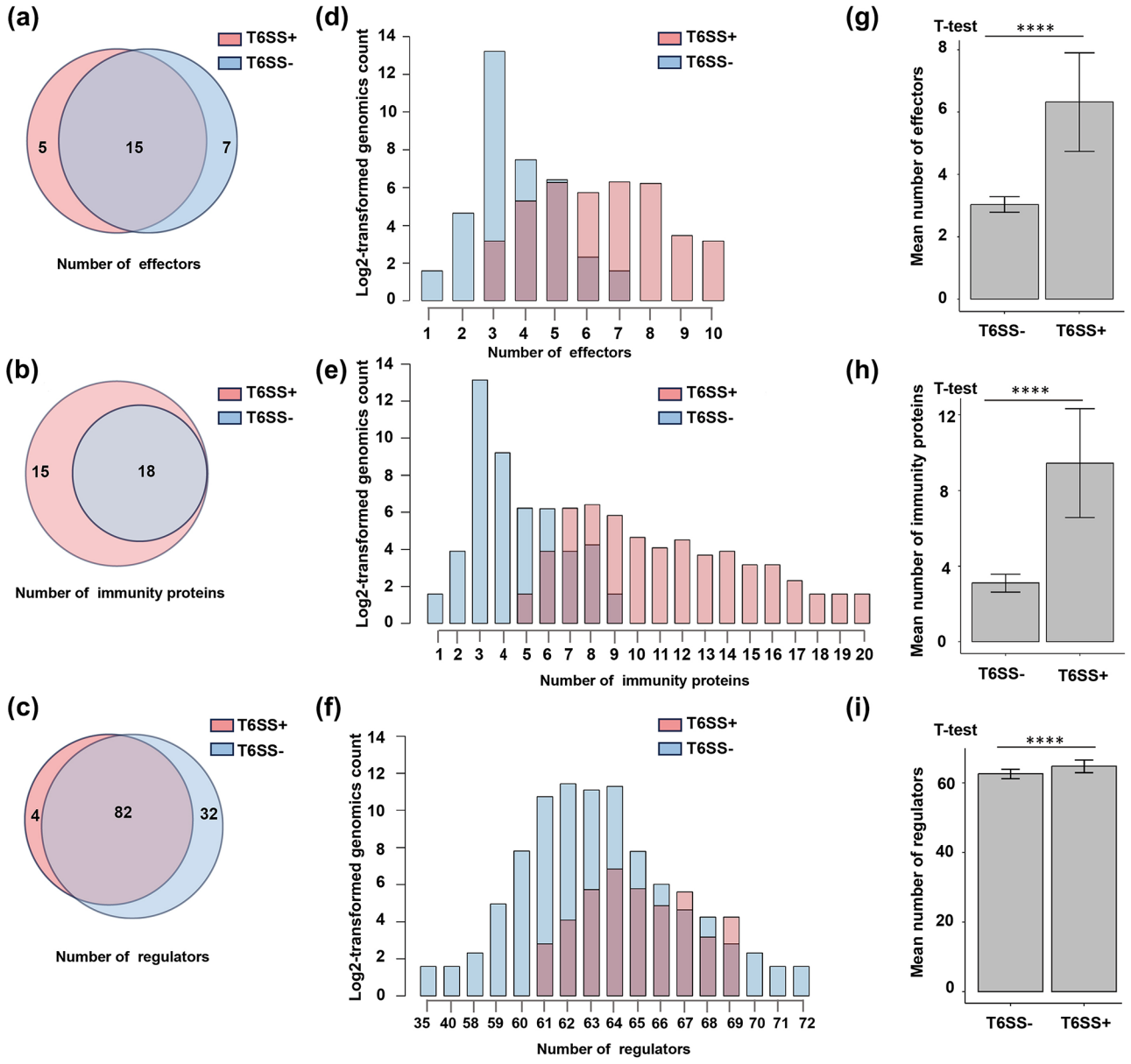

Fig. 4 Differences in the distribution of known T6SS-associated proteins in *Neisseria* species with and without complete T6SS gene clusters. The Venn diagram shows the types of immunity proteins (**a**), effector proteins (**b**), and regulatory proteins (**c**) in *Neisseria* species with complete T6SS gene clusters (T6SS+) and those lacking T6SS gene clusters (T6SS-). Normalized genomic counts corresponding to the types of effector (**d**), immunity (**e**), and regulatory proteins (**f**) present in the T6SS + and T6SS- groups. Statistical analysis (*t* test) comparing the normalized genomic counts of effector (**g**), immunity (**h**), and regulatory proteins (**f**) between the T6SS + and T6SS- groups

differences between the T6SS + and T6SS- strains were identified, resulting in the identification of 500 T6SS-related protein families, which included 9,963 protein families with significant correlations to T6SS loci (Supplementary Table 2). Among these 500 T6SS-related protein families, 54 are known T6SS-associated proteins, whereas 446 are novel and have not been studied in association with the T6SS.

To further investigate the function of these potential T6SS-associated proteins in *Neisseria*, we performed a network analysis to identify potential gene clusters or orphan genes whose presence is strongly associated with the T6SS and that may be functionally linked to the T6SS. A total of 8 tightly connected TAOGs were identified across various *Neisseria* genomes (Fig. 5).

Not surprisingly, two T6SS loci, T6SS^{nessi1} and T6SS^{nessi2}, are well identified from the results, with T6SS^{nessi2} separated into two parts, potentially reflecting evolutionary rearrangements or functional specialization. Among the T6SS-associated proteins that remain

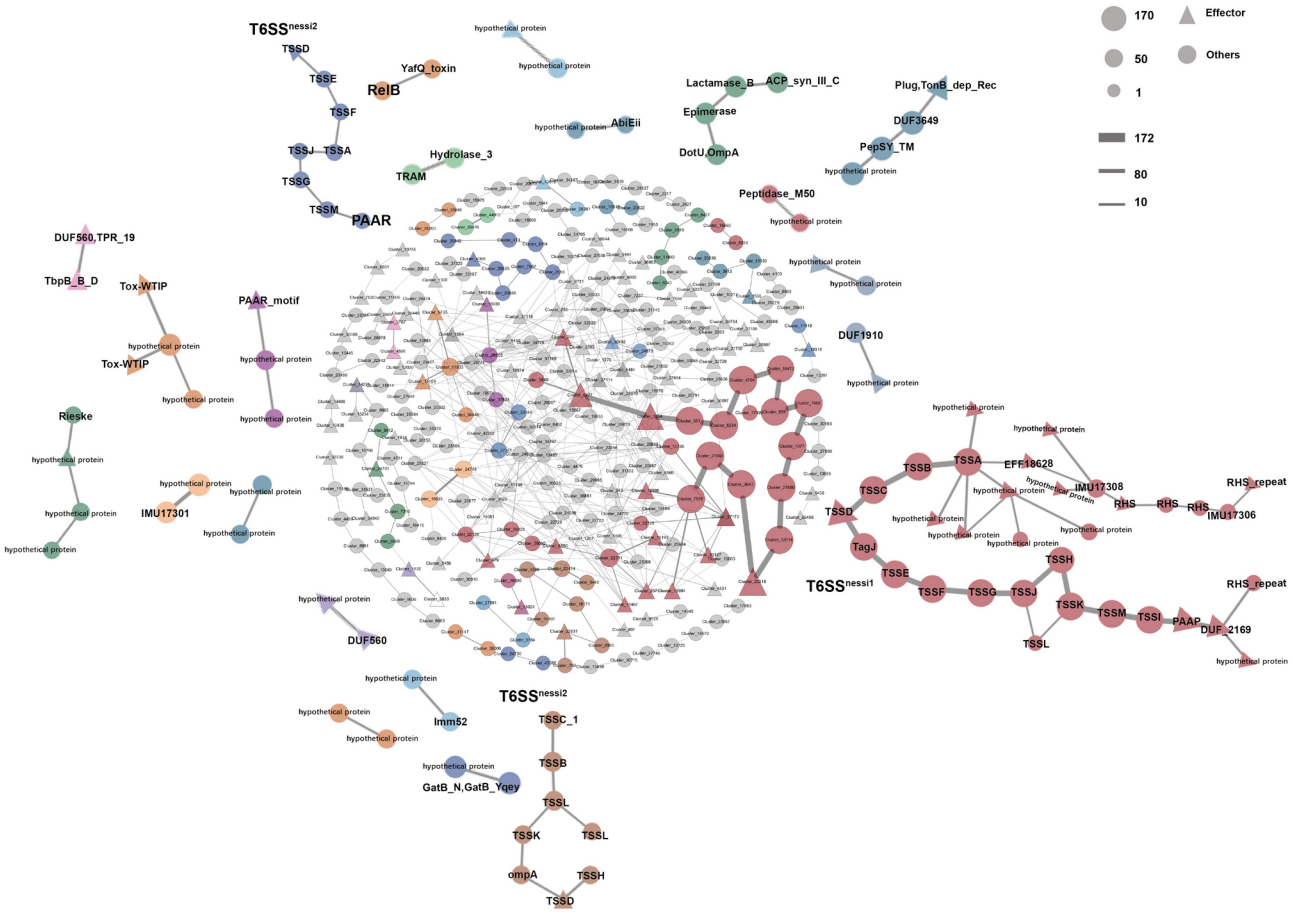

Fig. 5 Network analysis of T6SS-associated orthologous groups (TAOGs) in *Neisseria*. The network of 275 interconnected TAOGs with effector proteins is represented by triangles, and that of non-effector proteins are depicted as circles. The size of each triangle or circle reflects the frequency of occurrence of the corresponding protein family, with larger shapes indicating higher frequencies. The thickness of the connecting lines represents the number of associations between protein families, with thicker lines indicating stronger connections. Protein families with more than 10 associations were selected, annotated, and highlighted in different colors for detailed presentation

uncharacterized, several protein families, including phage-associated proteins (e.g., Cluster 34777), endonucleases (Cluster 24877), proteases (Cluster 32942), and proteins of unknown function (Cluster 23215), have been identified. These protein families not only underscore the structural and functional parallels between the T6SS and phage mechanisms but also highlight their potential roles in toxin delivery, microbial competition, and niche adaptation. Finally, we integrated the results from the Bastion6 database annotations, eggNOG-Mapper functional annotations, Pfam family annotations, and Prokka annotations for protein families with more than 10 connections (98 in total). After excluding validated T6SS components, we identified 64 proteins (Supplementary Table 3) that are highly likely to be associated with the *Neisseria* T6SS, thereby expanding the repertoire of T6SS-associated proteins in *Neisseria*.

Discussion

The T6SS is a widely distributed protein export mechanism in gram-negative bacteria that is involved primarily in bacterial competition [40], pathogenicity and environmental interactions [41], with different associated compositions and functions among different species [42, 43]. In our study, we analyzed 5,067 genomes across the entire *Neisseria* genus and identified two types of T6SS loci within the genus, with T6SS^{nessi2} being divided into two different sections (Fig. 1a). Although only a small fraction of the genomes contained complete T6SS gene clusters, more than 50% of the genomes of the majority of the species (12 out of 21) within the genus *Neisseria* contained a complete T6SS (Fig. 1b, c and d). This pattern is largely skewed by the large number of pathogenic *N. meningitidis* and *N. gonorrhoeae* genomes, most of which lack a complete T6SS, indicating that the T6SS might play a role in ecological adaptations and bacterial interactions within nonpathogenic or commensal species. These findings also suggest that these two species have

alternative competitive or pathogenetic virulence mechanisms, such as type IV pili and lipo-oligosaccharides, to survive in their specific niches [44].

On the other hand, our findings indicate that a complete T6SS gene cluster is present primarily in commensal *Neisseria* species, such as *N. mucosa*, *N. canis*, *N. flavescens*, *N. dumasiana*, and *N. zalophi*. These bacteria typically inhabit environments such as the human respiratory tract, where microbial diversity necessitates interspecies competition and where the T6SS acts as a bacterial weapon to outcompete other microbes [45]. Thus, we hypothesize that the T6SS in this genus primarily facilitates interactions with other microorganisms in the environment rather than being directly tied to host–pathogen dynamics. Such interactions may include microbial competition or niche establishment, highlighting the ecological relevance of the T6SS across the genus.

The functions of the T6SS are mediated primarily by effector, immune, and regulatory proteins [46]. Effector proteins serve as the principal "weapons" of the T6SS and are secreted into target cells to exert specific effects, such as cytotoxicity, cell wall degradation, and disruption of host functions [47]. Immune proteins protect bacteria from self-inflicted damage by neutralizing the toxicity of their effector proteins [48]. Simultaneously, the expression and activity of the T6SS are meticulously regulated by regulatory proteins [49], which oversee the system's expression, assembly, and activation through various mechanisms, including environmental response, gene regulation, and signal transduction [50]. In this study, we retrieved validated effector, immunity, and regulatory proteins from the SecReT6 database and conducted a homologous alignment to identify T6SS-related proteins in *Neisseria*. At the species level, we observed that *Neisseria* species with a complete T6SS locus presented a relatively high abundance of immunity proteins and effector proteins (Fig. 2a, b and c). We subsequently analyzed the effector proteins downstream of VgrG in *Neisseria* to investigate their diversity and distribution (Fig. 3). In addition to the effectors that have been characterized in *Neisseria* [24], we have also identified potential novel effectors containing RHS, DUF, DNase, and metallophosphatase domains, which have extended our knowledge of the effector repository of the genus. However, these prediction results need to be further verified by molecular experiments.

Our analysis revealed that genomes with a complete T6SS locus contained more effector proteins, immunity proteins, and regulatory proteins than did genomes lacking the T6SS locus, and these differences were statistically significant (Fig. 4). Hence, we conducted a comparative genomic analysis of all 5,067 genomes, examining all protein families and analyzing the differences in the distribution of these proteins in the presence and absence of the T6SS locus (T6SS+/T6SS-) to identify protein families that are functionally linked to the T6SS. Among these T6SS-associated but uncharacterized proteins, a substantial number of protein families presented high homology to bacteriophage tail proteins (e.g., Cluster 26661, Cluster 26832). The T6SS and bacteriophage tail structures are similar, both comprising a baseplate, sheath, and tail, and some studies suggest that the T6SS may have originated from bacteriophages [51, 52], implying a common evolutionary ancestor. For example, the T6SS effector protein VgrG is homologous to the bacteriophage tail proteins gp27/gp5, both of which have membrane-piercing capabilities. This evolutionary connection suggests that the T6SS represents a case of exaptation, where a viral infection apparatus was repurposed into a bacterial competition system.

Furthermore, certain families of nucleases (Cluster 13191), proteases (Cluster 19973), and proteins of unknown function (Cluster 31941) also present promising research potential. In further studies, we annotated and analyzed protein families that cooccurred more than 10 times (Fig. 5). These multiple occurrences and linkages totaled 98 protein families, of which 64 proteins were newly identified and distinct from the known T6SS-associated proteins cataloged in the database (Supplementary Table 3). These proteins, which were previously not associated with T6SS functions, open new avenues for studying the molecular mechanisms of bacterial competition and adaptation. For example, RHS proteins have been widely recognized as T6SS effectors in other bacterial genera and provide a competitive advantage. Similarly, ferripyoverdine receptor proteins have been implicated in the acquisition of iron, a critical resource in host-associated environments, suggesting a potential role for the T6SS in resource competition within *Neisseria*. The putative deoxyribonuclease and AbiEi RHS-containing proteins may function as T6SS effectors involved in delivering toxic effectors to competing bacteria. Although the functional roles of these newly identified T6SS-associated proteins require further experimental validation, our research provides novel insight for future research into the functional diversity of the T6SS in this genus.

In summary, our study provides a comprehensive investigation into the diversity, conservation, and functional significance of the T6SS, highlights the critical role of the T6SS in shaping microbial interactions and adaptations, broadens our understanding of T6SS-associated proteins and lays a foundation for future research into the ecological and evolutionary significance of the T6SS in *Neisseria*.

Supplementary Information

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Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

Supplementary Material 4

Supplementary Material 5

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Author contributions
QTG, SCH, and LS conceptualized the study, obtained funding, and supervised the work. TTD, WJY, NZ, and ZHP performed the data analysis, while MZ, CYW, and JJ validated the methodology. LLJ curated the data. TTD prepared the initial draft of the manuscript, with subsequent edits by QTG, SL, and SCH All authors reviewed the manuscript, and QTG integrated the comments to finalize it.

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Data availability
All genomic data used in this study were obtained from NCBI database. Detailed information, including accession numbers for each genome, is provided in Supplementary Table 1. The datasets supporting the conclusions of this article are included within Supplementary Table 2, Supplementary Tables 3 and Supplementary Table 4.

Declarations

Competing interests
The authors declare no competing interests.

Conflict of interest
We declare that we have no conflict of interest.

Ethics
This work did not require ethical approval from a human subject or animal welfare committee.

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