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The type IX secretion system: Insights into its function and connection to glycosylation in *Cytophaga hutchinsonii*

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ABSTRACT

The recently discovered type IX secretion system (T9SS) is limited to the Bacteroidetes phylum. *Cytophaga hutchinsonii*, a member of the Bacteroidetes phylum widely spread in soil, has complete orthologs of T9SS components and many T9SS substrates. *C. hutchinsonii* can efficiently degrade crystalline cellulose using a novel strategy, in which bacterial cells must be in direct contact with cellulose. It can rapidly glide over surfaces via unclear mechanisms. Studies have shown that T9SS plays an important role in cellulose degradation, gliding motility, and ion assimilation in *C. hutchinsonii*. As reported recently, T9SS substrates are *N*- or *O*-glycosylated at their C-terminal domains (CTDs), with *N*-glycosylation being related to the translocation and outer membrane anchoring of these proteins. These findings have deepened our understanding of T9SS in *C. hutchinsonii*. In this review, we focused on the research progress on diverse substrates and functions of T9SS in *C. hutchinsonii* and the glycosylation of its substrates. A model of T9SS functions and the glycosylation of its substrates was proposed.

1. Introduction

C. hutchinsonii is a cellulolytic bacterium belonging to the Bacteroidetes phylum [1]. Despite the lack of both cellobiohydrolases (CBHs) and lytic polysaccharide monooxygenases (LPMOs), which are essential for cellulose degradation [2,3], in its genome, *C. hutchinsonii* can grow on crystalline cellulose [4]. In contrast with the free cellulase-based mechanism of aerobic bacteria [5,6] and the cellulosome mechanism of anaerobic bacteria [7–9], *C. hutchinsonii* cells are orderly arranged on cellulose [1,10], revealing its contact-dependent cellulose degradation mode. Consequently, a novel mechanism of cellulose degradation is employed by *C. hutchinsonii*, which appears to occur via a coordinated degradation process in the outer membrane and periplasmic space [11,12]. In addition, despite its lack of organelles, such as flagella or type IV pili, *C. hutchinsonii* can glide over surfaces [13], although its motion mechanism is also a mystery.

In Gram-negative bacteria, the first protein-secretion system was described in *E. coli* regarding hemolysin A secretion [14]. Subsequently, 10 additional protein-secretion systems, termed TxSSs (T1SS–T11SS), have been discovered and characterized gradually [15–23]. They consist of one-step secretion systems and two-step secretion systems. T1SS, T3SS, T4SS, and T6SS are one-step secretion systems. In this case, substrates are translocated from the cytoplasm to the cell surface or extracellular milieu through a complete cell envelope-spanning structure. In two-step secretion systems, substrates are first transported via general secretory pathways, such as the Sec, Tat, and holin systems. Then the outer membrane transport system translocates the substrates from the periplasmic space across the outer membrane. T2SS, T5SS, T7SS, T8SS, T9SS, T10SS, and T11SS are the terminal branches in the two-step secretion system [15,22-24].

The type IX secretion system (T9SS) only exists in Bacteroidetes organisms. It was first detected in the Gram-negative anaerobic bacterium *Porphyromonas gingivalis* [25,26], which is a major causative agent of periodontitis [27]. Gingipains Kgp, RgpA, and RgpB are virulence factors of *P. gingivalis*. Because of the inactivation of *porT* (*sprT*) and *sov* (*sprA*), gingipains cannot be secreted to the cell surface; instead, they accumulate in the periplasmic space [25,28]. Later, genomic analyses and gene manipulation revealed another 10 proteins involved in the secretion of gingipains (PorKLMNPQUWXY) [26]. These proteins differ in sequence from the eight known protein-secretion systems, thus forming a new protein-secretion system, which was first termed PorSS but is now called the type IX secretion system (T9SS) [26,29,30]. All gingipains (Kgp, RgpA, and RgpB) have an N-terminal signal peptide and a C-terminal domain (CTD). Further analysis found that the CTD sequence consisting of 60–100 amino acids was the outer membrane export signal for T9SS substrates [31–33]. The CTDs of T9SS substrates (also called CTD proteins here) have different conserved domains. Most T9SS substrates have classical type A CTDs (TIGR04183), whereas others have type B CTDs (TIGR04134) [33].

T9SS is exclusively present in the majority of families of the Bacteroidetes phylum, including the *Phodothermaceae* family of the Bac-

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teroidetes Order II class, the *Prevotellaceae* family, the *Porphyromonadaceae* family of the Bacteroidia class, the *Flavobacteriaceae* family of the Flavobacteriia class, the *Chitinophagaceae* family and *Sphingobacteriaceae* family of the Sphingobacteriia class, and the *Cytophagaceae* family of the Cytophagia class. However, T9SS is not distributed in the *Bacteroidaceae* family of the Bacteroidia class [30,34]. Although T9SS has been known for more than a decade, its function has only been studied in a few species of the *Porphyromonadaceae, Flavobacteriaceae*, and *Cytophagaceae* families. *P. gingivalis* and *Tannerella forsythia* belong to the *Porphyromonadaceae* family and are keystone pathogens for periodontitis. *Flavobacterium columnare* and *Flavobacterium psychrophilum* are fish pathogens of the *Flavobacteriaceae* family. These four pathogenic bacteria utilize T9SS to secrete virulence factors that target a variety of host cells [29,35-37]. Another species of the *Flavobacteriaceae* family, *Flavobacterium johnsoniae*, is an aerobic, non-pathogenic environmental microorganism in which adhesins are transported via T9SS for gliding motility [38]. Studies have been extensively carried out, mainly in *P. gingivalis* to determine the components and functions of T9SS. For the class Cytophagia, some studies about T9SS were performed exclusively in *C. hutchinsonii*, which utilizes unique cellulose degradation and gliding mechanisms. Recently, T9SS was found to play important roles in cellulose degradation, gliding, and ion assimilation in *C. hutchinsonii* [39–43]. The study of T9SS in *C. hutchinsonii* can provide a significant reference for other strains in the class Cytophagia.

T9SS is robust and important in *C. hutchinsonii* because of its extensive substrates. At least 157 proteins are predicted to have CTDs in *C. hutchinsonii* [40,44,45]. Moreover, twelve of the 18 predicted cellulases have CTDs [46]. The T9SS component mutants Δ*porU* and Δ*sprP* are defective in cellulose degradation and gliding [39,40]. However, none of the other T9SS component genes have been deleted and reported for a long time in *C. hutchinsonii*. Recent studies found that disruption of T9SS results in the failure of the assimilation of several metal ions in *C. hutchinsonii* [41]. Upon this recognition, a modified complex medium with additional ions was applied, and multiple T9SS components were successfully deleted and studied [42,43]. In *P. gingivalis*, the ability to intercept substrate proteins on the cell surface by binding to anionic lipopolysaccharide (A-LPS) is a prominent feature of T9SS [47–51]. As reported by Tan et al., after translocating to the cell surface, CTD proteins might be anchored by LPS, similar to that observed in *P. gingivalis* [52]. Moreover, Xie found that T9SS substrates were modified in the periplasmic space and outer membrane [53]. The modification in the periplasmic space occurs on the CTD sequence of CTD proteins. It is related to the translocation and anchoring of T9SS substrates in *C. hutchinsonii*, as discussed in detail below.

The genetic tools and cellulose degradation mechanisms of *C. hutchinsonii* were extensively reviewed by Zhu in 2017 [13]. This current review will mainly focus on the recent advances in identifying the components, substrates, and functions of T9SS, as well as the modification of T9SS substrates in *C. hutchinsonii*.

2. T9SS components

2.1. T9SS components in P. gingivalis

T9SS has been extensively studied in *P. gingivalis*. T9SS has at least 21 components, which are unnecessary for survival because they can all be deleted in *P. gingivalis*. The components and structure of T9SS in *P. gingivalis* can be roughly described as: PorL and PorM located in the inner membrane and interacting via their transmembrane segments, forming a protein complex that functions as a molecular motor [26,54-56]; PorK and PorN located in the periplasmic space, forming a large ring complex [54,57,58]; and a periplasmic space protein PorW (SprE) anchored to the OM and interacting with Sov. The C-terminal domain of PorW interacts with PorN [59]. Sov and PorW are associated with another periplasmic protein, PorD. Therefore, PorW and PorD act as “bridges” to connect the protein complex PorKN and the translocon Sov, thereby ensuring maximum secretion efficiency [59]. Thus, a core complex across the cell membrane is formed by PorLMWDKN, whereas PorFGVQT and Sov form outer membrane beta-barrel structures [60]. After passing through the core channel, the substrate proteins reach the outer membrane channel for transportation across the outer membrane [57,61-63]. Moreover, an attachment complex composed of PorVUZQ exists on the cell surface [50], where the substrates bind to a shuttle protein, PorV, which delivers them to the attachment complex, and then the CTDs are cleaved by the PorU-a protease [50,64,65]. A-LPS is attached to the newly formed C terminus for anchoring substrates to the cell surface, whereas some substrates are released into the culture medium [66]. PorX, PorY, SigP, and the newly discovered PorA are transcription regulators that modulate the transcription of T9SS components [67,68]. PorA is a CTD-containing protein that regulates the PorXY system by activating the PorY sensor kinase directly or indirectly [69].

2.2. T9SS components identified in C. hutchinsonii

C. hutchinsonii possesses a complete set of T9SS-coding genes in its genome [30]. SprP was the first T9SS component to be identified in *C. hutchinsonii*. The Δ*sprP* mutant fails to digest cellulose and is defective in gliding [40]. *porU* was deleted by a linear DNA double-crossover system [39]. The ability of the Δ*porU* mutant to degrade cellulose and glide becomes weaker. Moreover, several CTD proteins are not secreted in the Δ*porU* mutant, including the main extracellular protein CHU_0344 [39]. However, in *C. hutchinsonii*, many attempts to delete other components of T9SS have failed. Zhu speculated that these components might be essential for growth and survival; therefore, their loss would be lethal [13]. In 2020, Gao found that T9SS was involved in assimilating trace ions. Mutant Δ*gldN* exhibited a growth defect in PY6 medium without the addition of ions, and the appropriate addition of Mg^{2+} and Ca^{2+} to the PY6 medium effectively made up for the growth defect of the mutant strain. Subsequently, by improving the culture conditions, *sprA, sprT*, and *porV* were all successfully deleted [42,43]. To date, most of the T9SS component genes of *C. hutchinsonii* have been deleted (shown in Table 1) under different complex culture conditions. The Δ*gldN*, Δ*sprA*, Δ*sprT*, and Δ*porV* mutants lead to the degradation of filter paper and gliding defects [41–43]. The Δ*porG*, Δ*porW*, Δ*porZ*, and Δ*porQ* mutants affect cellulose utilization and/or gliding (unpublished data from our lab). Before T9SS was discovered, only T6SS appeared to be involved in ion assimilation. Some substrates secreted by T6SS can chelate manganese, zinc, and iron and transport them to the intracellular space for various physiological processes [70–72]. Nevertheless, the core T9SS components, GldM, GldK, and GldL of *C. hutchinsonii* cannot be deleted in our laboratory, which suggests the functional diversity of the different T9SS components. Culture conditions need to be further optimized to obtain deletion strains and conduct functional studies of the core T9SS components in *C. hutchinsonii*.

3. T9SS functions in *C. hutchinsonii*

3.1. T9SS is related to cellulose degradation

Many microbes use different strategies to degrade cellulose in the environment. The free cellulases that form the collaborative digestion mechanism and cellulosome mechanism have been well studied. Some aerobic microbes, such as *Tichoderma reesei*, digest cellulose using free cellulases. They secret exoglucanase, endoglucanases (EGs), and β-glucanases (BGs) into the extracellular space. These synergize in the environment, degrading insoluble cellulose into oligosaccharides or glucose and then transporting them to the intracellular space [73]. Some anaerobic microbes from various *Clostridium* spp. digest cellulose using cellulosomes [74]. In this case, dockerin/cohesin domains and scaffolding proteins are required to organize multiple cellulase enzymes and cellulose-binding modules into complexes [7-9,74,75].

Table 1
Research status on the T9SS components of *C. hutchinsonii*.

Component	*P. gin*	*C. hut*	Function [a]	Study in *C. hutchinsonii*	Phenotypes of the deletion mutant in *C. hutchinsonii*	Reference
PorL (GldL)	PGN_1675	CHU_0172	IM. Forms Molecular Motors with			
PorM	Failed to delete with PY6 or complex medium		Unpublished			
PorM (GldM)	PGN_1674	CHU_0173	IM protein. Rotor and shaft/pinion	Failed to delete with PY6 or complex medium		Unpublished
PorK (GldK)	PGN_1676	CHU_0171	OM. Forms ring structure with PorN	Failed to delete with PY6 or complex medium		Unpublished
PorN (GldN)	PGN_1673	CHU_0174	OM/P. Forms ring structure with PorK	Deleted with complex medium	Defective in degrading filter paper and spreading on agar	[41]
PorG	PGN_0297	CHU_3410	OM. Binds to the PorK/N	Deleted with complex medium	Defective in degrading filter paper and spreading on agar	Unpublished
PorT (SprT)	PGN_0778	CHU_2709	OM	Deleted with complex medium	Defective in degrading filter paper and spreading on agar	[42]
PorW (SprE)	PGN_1877	CHU_0177	Forms “bridge” with PorD to connect protein complex PorKN and the Sov	Deleted with complex medium	Defective in degrading filter paper and spreading on agar	Unpublished
PorD	PGN_1783	CHU_3014	OM	Not deleted yet		
Sov (SprA)	PGN_0832	CHU_0029	OM. Translocon	Deleted with complex medium	Defective in degrading filter paper and spreading on agar	[42]
PorP (SprP)	PGN_1677	CHU_0170	OM. Binds to PorE and PG1035	Deleted with PY10 medium	Defective in degrading fifter and spreading on agar	[40]
PorE	PGN_1296	Many	P. Binds to the peptidoglycan, PorP and PG1035	Not deleted yet		
PorU	PGN_0022	CHU_3237	OM. Sortase	Deleted with PY6 medium	Defective in degrading filter paper and spreading on agar	[39]
PorV	PGN_0023	CHU_3238	OM. Shuttle protein	Deleted with complex medium	Defective in degrading filter paper and spreading on agar	[43]
PorZ	PGN_0509	CHU_3302	OM. Forms attachment complex with PorU, PorV, PorQ	Deleted with PY6 medium	Defective in degrading filter paper and spreading on agar	Unpublished
PorQ	PGN_1645	CHU_2991	OM. Forms attachment complex with PorU, PorV, PorZ	Deleted with complex medium	Defective in degrading filter paper and spreading on agar	Unpublished
PorA	PGN_0123		OM. Activate the PorY sensor kinase	No credible homologues of PorA		
in *C. hutchinsonii*						
PorX	PGN_1019	CHU_1040	Transcription regulator	Deleted with complex medium	Decreased degrading filter paper. Unaffected spreading on agar	Unpublished
PorY	PGN_2001	CHU_0334	Transcription regulator	Deleted with complex medium	Decreased degrading filter paper. Unaffected spreading on agar	Unpublished
SigP	PGN_0274	CHU_3697	Transcription regulator. Enhances the *por* genes	Deleted with PY6 medium	Unaffected degrading filter paper and spreading on agar	Unpublished
Plug	PGN_0144	CHU_2217	Seals the periplasmic face of Sov	Not deleted yet		
PorF	PGN_1437		Forms outer membrane beta-barrel structures with PorG, PorP, Sov, PorV, PorQ, and PorT	No credible homologues of PorF in *C. hutchinsonii*		

[a]IM means that the protein locates in the inner membrane, OM means that the protein locates in the outer membrane, P means that the protein locates in the periplasmic space.

In the genome of *C. hutchinsonii*, nine EGs and four BGs were predicted to be involved in cellulose utilization [2]. Surprisingly, *C. hutchinsonii* lacks obvious CBHs and LPMOs, essential for crystalline cellulose hydrolysis. Moreover, no cellulosome genes have been identified in the *C. hutchinsonii* genome [2]. Therefore, a novel cellulose degradation strategy is employed in *C. hutchinsonii*. Wilson proposed a cellulose degradation hypothesis for *C. hutchinsonii* [12]; i.e., that efficient cellulose degradation is a synergistic process of the outer membrane and vital periplasmic space proteins in *C. hutchinsonii*. Complexes in the outer membrane can remove individual cellulose molecules from cellulose fibers and bind them to the specific outer membrane receptor. Then cellulose molecules transport across the outer membrane into the periplasmic space, where they are cleaved to glucose by endoglucanases [12]. The outer membrane complexes and transporters play key roles

in this process. However, the cellulose degradation mechanism of *C. hutchinsonii* remains a mystery.

Direct contact with cellulose is required for its degradation by *C. hutchinsonii* [10]. Bai first demonstrated the surface degradation pathway in *C. hutchinsonii* [76]. In this case, surface proteins secreted to the cell surface via T9SS, including cellulases, are important for cellulose degradation. An in-depth study of T9SS showed that it is related to cellulose degradation in *C. hutchinsonii*. As described previously, T9SS component mutants (Δ*porU*, Δ*gldN*, Δ*sprA*, Δ*sprT*, Δ*porV*, and Δ*sprP*) in *C. hutchinsonii* failed to utilize cellulose [39–43]. Furthermore, a re-analysis of the *C. hutchinsonii* genome [46] revealed that the carbohydrate-active enzyme (CAZyme) repertoire of *C. hutchinsonii* consists of 189 unique proteins, including five *β*-glucosidases (BGs), five GH5, six GH8, and seven GH9. Twelve of the eighteen predicted cellulases had CTDs (Cel5A, Cel5E, all of GH8, CHU_0961, Cel9A, Cel9B, and Cel9D) and may be transported via T9SS [46]. However, in *C. hutchinsonii*, the mutant lacking seven EGs (Cel5A (CTD), Cel5B, cel5C, Cel9A (CTD), Cel9B (CTD), Cel9E, and Cel9F) could still utilize cellulose nearly as well as did the wild-type counterpart [77], which may be attributable to the functional redundancy of multiple cellulases. Nevertheless, several novel T9SS substrates essential for cellulose degradation have been identified and characterized in *C. hutchinsonii*, including the type B CTD protein CHU_3220, CHU_1557, and the type A CTD protein CHU_0922. Both the Δ*3220* mutant and Δ*0922* mutant cannot degrade filter paper, exhibiting a low cellulose-binding degree and weak degradation capabilities for the crystalline cellulose region, indicating that these two T9SS substrates are critical for cellulose degradation [45,78]. CHU_1557 is involved in using the crystalline cellulose region in *C. hutchinsonii*. Further studies found that CHU_1557 affects the degradation of the crystallization region by affecting glucose absorption [45,78,79].

By analyzing the localization of the cellulases encoded in its genome, several cellulases without CTDs were predicted to be localized in the periplasmic space [46]. Therefore, there might be a periplasmic pathway to degrade cellulose in *C. hutchinsonii*. The periplasmic space of *C. hutchinsonii* has four EGs (Cel5B, Cel5C, Cel9C, and Cel9F) and four BGs (BglA, BglB, BglC, and BglD) [46,77]. The mutant Δ*cel5B*/Δ*cel9C* is seriously defective in the degradation of crystalline cellulose and amorphous cellulose [77]. The Δ*bglA*/*bglB* mutant cannot utilize cellobiose and cellulose [76]. These findings indicate that *C. hutchinsonii* has a periplasmic space degradation pathway that is essential for the degradation of crystalline cellulose by this bacterium. Endonuclease cellulases alone (and not exonuclease cellulases) exist in *C. hutchinsonii* [2]. Some processive endoglucanases have partial exoglucanase activity [80,81]. However, controversy remains about whether processive endoglucanases exist in *C. hutchinsonii*. As reported by Zhang et al., Cel5B and Cel9C from *C. hutchinsonii* can hydrolyze amorphous cellulose (RAC), and many reducing sugars stem from the soluble products [82,83]. Moreover, Cel5B and Cel9C can hydrolyze *pNPC* and cellotriose, respectively, whereas most of the typical endoglucanases cannot [82,83]. It suggested that Cel5B and Cel9C from *C. hutchinsonii* might be processive endoglucanases. However, as reported by Zhu et al., the ratios of soluble to insoluble reducing sugars produced by Cel5B and Cel9C were very low, indicating that Cel5B and Cel9C are unlikely to be processive [77]. Therefore, further research is needed to elucidate whether processive endoglucanases exists in *C. hutchinsonii*.

Other proteins involved in cellulose degradation were gradually characterized to examine the novel cellulose degradation strategy in *C. hutchinsonii*, including CHU_0134, CHU_1276, CHU_1277, CHU_1719, CHU_1797, CHU_1798, and CHU_2981. They were described and discussed in a previous review [13]. Subsequently, an outer membrane protein, the putative peptidoglycan-associated lipoprotein CHU_0125, was identified [84]. CHU_0125 affects the integrity of the outer membrane, resulting in the improper localization of several outer membrane proteins. The Δ*0125* mutant cannot degrade crystalline cellulose [84]. The periplasmic space proteins CHU_1165 and CHU_0052 control protein folding and maturation, thereby affecting cellulose degradation. CHU_1165 is a putative disulfide oxidoreductase closely related to outer membrane proteins' stability, activity, and folding [85]. The serine protease CHU_0052 (DegQ) acts as a chaperone-protease to facilitate the refolding or degradation of misfolded periplasmic proteins [86]. Several cytoplasmic proteins modulate cellulose degradation by affecting the anchoring or modification of CTD proteins, such as CHU_2177, an O-antigen ligase, concerning cellulose degradation. It regulates the anchoring of CTD proteins in the outer membrane and the integrity of the biofilm [52]; moreover, a glycosyltransferase (CHU_3842) is employed for the *N*-glycosylation of T9SS substrates. The Δ*3842* mutant has a weak cellulose degradation ability [53]. In addition, two regulators were identified as being involved in cellulose degradation in *C. hutchinsonii*. Wang found that a Sigma Factor (CHU_3097) can control cellulose degradation by regulating the expression of CHU_1276 [87], which is a histone-like HU protein, a transcriptional regulator that modulates the transcription of genes involved in cellulose degradation, gliding, and biofilm formation [88]. Except for T9SS, the type II secretion was investigated by Wang in *C. hutchinsonii*. Three orthologous genes encoding the major components CHU_3195 (T2S-D), CHU_3198 (T2S-G), and CHU_3199 (T2S-F) are involved in cellulose degradation [89].

Numerous studies and findings of important proteins have provided evidence of and insights into the complex mechanism of cellulose degradation in *C. hutchinsonii*. Table 2 summarizes these proteins. The key step in the predicted *C. hutchinsonii* cellulose degradation mechanism encompasses the outer membrane transporter proteins that transport individual cellulose molecules into the periplasmic space for further degradation. However, the key outer membrane transporters performing this function have not yet been identified. In *C. hutchinsonii*, the transport of individual cellulose molecules from the outer membrane into the periplasm may be similar to the starch degradation system of *Bacteroides thetaiotaomicron*. In *B. thetaiotaomicron*, SusD and SusC are employed to bind and transport the starch oligosaccharide chain across the outer membrane [90]. Nevertheless, neither SusD-like nor SusC-like proteins in *C. hutchinsonii* are necessary for cellulose degradation [91]. Therefore, to the mechanism underlying the transport of individual cellulose molecules across the outer membrane in *C. hutchinsonii* warrants further study.

3.2. T9SS affects gliding motility

Many microbes in the Bacteroidetes phylum have no flagella or type IV fimbriae, but can rapidly move on solid surfaces. This process is called gliding motility. *F. johnsoniae* is a model strain of Bacteroidetes, and its gliding mechanism has been studied for decades. *F. johnsoniae* can glide on glass, polystyrene, polytetrafluoroethylene, and agar surfaces [92,93]. At least 20 genes are involved in the cell motility of *F. johnsoniae*, including the 12 *gld* genes required for gliding, 7 *spr* genes required for colony spreading but not for the gliding of individual cells, and several *rem* genes that are functionally redundant for gliding [94,95]. Many mutations in T9SS components disrupt gliding and secretion, which suggests that the gliding motility machines are intertwined with T9SS in *F. johnsoniae* [38,96]. The gliding of *F. johnsoniae* is caused by the adhesins SprB and RemA in a left-handed helical manner [93]. Both SprB and RemA are T9SS substrates [38]. SprB is a filamentous macromolecular protein with a molecular weight of 669 kDa. Like SprB, RemA is also a cell surface protein driven to move on the cell surface [97].

Like other Bacteroidetes species, *C. hutchinsonii* does not have flagella or type IV fimbriae, but can rapidly move on solid surfaces [13]. The power provided by T9SS and the cell motility driven by T9SS substrate adhesins are the two factors that promote *F. johnsoniae* gliding. *C. hutchinsonii* has all the T9SS homologous genes and the movement-related homologous genes, indicating that the gliding mechanism of *C. hutchinsonii* may be similar to the dynamic system of *F. johnsoniae*. The deletion of T9SS components led to defective gliding in *C. hutchinsonii*. The Δ*sprP* mutant cannot spread on agar [40]. Δ*3237* (*porU*) appears defective on colony spreading. The deletion of *gldN, sprA, sprT*, and *porV*

Table 2
Genes demonstrated to be involved in cellulose utilization and/or gliding motility in *C. hutchinsonii*.

Locus	Phenotypes of mutants		Function	Reference
	Cellulose utilization	Spreading on agar		
OM proteins				
CHU_0922	No	No	Hypothetical protein with type A CTD. Participate in the degradation of crystalline cellulose and binding with cellulose	[78]
CHU_1557	Decreased		Hypothetical protein with type B CTD. Involved in the uptake of glucose at low concentrations	[79]
CHU_0125	Decreased		Peptidoglycan-associated lipoprotein. Influenced the integrity of outer membrane	[84]
CHU_3195	Decreased		T2SS D protein	[89]
CHU_3198	Decreased		T2SS G protein	[89]
CHU_3199	Decreased		T2SS F protein	[89]
Periplasmic proteins				
CHU_1165	No	No	Disulfide Oxidoreductase	[85]
CHU_0052	No	No	DegQ, chaperone-protease. Facilitate refolding or degradation of misfolded proteins	[86]
Cytoplasmic proteins				
CHU_2750	Decreased	Decreased	Histone-like protein HU. A transcriptional regulator for modulating transcription of cellulose degradation, cell motility, and biofilm formation related genes	[88]
CHU_3097	Decreased	No	Sigma factor. Regulates expression of outer membrane proteins.	[87]
CHU_2177	No	No	O-antigen ligase. Affects the localization of T9SS substrates.	[52]
CHU_3842	Decreased	Decreased	Glycosyltransferase. Involved in the *N*-glycosylation of *C. hutchinsonii*	[53]

causes defective gliding, proving that T9SS is associated with gliding in *C. hutchinsonii* [41–43]. Nevertheless, PorU is not required for the secretion of RemA and SprB in *F. johnsoniae*, and the Δ*porU* mutant has the same diffusion and gliding ability as the wild-type counterpart [98]. The Δ*porV* mutant has gliding motion and forms diffuse colonies, but lacks attachment to glass in *F. johnsoniae* [98]. The Δ*porV* mutant in *F. columnare* also retains its gliding ability [37]. In contrast, the deletion of PorV and PorU in *C. hutchinsonii* led to defective gliding, suggesting that the gliding mechanisms may differ in different species.

In *C. hutchinsonii*, the adhesins that drive gliding may not be the same as those of *F. johnsoniae*. The absence of SprB in *F. johnsoniae* leads to the formation of non-diffusible colonies on agar surfaces [99], whereas *chu_2225,* the homologous gene of *sprB*, does not affect gliding on the surface of the medium in *C. hutchinsonii* (unpublished data). There are several genes with high homology to *chu_2225* in *C. hutchinsonii* (*chu_0597*: coverage = 71%, identity = 34%; *chu_2925*: coverage = 99%, identity = 37%; *chu_2528*: coverage = 94%, identity = 28%; and *chu_2924*: coverage = 71%, identity = 34%), all of which are type B CTD proteins that may lead to functional redundancy in *sprB*. A Blast search showed that there are no credible homologues of RemA in *C. hutchinsonii*. It is possible that other adhesins in *C. hutchinsonii* that affect its gliding motility.

Moreover, lacking many other genes could lead to defective gliding in *C. hutchinsonii*. The Δ*0922*, Δ*1165*, Δ*0052*, Δ*3097*, Δ*2750*, Δ*2177*, Δ*3842*, Δ*3195*, Δ*3198*, and Δ*3199* mutants (shown in Table 2), as well as the Δ*1719*, Δ*1797*, and Δ*1798* mutants, do not form diffuse colonies [100–102], indicating that the gliding motility mechanism of *C. hutchinsonii* is very complex.

3.3. T9SS is important for ion assimilation

GldN is a core T9SS component. In *C. hutchinsonii*, the inactivation of *gldN* causes a significant growth defect in PY6 medium. Adding 0.8 mM Ca^{2+} or 0.9 mM Mg^{2+} to PY6 medium can make up the growth defects of the Δ*gldN* mutant [41]. This implies that incorporating Ca^{2+} and Mg^{2+} in *C. hutchinsonii* relies on T9SS integrity. Ca^{2+} is essential for chemotaxis, cell differentiation, twitching, swarming, gliding, transcriptional regulation of genes, and pathogenicity [103]. In turn, Mg^{2+} acts as a cofactor of hundreds of enzymes that maintain the function of ribosomes and interact very strongly with nucleic acids [104]. Most metal ions can pass through the outer membrane via passive diffusion through pore proteins. However, when the extracellular metal ion concentration is low, bacteria need to replace passive diffusion with energy-dependent methods to obtain scarce metal ions efficiently [105,106]. T6SS secrets the effector proteins TseF, TseZ, and TseM to chelate heme iron, zinc, and manganese. Subsequently, the effector proteins bind to the heme transporter HmuR, MnoT, the Fe(III)-pyochelin receptor FptA, and the porin OprF, respectively these ions are passed through the outer membrane for cell antioxidation [70–72]. In the Δ*gldN* mutant, the intracellular contents of Ca^{2+} and Mg^{2+} were reduced obviously, which further demonstrated that GldN is involved in the assimilation of trace amounts of Ca^{2+} and Mg^{2+} in *C. hutchinsonii* [41]. This may be the key reason for the difficulty in obtaining the mutants of T9SS core components in *C. hutchinsonii*.

Different T9SS component proteins have various effects on ion assimilation. The intracellular concentration of Mg^{2+} in the Δ*sprT* mutant is nearly consistent with the that of the wild-type counterpart, and the concentration of Mg^{2+} in Δ*sprA* is only slightly reduced compared with the wild-type, indicating that SprA and SprT are not essential for the assimilation of Mg^{2+} [42]. Regarding the Δ*porV* mutant, Ca^{2+} and K^{+} are required to shorten the lag phase or improve the biomass in PY6 medium [43]. The inactivation of the *porU* homolog (*chu_3237*) and the *sprP* homolog (*chu_0170*) in *C. hutchinsonii* does not cause growth defects [39,40]. The differences in ion assimilation of these T9SS components indicate the diversity of their functions. To the best of our knowledge, no study has reported that the inactivation of T9SS components affects the growth of other Bacteroidetes phylum species. Therefore, T9SS has a more extensive function in *C. hutchinsonii*.

Regarding the effect of T9SS on ion assimilation, some ion-chelating proteins might be T9SS substrates. Proteins containing the immunoglobulin (BIg-like) domain are presumed to be novel calcium-binding proteins (CaBP) [103]. The T9SS substrates CHU_0938, CHU_0939, CHU_1221, CHU_2040, and CHU_2922 in *C. hutchinsonii* are BIG-like proteins. All have a CTD and are absent on the cell surface of Δ*gldN* (unpublished data). However, the single deletion of these genes does not affect the growth state in PY6 medium, which may be due to functional redundancy. The deficiency of ion assimilation caused by the deletion of the genes mentioned above can be completely compensated.

CHU_2807 is a putative outer membrane transporter in *C. hutchinsonii*, as it contains a TolC domain. TolC can form a trimeric channel structure and is required for the secretion of various T1SS substrates, especially multidrug substrates [107,108]. The expression of CHU_2807 in the Δ*gldN* mutant is significantly decreased, and the Δ*2807* mutant has defective growth in PY6 medium [41]. Similarly, adding Ca^{2+} or

Mg^{2+} can make up for this growth defect. Therefore, CHU_2807 plays a role in ion assimilation as an outer membrane transporter; nevertheless, further research is needed to confirm this observation. Moreover, searching for metal-ion-binding proteins is necessary to determine how T9SS affects the assimilation of ions in *C. hutchinsonii*.

4. T9SS substrates

4.1. Characteristics of T9SS substrates

The C-terminal signal is a sequence of ~80 amino acids required for the secretion of T9SS substrates across the outer membrane [31–33]. The structure of the CTD includes a cleavage motif; an N-terminal Spacer region; and B, D, and E motifs [44]. According to the atomic structure analysis of RgpB and PorZ in *P. gingivalis*, the recognition signal of T9SS is not the amino acid sequence but the folded structure of CTD [109,110]. The CTD signal of RgpB comprises seven strands and is divided into two sheets. These two sheets exhibit the sandwich-like fold typical of an immunoglobulin-superfamily (IgSF) domain. PorZ is also a substrate of T9SS. The CTD of RgpB is cleaved, whereas PorZ retains its CTD. Despite the low amino acid sequence similarity between the CTDs of PorZ and RgpB, PorZ still shares the same IgSF domain as RgpB [110], which suggests that T9SS recognizes the signal is in the tertiary structure of the CTD. This conclusion may be applicable to universal CTD proteins.

Most T9SS substrates carry the classic C-terminal conserved domain Por_Secre_Tail (TIGR04183); however, some substrate proteins have different domains [33]. The adhesin SprB in *F. johnsoniae* has a Bac_Flav_CTERM domain (TIGR04131) [111]. The characterized large protein CHU_3220, which is related to the degradation of the cellulose crystallization zone in *C. hutchinsonii*, carries the CHU_C family (PFAM13585) [45]. The Bac_Flav_CTERM domain (TIGR04131) and CHU_C family (PFAM13585) are closely related. Therefore, the CTDs of the Por_Secre_Tail domain are termed type A (TIGR04183), and the CTDs with the Bac_Flav_CTERM domain and the CHU_C domain are termed type B (TIGR04131) [13,30]. In *C. hutchinsonii*, Type B CTDs are sometimes labeled as the FlgD_ig super family (CL21544).

The type A and type B CTDs in *F. johnsoniae* have different conserved motifs, with type A CTDs being conserved to a greater extent. Different conserved motifs may determine the different fates of type A and type B CTD proteins after transport. Type A CTD proteins are bound to PorP, and then delivered to the outer membrane attachment complex by the shuttle protein PorV, followed by attachment to A-LPS. Type B CTD proteins may anchor to the cell surface by binding to PorP-like proteins and may be directed to the motility apparatus [112,113]. It seems reasonable that type B CTD proteins tend to participate in gliding motility, because the adhesin sprB of *F. johnsoniae* has a type B CTD, and the genome of *F. psychrophilum* is predicted to encode 10 type B CTD proteins, nine of which are annotated as putative adhesins [37].

4.2. T9SS substrates in C. hutchinsonii

T9SS has been hailed as one of the most robust secretion systems, mainly because very large amounts of substrates are secreted via the T9SS system. The genomes of 104 different species were analyzed by Surashree, 90 of which express CTD proteins, with multiple species having more than 100 CTD proteins [33]. *C. hutchinsonii* has the greatest number of T9SS substrates in the class Cytophagia. The wide range of the molecular weight of substrate proteins also suggests the uniqueness of T9SS. Proteins as small as 7 kDa (CHU_1169) and as large as 669 kDa (Fjoh_0979, SprB) all belong to the substrates of T9SS. The number of CTD proteins encoded by the genomes and those with a molecular weight greater than 100 kDa in six species of T9SS were counted. The quantitative statistics of CTD proteins in different species are based on the published literature [36-38,114]. As predicted by Zhu, *C. hutchinsonii* carries 147 CTD proteins. Later, Veith identified 95 CTD proteins in *C. hutchinsonii* [40,44]. These two data partially overlap. Taken together, we suggest that the number of CTD proteins in *C. hutchinsonii* is 157. In *T. forsythia* ATCC43037, 26 CTD proteins were predicted by Friedrich [115]. Subsequently, six KLIKK proteases were added as CTD proteins by Ksiazek [116]. Here we suggest *T. forsythia* ATCC43037 carries 32 CTD proteins. The molecular weight (including the CTDs) was predicted using the https://web.expasy.org/compute_pi/ website. Apparently, among these strains, T9SS in C. hutchinsonii has the greatest number of substrate proteins, up to 157. Moreover, as shown in Fig. 1, *C. hutchinsonii* has the greatest number of CTD proteins larger than 100 kDa. This suggests that T9SS is powerful in *C. hutchinsonii* and allows the translocation of a large amount of macromolecular substrates across the outer membrane.

The functions of CTD proteins are important and diversified. In *P. gingivalis*, the T9SS substrates PorU and PorZ also function as T9SS components. Moreover, the T9SS substrates gingipain RgpB, HBP35, HagA, Kgp, and RgpA are effector proteins for periodontitis [29]. Porphyromonas peptidylarginine deiminase is also a T9SS substrate protein [29]. In *T. forsythia*, T9SS transports the S-layer proteins TfsA and TfsB to form an electron-dense surface layer, and various proteases, metalloproteases, and serine proteases contribute to virulence [35,116]. In the fish pathogen *F. columnare*, the T9SS substrates chondroitin sulfate lyases CslA and CslB, peptidases, and thiol-activated cytolysins act as virulence factors for columnaris disease [37,117,118]. Another fish pathogen, *F. psychrophilum*, causes bacterial cold-water disease, in which T9SS secretes various peptidases and nucleases as weapons for bacterial cold-water disease and adhesins, as well as motility proteins for gliding motility [37]. In *F. johnsoniae*, the SprB and RemA CTD proteins drive gliding motility [38]. The chitinase used for chitin degradation is also transported by T9SS in *F. johnsoniae* [119].

The function of CTD proteins in *C. hutchinsonii* was predicted and clustered based on COG (http://www.ncbi.nlm.nih.gov/COG/old/xognitor.html). As shown in Fig. 2, many CTD proteins in C. hutchinsonii were predicted to be carbohydrate transport- and metabolism-related proteins, which indicated that T9SS is essential for cellulose degradation and biomass conversion. Moreover, the functions of CTD proteins in *C. hutchinsonii* are complex and diversified. They were predicted to be involved in posttranslational modification and protein maturation; to participate in genetic activity in cells, such as cell-cycle control, division, chromosome partitioning; and to act as transporters that promote the transportation of nucleotides, lipids, amino acids, as well as intracellular trafficking, secretion, and vesiculation. Some CTD proteins were even predicted to be involved in signal transduction. To date, CTD proteins have only been verified to be associated with cellulose degradation and gliding motility in *C. hutchinsonii* [45,78,79]. Although confirming these predicted functions requires further experiments, these findings suggest that T9SS is significant for the Bacteroidetes phylum. In addition, there are a large number of T9SS substrates with functions that have not been characterized and predicted. The role of these proteins is worthy of further study, which may provide additional evidence to reveal the unique cellulose degradation strategy, gliding mechanism, and even other important physiological processes of *C. hutchinsonii*.

4.3. The CTDs of T9SS substrates are glycosylated in the periplasm

As the signal for T9SS, CTDs contribute to the secretion and localization of CTD proteins. The green fluorescent protein (GFP) and the CTD of CHU_2708 were fused to a recombinant protein to examine the function of the CTD [53]. CTD_{CHU_2708} was shown to be *N*-glycosylated in the periplasm before being transported across the outer membrane [53]. Although its molecular weight was predicted at 35 kDa, a band of about 40 kDa was detected in the periplasmic space. The protein observed at 40 kDa was determined to be glycosylated by lectin ConA and periodic acid-Shiff staining. Peptide-*N*-glycosidase F (PNGase F) can hydrolyze *N*-linked oligosaccharides by cleaving between the innermost GlcNAc

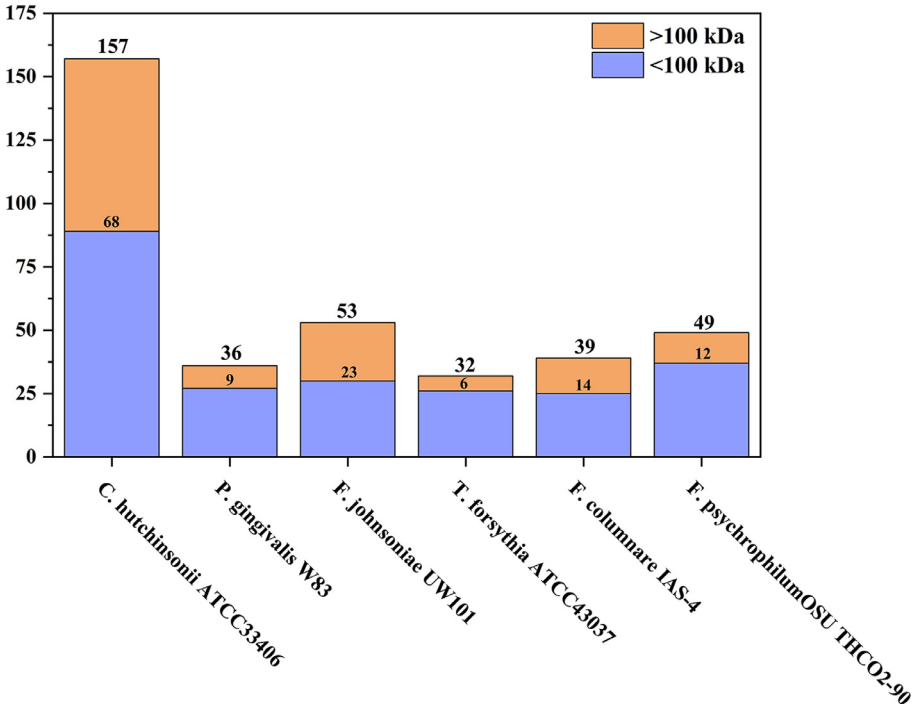

Fig. 1. Molecular weight statistics of CTD proteins in various species. The figures above the columns are the number of total CTD proteins in different species, the figures in the orange columns are the number of CTD proteins with a theoretical molecular weight larger than 100 kDa.

and asparagine. The glycosylated GFP-$CTD_{2708CTD}$ protein is sensitive to PNGase F, indicating that the fusion protein is *N*-glycosylated [53]. The glycosylation site of GFP-$CTD_{2708CTD}$ conforms to the N-X-S/T motif residue in the CTD and is in the flexible region of the CTD structure, which is consistent with previous reports on *N*-glycosylation sites in eukaryotes [120]. The glycosyltransferase *chu_3842* is homologous to *pglA* and was involved in the *N*-glycosylation of GFP-$CTD_{2708CTD}$. *pglA* is a member of the *pgl* gene cluster and contributes to the *N*-glycosylation of *Campylobacter jejuni* [121]. The Δ*3842* mutant is defective in cellulose degradation and cell motility, and the bacterial stress resistance of the mutant is weakened compared with that of the wild-type. DegQ can degrade misfolded proteins in the periplasmic space [86]. The transcription level of DegQ is significantly increased in *N*-glycosylation site mutants, suggesting that *N*-glycosylated modification affects the folding and stability of proteins in *C. hutchinsonii*. Furthermore, the mutation of the *N*-glycosylated site results in the absence of the fusion protein on the outer membrane, indicating that the *N*-glycosylation of the CTD may be linked to the translocation of CTD proteins to the cell surface.

According to previous reports, the N-glycosylation systems of bacteria are specific to Proteobacteria and Campylobacterota [122]. It was first identified in *C. jejuni* as lipid carrier-mediated en bloc protein glycosylation [123]. *N*-glycosylation of *C. jejuni* was achieved via the *pgl* gene cluster to synthesize a heptaxyoligosaccharide [124]. After LLO is transferred to the periplasmic space by the PglK flippase, the PglB oligosaccharyltransferase releases oligosaccharides into the periplasm or transfers them to Asn-residues of the receptor protein sequence D/E-X1-N-X2-S/T (X1 and X2≠Pro) [125]. The destruction of *N*-glycosylation pathways affects the pathogenicity, nitrate reductase activity, protein quality and stability, nutrient transfer, stress reactions, and antibiotic resistance of *C. jejuni* [126,127].

N-glycosylated fusion proteins are 5 kDa heavier than the theoretical molecular weight of the non-glycosylated counterparts in *C. hutchinsonii*, suggesting that complex glycans might be transferred to proteins in *C. hutchinsonii*. Therefore, *N*-glycosylation in *C. hutchinsonii* is similar to that detected in *C. jejuni*, but different from the disaccharide modification by the stepwise *N*-glycosylation pathway in *Haemophilus influenzae* [128,129]. Moreover, *N*-glycosylation of both *C. hutchinsonii* and *C. jejuni* occurs in the periplasmic space. However, there are some differences between *C. hutchinsonii* and *C. jejuni*. First, there is no *pgl* gene cluster in the genome of *C. hutchinsonii*, and *N*-glycosylation has different oligosaccharide chains compared with *C. jejuni* [53]. PNGase F cannot eliminate the oligosaccharide chains of *C. jejuni* because of the unique linking sugar bacillosamine [130]. However, the *N*-glycosylation of *C. hutchinsonii* can be cleaved by PNGase F, indicating that the first sugar structure linked to Asn-in *C. hutchinsonii* is GlcNAc. Second, the *N*-glycosylation motif of *C. hutchinsonii* is consistent with the motif recognized by the eukaryotic *N*-glycosylation system, but different from the D/E-X1-N-X2-S/T sequon of *C. jejuni*.

Notably, no glycosylation on the CTDs has been found in other species of Bacteroidetes. Except for D696A and Δ692–702, the mutation of all potential glycosylation sites in the CTD of RgpB had no effect on its secretion and anchor in *P. gingivalis* [131]. When GFP fused with the CTD of HBP35 was expressed, no GFP-related proteins were detected in the cytoplasm/periplasmic fraction in *P. gingivalis* [32]. In *F. johnsoniae*, the CTD of RemA has no obvious modification [33]. Therefore, additional studies are needed to demonstrate whether *N*-glycosylation systems are universal in the Bacteroidetes phylum. Nevertheless, studies on *N*-glycosylation in *C. hutchinsonii* extend the *N*-glycosylation system from Proteobacteria and Campylobacterota to Bacteroidetes. Generally, mature T9SS substrate proteins are removed from the CTD after being transported to the cell surface. Therefore, glycosylation of the CTD may contribute to the recognition and translocation of CTD proteins by T9SS. In eukaryotes, glycans of proteins act as labels to help the recognition between proteins [132,133], but no similar function has been reported in prokaryotes. Xie proved that *N*-glycosylation in the CTD is essential for the secretion and localization of *C. hutchinsonii* CTD proteins. Moreover, *N*-glycosylation profoundly impacts cellulose degradation, cell motility, and stress resistance in *C. hutchinsonii* [53]. The wide range of reported phenotypic characteristics enriched our understanding of the bacterial *N*-glycosylation system.

O-glycosylation in the Bacteroidetes phylum was first described in *Bacteroides fragilis* [134], and was then detected in diverse families of this phylum [135]. The enzymes involved in this *O*-glycosylation system are encoded by the *lfg* gene cluster [136]. In *B. fragilis*, the glycosylation site motif was identified as (D)(S/T)(A/I/L/V/M/T), with the glycan linked to S or T [136]. According to the conserved motifs, a large num-

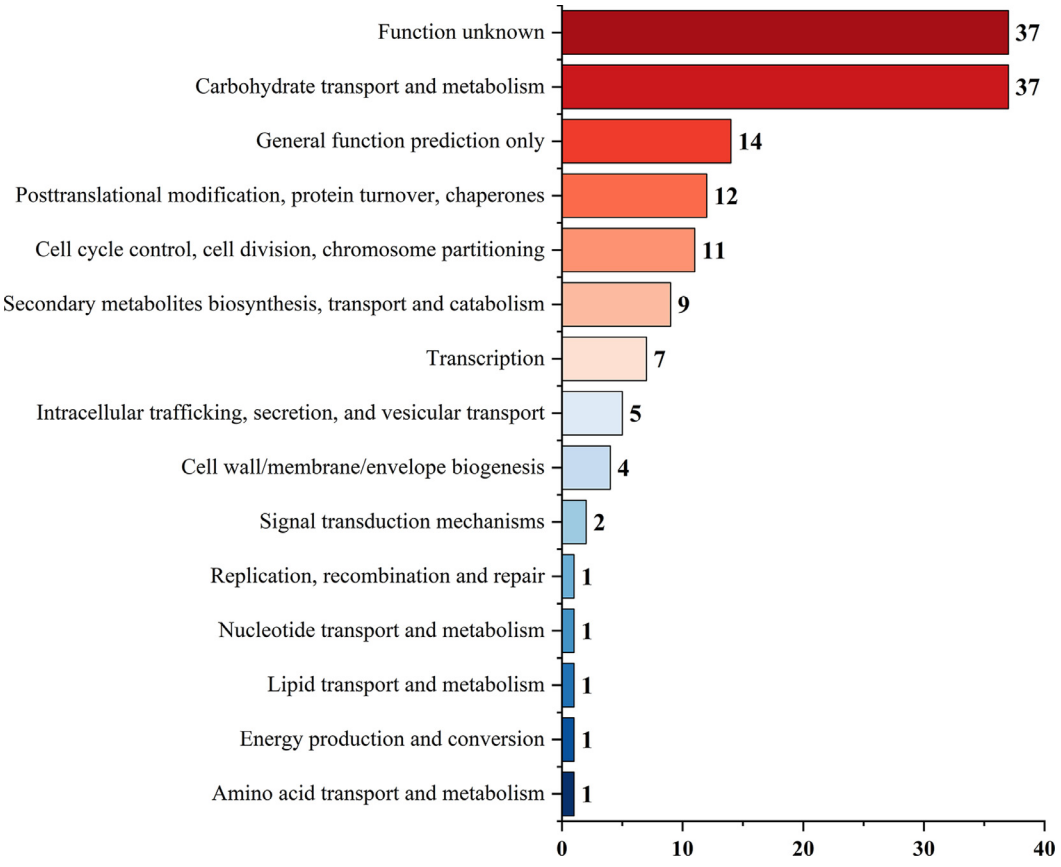

Fig. 2. Histogram of CTD protein functions based on COG annotation and Clustering in *C. hutchinsonii*.

ber of glycoproteins were predicted, and 20 glycoproteins have been identified in *B. fragilis*. *O*-glycosylation may contribute to protein folding, protein-protein interactions, and peptide degradation. The latest research reported by Veith identified 257 putative glycosylation sites in 145 glycoproteins [137]. Moreover, 312 glycosylated sites in 145 glycoproteins were identified in *T. forsythia*, and their glycosylated motifs were expanded to (D)(S/T)(A/I/L/V/M/T/S/G/C/F) [138].

T. forsythia is covered with a two-dimensional crystal surface layer (S-layer) composed of TfsA (Tanf_03370) and TfsB (Tanf_03375) [139]. The S-layer can delay the recognition of the bacterium by the host innate immune system [140,141]. In *T. forsythia*, T9SS mutants lack a surface layer, confirming that TfsA and TfsB are T9SS substrates [35,142,143]. In T9SS mutants, TfsA and TfsB are trapped within the periplasm and are *O*-glycosylated via a general glycosylation pathway operating in Bacteroidetes [143,144]. A recent study in our laboratory showed that the CHU_1107 cellulase, a type A CTD protein, was *O*-glycosylated at CTD (unpublished data). This indicates that the CTDs of T9SS substrates undergo not only *N*-glycosylation, but also *O*-glycosylation.

4.4. The anchoring of T9SS substrates is related to LPS

After CTD proteins pass through the outer membrane, they are anchored to the cell surface or secreted into the extracellular milieu. The anchoring of CTD proteins has been studied well in *P. gingivalis*. T9SS substrates have a unique anchoring mode in *P. gingivalis*, which possesses two different lipopolysaccharides: the conventional LPS containing an *O*-polysaccharide and A-LPS with an anionic polysaccharide repeated unit; moreover, A-LPS carries a phosphorylated branched chain mannan [48,145]. The epitope for the MAb-1B5 antibody includes the Manα1–2Manα1-phosphate portion of APS. MAb-1B5 has specific immune responses to the CTD protein RgpB, indicating that RgpB is modified with A-LPS in *P. gingivalis* [47,48]. RgpB is transported across the outer membrane via T9SS, the Gram-negative sortase PorU recognizes and cuts the CTD sequence, and mature substrates are bound to A-LPS by an unknown structural short peptide of 648 Da at the cleavage site and anchored to the cell surface [66]. A-LPS yields a diffuse modified band that is 20 kDa larger than the theoretical molecular weight of the protein [146]. In specific mutants, O-LPS is produced, and A-LPS is absent, but CTD proteins are not attached to the cell surface, suggesting that CTD proteins are not modified with O-LPS [147]. These results suggest that A-LPS is the anchor that links CTD proteins to the outer membrane.

According to the latest study on *C. hutchinsonii*, CHU_2177 was identified as an O-antigen ligase. In the Δ*2177* mutant, the O-antigen of LPS is absent. Moreover, 67% of CTD proteins are not properly anchored to the outer membrane in the Δ*2177* mutant compared with that observed for the wild-type counterpart, including six speculated endocellulases (CHU_3727, CHU_1240, Cel9A, Cel9B, Cel9D, and Cel9E) and three glycosidehydrolase-family proteins (CHU_1155, CHU_0353, and CHU_2379) [52]. As detected by Western blotting, the putative cell surface endoglucanase Cel9A is absent on the cell surface but present in the culture medium of the Δ*2177* mutant. Concomitantly, the theoretical molecular weight of Cel9A is predicted at 105 kDa, but the mature Cel9A has a molecular weight greater than 130 kDa [41]. Several other cellulases also have a molecular weight of 20–30 kDa larger than the predicted values in the outer membrane of *C. hutchinsonii*. Moreover, in the recombinant protein GFP-CTD$_{2708CTD}$, the membrane-associated CTD has a new modification that differs from *N*-glycosylation because the molecular weight is higher than the theoretical value. However, the modified GFP cannot be digested by PNGase F [53]. Therefore, the molecular weight increase of these CTD proteins that occurs on the outer membrane is closely related to the O-antigen. However, there is currently no evidence of the presence of A-LPS in *C. hutchinsonii*. Previous studies have shown that the antibody of A-LPS has a very weak cross-reaction with proteins from *C. hutchinsonii*, indicating that it may not

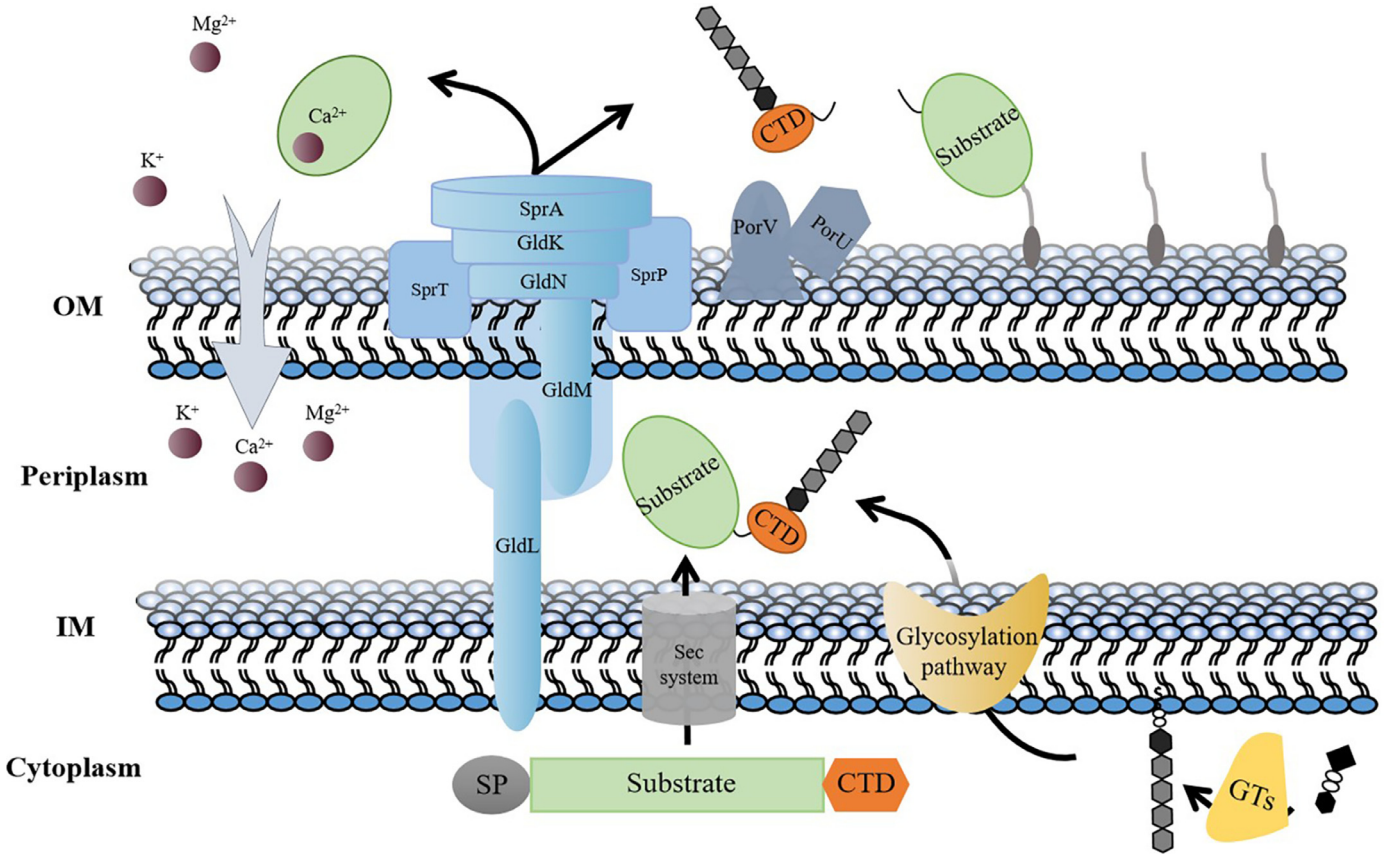

Fig. 3. Model of T9SS functions and the glycosylation of T9SS substrates in *C. hutchinsonii*. The Sec system transports the CTD protein to the periplasmic space, where CTDs are *N*- or *O*-glycosylated. Then, the T9SS recognizes the CTD signals and transports substrates across the outer membrane. PorU cleaves the CTDs, and the mature CTD proteins are linked to the cell surface by LPS. Some substrates chelate metal ions and deliver them to receptor proteins on the outer membrane.

have A-LPS or has an A-LPS that is different from that of *P. gingivalis* [44]. The exact anchoring mechanism of CTD proteins in *C. hutchinsonii* deserves further study.

5. Summary and future directions

Multiple component genes of T9SS have been deleted and characterized based on the development of genetic manipulation techniques and the optimization of culturing methods (Table 1). It has been suggested that T9SS is involved in cellulose degradation, gliding motility, and ion assimilation in *C. hutchinsonii*. The transport of cellulase and other important outer membrane proteins to the cell surface via T9SS also demonstrated the uniqueness of the degradation mechanism of *C. hutchinsonii* cells, which occurs via direct contact with cellulose. T9SS in *C. hutchinsonii* has abundant substrate proteins, and more than 43% of the CTD proteins have molecular weights greater than 100 kDa (Fig. 1), indicating that T9SS is powerful in *C. hutchinsonii.* These CTD proteins in *C. hutchinsonii* may participate in various functions, such as posttranslational modification, transportation, and signal transduction (Fig. 2), which was a surprising and unexpected finding. Moreover, in addition to the anchoring and accompanying modification of mature CTD proteins by LPS on the cell surface, *N*-glycosylation or *O*-glycosylation of substrate proteins in the periplasmic space was also observed. Furthermore, CTDs act as a signal recognized by T9SS. The glycosylation that occurs on CTDs may contribute to the recognition and transport of CTD proteins, which has not been reported in prokaryotes. These studies propose a model of T9SS functions and the glycosylation of T9SS substrates in *C. hutchinsonii* (Fig. 3). However, many questions remain that need to be solved in the future. First, there seems to be a variety of modification and anchoring forms of CTD proteins in *C. hutchinsonii*. What are the effects of these modifications on the function of CTD proteins? Is *N*-glycosylation common in *C. hutchinsonii* and in other Bacteroidetes? Second, a large proportion of CTD proteins in *C. hutchinsonii* are uncharacterized. Studying the functions of these substrates will provide tremendous help to understand the unique physiological functions of *C. hutchinsonii*. Moreover, in *C. hutchinsonii*, the outer membrane transporter that translocates individual cellulose molecules into the periplasmic space, the adhesins involved in gliding motility, and the outer membrane receptor proteins involved in ion assimilation remain unidentified. Mass spectrometry techniques and proteomics analyses would be particularly helpful for elucidating these questions fully.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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