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N-Glycosylation of a Cargo Protein C-Terminal Domain Recognized by the Type IX Secretion System in *Cytophaga hutchinsonii* Affects Protein Secretion and Localization

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ABSTRACT *Cytophaga hutchinsonii* is a Gram-negative bacterium belonging to the phylum *Bacteroidetes*. It digests crystalline cellulose with an unknown mechanism and possesses a type IX secretion system (T9SS) that can recognize the C-terminal domain (CTD) of the cargo protein as a signal. In this study, the functions of the CTD in the secretion and localization of T9SS substrates in *C. hutchinsonii* were studied by fusing the green fluorescent protein (GFP) with the CTD from CHU_2708. The CTD is necessary for the secretion of GFP by *C. hutchinsonii* T9SS. The GFP-CTD_{CHU_2708} fusion protein was found to be glycosylated in the periplasm, with a molecular mass about 5 kDa higher than that predicted from its sequence. The glycosylated protein was sensitive to peptide-*N*-glycosidase F, which can hydrolyze *N*-linked oligosaccharides. Analyses of mutants obtained by site-directed mutagenesis of asparagine residues in the N-X-S/T motif of CTD_{CHU_2708} suggested that *N*-glycosylation occurred on the CTD. CTD *N*-glycosylation is important for the secretion and localization of GFP-CTD recombinant proteins in *C. hutchinsonii*. Glycosyltransferase-encoding gene *chu_3842*, a homologous gene of *Campylobacter jejuni pglA*, was found to participate in the *N*-glycosylation of *C. hutchinsonii*. Deletion of *chu_3842* affected cell motility, cellulose degradation, and cell resistance to some chemicals. Our study provided evidence that the CTD as the signal of T9SS was *N*-glycosylated in the periplasm of *C. hutchinsonii*.

IMPORTANCE The bacterial *N*-glycosylation system has previously been found only in several species of *Proteobacteria* and *Campylobacterota*, and the role of *N*-linked glycans in bacteria is still not fully understood. *C. hutchinsonii* has a unique cell contact cellulose degradation mechanism, and many cell surface proteins, including cellulases, are secreted by the T9SS. In this study, we found that *C. hutchinsonii*, a member of the phylum *Bacteroidetes*, has an *N*-glycosylation system. Glycosyltransferase CHU_3842 was found to participate in the *N*-glycosylation of *C. hutchinsonii* proteins and had effects on cell resistance to some chemicals, cell motility, and cellulose degradation. Moreover, *N*-glycosylation occurs on the CTD translocation signal of T9SS. The glycosylation of the CTD appears to play an important role in affecting T9SS substrate transportation and localization. This study enriched our understanding of the widespread existence and multiple biological roles of *N*-glycosylation in bacteria.

KEYWORDS *Cytophaga hutchinsonii*, T9SS, glycosylation, CTD, protein secretion

Protein secretion systems are crucial for bacteria to grow and survive, including getting nutrients and responding to various extracellular milieux (1). The recently discovered type IX secretion system (T9SS) is exclusively widespread in the *Bacteroidetes* phylum (2). It involves the secretion of abundant proteins that play important roles in the survival of bacteria, such as pathogenicity related to soluble or cell-associated peptidases in *Porphyromonas gingivalis*, motility required for cell surface adhesins in *Flavobacterium johnsoniae*, and obtaining nutrition from the environment through other hydrolytic enzymes (3). It

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is a common feature that most cargo proteins of the T9SS have an N-terminal signal peptide (SP) and a conserved C-terminal domain (CTD) (4). The N-terminal signal peptide allows substrates to be transported across the plasma membrane by the Sec system. The CTD, the signal recognized by the T9SS, assists cargo proteins across the outer membrane (5). The majority of T9SS CTDs are divided into two protein domain families, the family TIGR04183 (type A CTDs) and the family TIGR04131 (type B CTDs) (6, 7). Many studies have focused on type A CTDs of proteinaceous virulence factors of *P. gingivalis* (8, 9). Most CTDs are removed by PorU sortase during or after secretion in *P. gingivalis* (10). After CTDs detach from the cargo proteins, some cargo proteins are anchored to the cell surface by anionic lipopolysaccharide (A-LPS) modification, while other T9SS substrates are released into the extracellular space in soluble form (11). Type A CTDs of *P. gingivalis* contain approximately 80 amino acids and have five conserved sequential motifs (2, 4). A recent study has shown that the two-terminal-β-strand structure of the type A CTD of RgpB in *P. gingivalis* may contain the signal recognized by the T9SS (5). Type B CTDs are very different in sequence from type A CTDs, and there have been relatively few studies on them (9). The C-terminal region (218 amino acids) of the type B CTD of gliding motility adhesin SprB is capable of targeting proteins to be secreted by the T9SS in *F. johnsoniae* (12).

Cytophaga hutchinsonii is an aerobic Gram-negative cellulolytic bacterium and widely distributed in the soil. Different from free cellulases or cellulosome strategies, its mechanism of cellulose hydrolysis is unique and mysterious (13). It relies heavily on cell surface-localized cellulases and other factors to digest crystalline cellulose (14, 15). *C. hutchinsonii* as a member of the *Bacteroidetes* has all homologous genes encoding T9SS component proteins (16). Deletion of T9SS component protein PorU or SprP causes defects in the secretion of CTD proteins, cellulose degradation, and gliding motility in *C. hutchinsonii* (17, 18). Recently, we found that the T9SS components GldN, SprA, and SprT of *C. hutchinsonii* also take part in ion assimilation (19, 20). Bioinformatic analysis showed that *C. hutchinsonii* has the largest amount of CTD proteins in the phylum *Bacteroidetes* (4). There are at least 147 CTD proteins processed by the T9SS, of which 118 contain type A CTDs, including carbohydrate binding proteins, cellulases, and other hypothetical proteins (18). CHU_3220, with a CHU_C domain closely related to type B CTDs, is secreted to the outer membrane by the T9SS and participates in utilization of the cellulose crystalline region (21). However, the features and functions of CTDs of T9SS substrates in *C. hutchinsonii* have rarely been reported. A previous study found that the cell envelope fraction of *C. hutchinsonii* has weak reactivity with the A-LPS antibody MAb-1B5 of *P. gingivalis* (4). How these T9SS substrates attach to the outer membrane of *C. hutchinsonii* is worthy of further study.

CHU_2708, with a typical type A CTD, is the only reported T9SS cargo protein with a clear CTD cleavage site in *C. hutchinsonii* (4). In this study, the CTD of CHU_2708 was linked to the C terminus of green fluorescent protein (GFP) to study its function in the secretion of T9SS cargo proteins. We found that CTD_{CHU_2708} in the fusion protein was glycosylated in the periplasm of *C. hutchinsonii* and that glycosylation affects the secretion and localization of the fusion protein. This study provides insights on the role of glycosylation of type A CTDs in *C. hutchinsonii* T9SS substrates, which may help to explain the recognition mechanism of T9SS in the phylum *Bacteroidetes*.

RESULTS

The CTD in the GFP-CTD_{CHU_2708} is necessary for its secretion by the T9SS in *C. hutchinsonii*. It is a common feature that N-terminal signal peptides assist T9SS cargo proteins across the cytoplasmic membrane by the Sec system, and CTDs target T9SS substrates to the outer membrane in the phylum *Bacteroidetes* (22). Though *C. hutchinsonii* has the largest number of CTD-containing proteins among members with the novel secretion system (2), the hypothetical protein CHU_2708 is the only reported T9SS substrate with a defined CTD cleavage site. The typical type A CTD of CHU_2708 contains 88 amino acid residues from the C terminus (4). Moreover, a previous study reported that the length of the CTD cleavage region affects the modification and anchorage of T9SS cargo proteins on the cell surface (23). In this study, the N-terminal

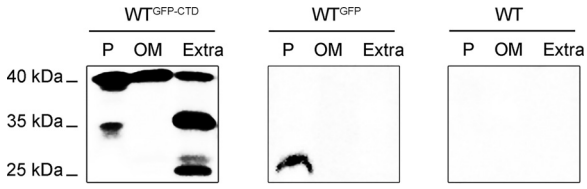

FIG 1 The CTD in GFP-CTD_{CHU_2708} is necessary for its secretion by the T9SS in *C. hutchinsonii*. The periplasmic (P), outer membrane (OM), and extracellular (Extra) proteins of the different *C. hutchinsonii* expression strains were identified by Western blotting with anti-GFP antibody. The wild type (WT) was used as the control. The amount of protein loaded in each lane came from cells of equal culture volumes.

signal peptide and C-terminal 110 amino acids from CHU_2708 were fused to the N and C termini of the green fluorescent protein (GFP), respectively, and the recombinant protein (signal peptide-GFP-CTD_{CHU_2708}) was expressed in *C. hutchinsonii* to explore the function of the CTD. GFP antibody was used to detect the secretion and localization of the GFP recombinant protein. Western blotting results showed that GFP-related proteins were found in the outer membrane and extracellular fraction of the wild-type (WT) strain containing $promoter_{CHU\_2708}$-$signal\ peptide_{CHU\_2708}$-GFP-$CTD_{CHU\_2708}$ ($WT^{GFP\text{-}CTD}$). However, when GFP only with the signal peptide was expressed in *C. hutchinsonii*, GFP (25 kDa) was detected only in the periplasm of the WT strain containing $promoter_{CHU\_2708}$-$signal\ peptide_{CHU\_2708}$-GFP ($WT^{GFP}$) (Fig. 1). These results showed that the CTD of CHU_2708 facilitated the translocation of GFP-related proteins across the outer membrane. Protein bands with a molecular size as predicted by the GFP-CTD_{CHU_2708} polypeptide sequence of 35 kDa were found in the periplasm and culture medium. They were designated GFP-CTD_{CHU_2708} fusion proteins. We previously found that PorU sortase of *C. hutchinsonii* is involved in removing CTDs of T9SS cargo proteins (17). Both the GFP-CTD_{CHU_2708} fusion protein and GFP were detected in the culture fluid of the $WT^{GFP\text{-}CTD}$ strain, revealing that the CTD of CHU_2708 was removed after secretion. It was unexpected to find that a GFP-related protein of about 40 kDa occurred in the periplasm of the $WT^{GFP\text{-}CTD}$ strain, which is about 5 kDa bigger than the GFP-CTD_{CHU_2708} fusion protein. Moreover, protein bands of approximately 40 kDa also appeared in the outer membrane and extracellular fraction of the $WT^{GFP\text{-}CTD}$ strain, suggesting that recombinant GFP might be in a modified form after secretion. So, we designated the protein (40 kDa) on the outer membrane modified GFP.

The GFP-CTD_{CHU_2708} fusion protein may be *N*-glycosylated in the periplasm. Previous studies reported that T9SS cargo proteins were extensively modified by A-LPS for anchoring after outer membrane translocation (2). It is surprising that the protein around 40 kDa was also detected in the periplasm of the $WT^{GFP\text{-}CTD}$ strain. In addition, this unique protein could not be detected in the cytoplasm of the $WT^{GFP\text{-}CTD}$ strain (Fig. S1). Pierce anti-GFP magnetic agarose beads were used to purify the GFP-related proteins from the periplasmic fraction of the $WT^{GFP\text{-}CTD}$ strain. Then the purified proteins were analyzed by liquid chromatography-tandem mass spectrometry. The results showed that both the proteins of 40 kDa and 35 kDa contained peptide fragments derived only from GFP and CTD_{CHU_2708}. Glycosylation is one of the most prevalent modification forms in protein processing (24). In order to determine whether the protein of 40 kDa in the periplasmic space of the $WT^{GFP\text{-}CTD}$ strain was glycosylated, the purified GFP-related proteins were assayed by periodate-Schiff staining. As shown in Fig. 2A, the protein of 40 kDa had a positive staining reaction, while the protein of 35 kDa was not stained. We also used the lectin affinity approach for further verification, and the results showed that only the protein of 40 kDa reacted with concanavalin A (ConA). These results proved that the GFP-CTD_{CHU_2708} fusion protein was glycosylated to 40 kDa in the periplasm of the $WT^{GFP\text{-}CTD}$ strain. Then the periplasmic space glycoprotein (40 kDa) was designated glycosylated GFP. Peptide-*N*-glycosidase F (PNGase F) is the most effective enzyme to remove almost all *N*-linked oligosaccharides from asparagine residues of glycoproteins (25). For further study, the periplasmic proteins of

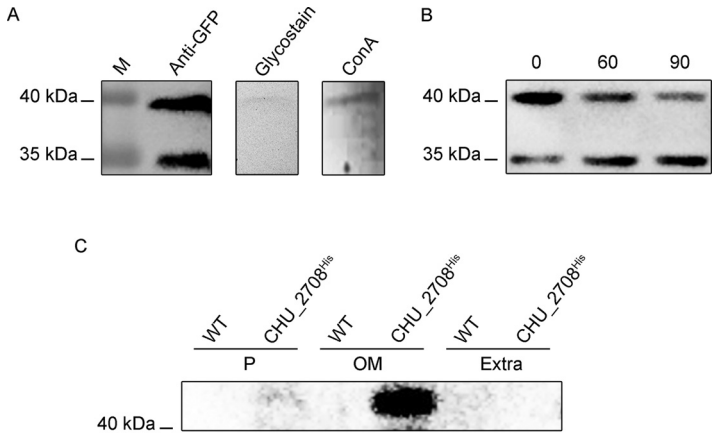

FIG 2 Glycosylation of the GFP-related proteins in the *C. hutchinsonii* $WT^{GFP\text{-}CTD}$ strain. (A) The purified proteins from the periplasmic fraction of $WT^{GFP\text{-}CTD}$ were separated by SDS-PAGE, detected with the anti-GFP antibody, stained with periodate-Schiff, or probed with ConA lectin. Lane M, molecular mass marker. (B) Periplasmic proteins of the $WT^{GFP\text{-}CTD}$ separated by SDS-PAGE after treatment with PNGase F for 0 min, 60 min, and 90 min and then detected with anti-GFP antibody. (C) The periplasmic, outer membrane, and extracellular proteins of the WT and CHU_2708^{His} strains were Western blotted with the anti-His tag.

the $WT^{GFP\text{-}CTD}$ strain were subjected to PNGase F treatment. Western blot results showed that the glycosylated GFP gradually decreased when being treated with PNGase F, while the content of the GFP-CTD_{CHU_2708} fusion protein (35 kDa) increased over time (Fig. 2B). Glycosylated GFP is susceptible to PNGase F enzymatic deglycosylation. The results not only confirmed that it is a glycoprotein but also suggested that it may be *N*-glycosylated.

To determine the mature form of T9SS substrate CHU_2708 in *C. hutchinsonii*, a His tag was added after its signal peptide. Different cellular fraction lysates were analyzed by Western blotting using antibody to His tag. CHU_2708 could only be detected in the outer membrane fraction, and its molecular weight was higher than the predicted 33 kDa, indicating that the mature CHU_2708 was modified on the outer membrane (Fig. 2C). The modified CHU_2708 could not react with PNGase F, suggesting that the modification form of T9SS substrate on the cell surface is different from that of the periplasm.

Potential *N*-glycosylation sites of CTD_{CHU_2708}. As shown in Fig. 1, glycosylation occurred only when GFP was fused to CTD_{CHU_2708}, so glycosylation may occur on the CTD region. Glycans are usually attached to asparagine residues in the N-X-S/T amino acid motif (X $\neq$ P) in the *N*-glycosylation system (26). Three asparagine residues (N273, N276, and N296) of CTD_{CHU_2708} are potential *N*-linked glycosylation sites conforming to the conservative N-X-S/T motif (Fig. 3A). Site-directed mutagenesis was then used to individually mutate asparagine to glutamine. The mutation of N276 in the NVT motif resulted in the apparent disappearance of the glycosylated GFP and GFP-CTD_{CHU_2708} fusion protein in the periplasm (Fig. 3B). None of the GFP-related proteins could be detected on the outer membrane or the extracellular fraction of the $N^{276}Q$ mutant strain (Fig. 3C and D). When N296 in the NGS motif was mutated, compared with the $WT^{GFP\text{-}CTD}$ strain, the glycosylated GFP in the periplasmic space was reduced (Fig. 3B). There was no modified GFP on the outer membrane of the $N^{296}Q$ strain and less GFP-CTD_{CHU_2708} fusion protein and GFP in the extracellular milieu (Fig. 3C and D). The changing of N273 in the NNS motif to glutamine had no significant effect on the glycosylated GFP in the periplasm of the $N^{273}Q$ strain (Fig. S2A). These results showed that the site N296 of CTD_{CHU_2708} had an effect on the glycosylated GFP. The destruction of site N296 of CTD_{CHU_2708} led to defective secretion and localization of GFP-related proteins, suggesting that the glycosylation of CTD_{CHU_2708} may play roles in substrate recognition and transportation of T9SS. Moreover, site N276 may also affect the stability of periplasmic space GFP-related proteins in *C. hutchinsonii*, because the mutation of

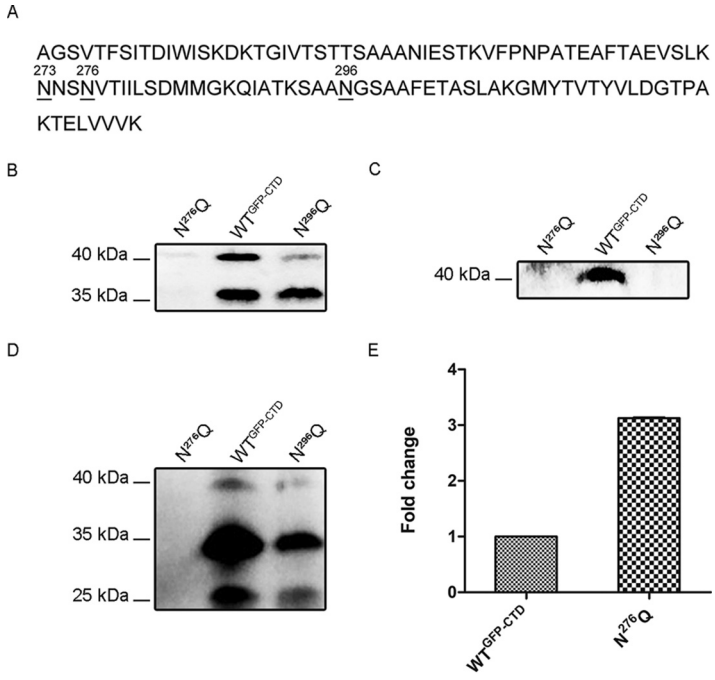

FIG 3 Site-directed mutagenesis of CTD_{CHU_2708} and transcription level of *C. hutchinsonii degQ* in the mutant. (A) Amino acid sequence of the CTD of CHU_2708. The residues underlined were mutated to glutamine. The asparagine residues within the conserved N-X-S/T amino acid motif were numbered. (B to D) The periplasmic protein (B), outer membrane proteins (C), and extracellular proteins (D) of the $WT^{GFP\text{-}CTD}$, $N^{276}Q$, and $N^{296}Q$ strains were detected with anti-GFP antibody. (E) Transcription level of *degQ* in the $WT^{GFP\text{-}CTD}$ and $N^{276}Q$ mutant strains. The mean values and standard deviations from three biological replicates are shown.

N276 caused disappearance of all GFP-related proteins in the periplasm of $N^{276}Q$. Proteins of the HtrA family are effective in removing misfolded or damaged proteins in the periplasm (27). We previously reported that CHU_0052 (DegQ) is involved in periplasmic protein quality control, and the high expression level of *degQ* reflects the protein folding pressure in the periplasmic space (20, 28). We found that the expression level of *degQ* in the $N^{276}Q$ mutant strain increased 3 times compared with that in the $WT^{GFP\text{-}CTD}$ strain when the unique asparagine site N276 of CTD_{CHU_2708} was mutated (Fig. 3E). The result supported that the site N276 of CTD_{CHU_2708} may contribute to the stability of GFP-related proteins in the periplasm.

CHU_3842 participated in the *N*-glycosylation system of *C. hutchinsonii* proteins. The mutation of the two asparagine residues in the N-X-S/T amino acid motif of CTD_{CHU_2708} led to the disappearance or decrease of glycosylation of the GFP-CTD_{CHU_2708} fusion protein. These phenomena hint that *C. hutchinsonii* has an *N*-glycosylation system. The gastrointestinal pathogen *Campylobacter jejuni* was the first bacterium demonstrated to have a well-characterized *N*-linked glycosylation pathway (29). A 16-kb *pgl* (protein glycosylation) gene cluster encodes enzymes that are required for *N*-glycan synthesis and protein linkage in *C. jejuni* (30). There are 5 glycosyltransferases involved in the *N*-glycosylation system of *C. jejuni*, PglA, PglC, PglH, PglI, and PglJ (31). Bioinformatics analysis showed that PglH and the key oligosaccharyltransferase PglB are not found in *C. hutchinsonii*. Moreover, the amino acid sequence identity of PglI and PglJ between *C. hutchinsonii* and *C. jejuni* is very low. In addition, the homologous genes of *C. jejuni pglA* and *pglC* were predicted to be *chu_3842* and *chu_1211* in *C. hutchinsonii*. However, the deletion of *C. hutchinsonii* CHU_1211, which has a 43% amino acid sequence identity with *C. jejuni* PglC, had no significant effect on *N*-glycosylated proteins (data not shown). PglA is responsible for adding *N*-acetylgalactosamine (GalNAc) residues on Und-P-diNAcBac in *C. jejuni* (29). There is a 28% amino acid sequence identity between *C. jejuni* PglA and CHU_3842 (Fig. 4A). In addition, glycosyltransferase CHU_3842 and *C. jejuni* PglA have the same conserved GT4_CapM-like functional domain.

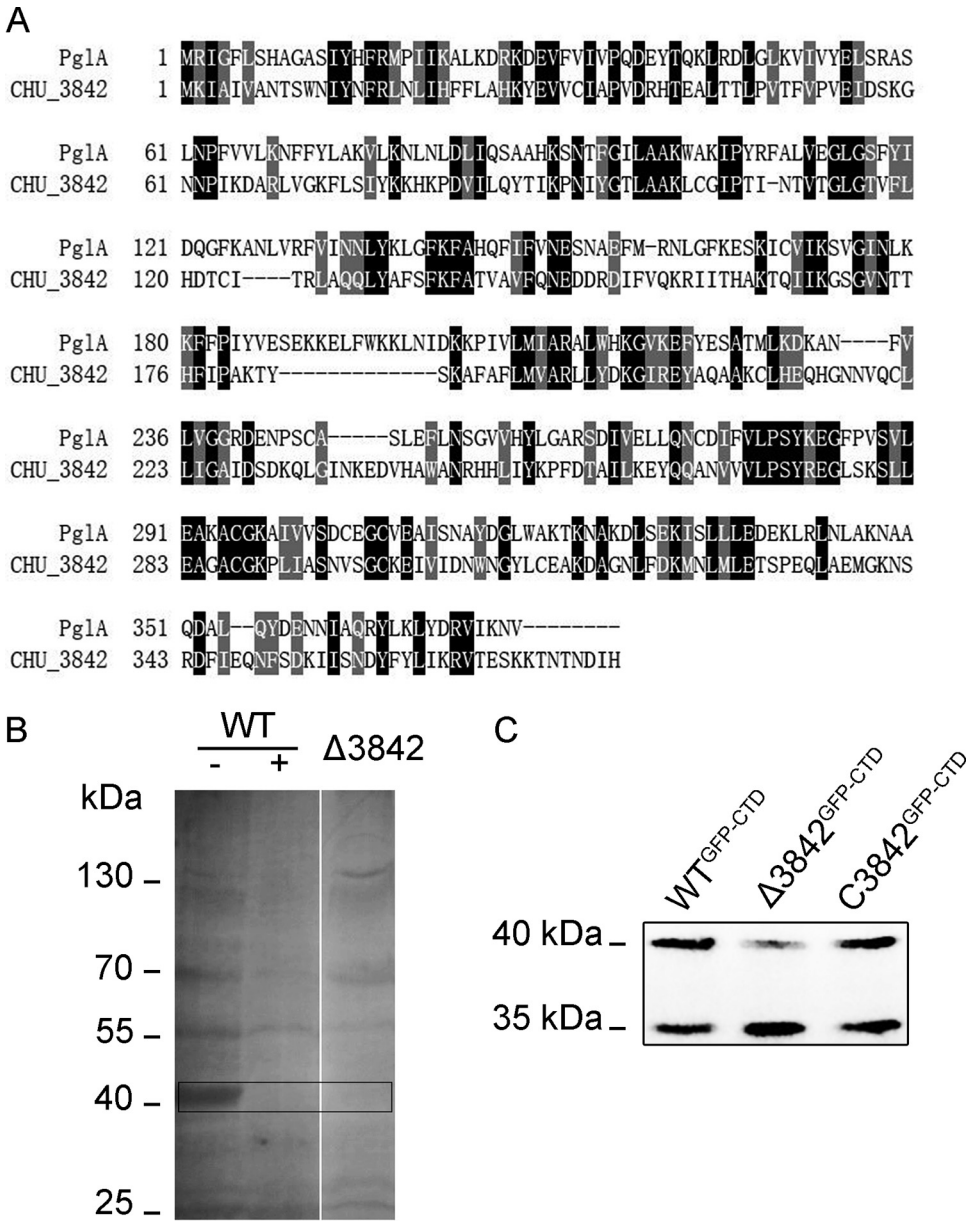

FIG 4 CHU_3842 is involved in the *N*-glycosylation of *C. hutchinsonii* proteins. (A) Sequence alignment of PglA from *C. jejuni* and CHU_3842. Black and gray shading shows identical and similar amino acid residues, respectively. The lines show the gaps between the two sequences. (B) The periplasmic glycoproteins of the Δ3842 mutant and WT. ConA lectin was used to enrich the periplasmic proteins of the WT and Δ3842 strains, treated with PNGase F (+) or not (-), then separated by SDS-PAGE, and probed with ConA lectin. (C) The periplasmic fraction proteins of the $WT^{GFP\text{-}CTD}$, $\Delta3842^{GFP\text{-}CTD}$, and $C3842^{GFP\text{-}CTD}$ strains were detected with anti-GFP antibody.

To explore the role of CHU_3842 in the glycosylation system of *C. hutchinsonii*, the linear DNA double-crossover method was used to knock it out. The periplasmic glycoproteins of the wild type and the Δ3842 mutant were enriched by ConA lectin affinity chromatography. As lectin Western blotting showed, there were many glycoproteins in the periplasmic space of the wild type, especially the glycoproteins around 40 kDa, and they disappeared after PNGase F treatment (Fig. 4B), indicating that these proteins may be *N*-glycosylated. The periplasmic glycoproteins of the Δ3842 mutant were much less than that of the wild type. No glycoproteins at 40 kDa were found in the Δ3842 mutant. When the recombinant GFP (GFP-CTD_{CHU_2708}) was expressed in the Δ3842 mutant, compared with the $WT^{GFP\text{-}CTD}$ strain, the glycosylated GFP (40 kDa) in the periplasm of the $\Delta3842^{GFP\text{-}CTD}$ mutant was decreased. After supplementing the

function of CHU_3842, glycosylated GFP returned to normal in the periplasm of the C3842$^{GFP\text{-}CTD}$ strain (Fig. 4C). All these results suggested that glycosyltransferase CHU_3842 is engaged in the *N*-glycosylation process of *C. hutchinsonii*.

Phenotypic properties of the Δ3842 mutant. *N*-Glycosylation was shown to be associated with stress resistance and motility in bacteria (32). The disk diffusion susceptibility test found that the cells of the Δ3842 mutant had bigger inhibition zone diameters than the wild type on sodium dodecyl sulfate, cumene hydroperoxide, and dithiothreitol, showing that the Δ3842 mutant was more sensitive to these agents than the wild type (Fig. 5A). Quantitative cell stress resistance to chemical analysis by absorbance changes found that the cell density of the Δ3842 mutant was more quickly reduced than that of the wild type in the early stages of detection (Fig. 5B). The results showed that the cell resistance to chemicals was affected by CHU_3842.

Previous reports showed that some cellulases containing type A CTDs are secreted and anchored to the outer membrane by the *C. hutchinsonii* T9SS (4, 18). We detected the capability for cellulose utilization after the deletion of CHU_3842. The growth rate of the Δ3842 mutant was lower than that of the wild type on the Whatman filter paper (Fig. 5C). Further assay of the cellulase activity, as in Fig. 5D, showed that the cell surface cellulase activity of Δ3842 was decreased in contrast to that of the wild type. These results suggested that CHU_3842 had effects on the cellulose degradation of *C. hutchinsonii*. Cells of the Δ3842 mutant formed smaller spreading colonies in contrast to the wild-type cells on soft agar, suggesting that the motility of cells of the Δ3842 mutant was deficient (Fig. 5E). These results indicated that the deletion of CHU_3842 caused the loss of *N*-glycosylation of some proteins, leading to pleiotropic defects on cell resistance, cell mobility, and cellulose degradation in *C. hutchinsonii*.

DISCUSSION

Protein glycosylation is the most common and complex posttranslational modification, which not only enlarges an organism's proteome but also affects many vital processes (26). For a long time, the protein glycosylation system was thought to be restricted to eukaryotes. It was not until the discovery of glycoproteins in *Halobacterium* and *Clostridium* that it was proved that protein glycosylation systems exist in all forms of life (33, 34). Several types of protein glycosylation systems have been identified in bacteria, including *N*-, *O*-, *C*-, and *S*-linked protein glycosylation systems (26). The enteropathogen *C. jejuni* was the first bacterium reported to have a general *N*-linked protein glycosylation (*pgl*) system (35). Further comparative genomic analysis showed that the conserved *pgl* locus is widespread in *Campylobacter* species (36). Other atypical bacterial *N*-glycosylation systems have also been uncovered and characterized (37–39). Many studies reported that bacterial *N*-glycosylation systems are limited within the species of *Proteobacteria* and *Campylobacterota* (formerly *Epsilonproteobacteria*) (40). Our study provides the evidence that *C. hutchinsonii*, a member of the phylum *Bacteroidetes*, also has an *N*-glycosylation system. The *N*-glycosylation system of *C. hutchinsonii* has some similarities to that of *C. jejuni*. First, glycosyltransferase PglA was shown to engage in *N*-glycosylation of the *C. jejuni* periplasmic lipoprotein AcrA (41). Bioinformatic analysis revealed that *chu_3842* is the homologous gene of *pglA*, and the deletion of CHU_3842 led to the disappearance of the periplasmic *N*-linked glycoproteins in *C. hutchinsonii* (Fig. 4). Second, the *N*-glycosylation of the GFP-CTD recombinant protein occurred in the periplasmic space of *C. hutchinsonii*, just as most *N*-glycosylated proteins in *C. jejuni*. Third, proteins are decorated with a heptasaccharide glycan in the *N*-glycosylation system of *C. jejuni*. Glycosylated GFP was 5 kDa higher than the theoretical molecular weight, indicating that there might also be complex glycans transferred to proteins of *C. hutchinsonii*, which is different from the monosaccharide or disaccharide modified protein in the atypical *N*-glycosylation system. However, there are also some differences between the *N*-glycosylation systems of *C. hutchinsonii* and *C. jejuni*. Our study showed that the glycosylated GFP is sensitive to PNGase F, while the *N*-linked glycoprotein of *C. jejuni* contains unique linking sugar bacillosamine,

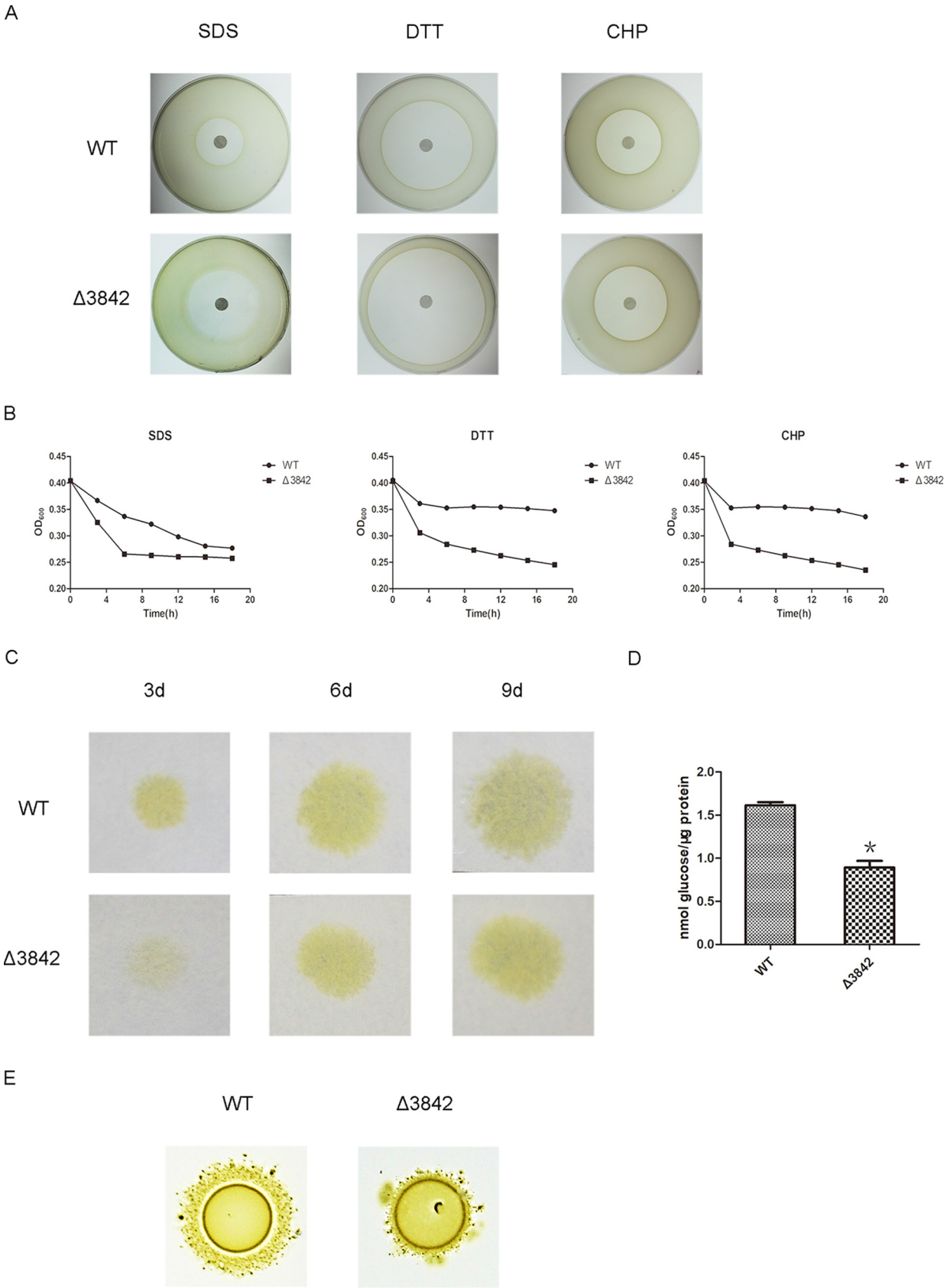

FIG 5 CHU_3842 has effects on cell resistance, cellulose degradation, and cell motility. (A) Disk diffusion susceptibility assay of the Δ3842 and WT strains to sodium dodecyl sulfate (SDS), cumene hydroperoxide (CHP), and dithiothreitol (DTT). (B) Resistance of the Δ3842 and WT strains to the agents. (C) Filter paper degradation ability of the Δ3842 and WT strains. (D) Cell surface cellulase activity analysis of the Δ3842 and WT strains. The standard deviations from three biological replicates are shown. *, $P < 0.05$. (E) Colony spreading ability of the Δ3842 and WT strains.

which makes it resistant to PNGase F (42). We also mutated T278 in the NVT motif of CTD_{CHU_2708} to alanine, and no glycosylated GFP was found in the periplasm of the mutant strain (Fig. S2B). The possible *N*-glycosylation sites N276 and N296 of CTD_{CHU_2708} are located in the N-X-S/T (X $\neq$ P) sequence, which is consistent with the motif recognized by the eukaryotic *N*-glycosylation system but different from the D/E-X-N-X-S/T sequon of *C. jejuni*. The characteristics of the *N*-glycosylation system in *C. hutchinsonii* need to be further studied.

The *O*-glycosylation system is extensively distributed in the phylum *Bacteroidetes* (43). Flagellins, pilins, and S-layer proteins are common *O*-glycosylated polymeric proteins (44). Plenty of studies have focused on the A-LPS glycosylation of T9SS substrates on the cell surface in *P. gingivalis* (45). Previous studies showed that the CTD signal is essential for the secretion, A-LPS modification, and attachment of T9SS substrates to the cell surface. In addition, the T9SS CTD signal was not the site of modification (3). *C. hutchinsonii* has the largest number of T9SS substrates in the phylum *Bacteroidetes*. There are many CTD proteins with type A CTD processing by the *C. hutchinsonii* T9SS (18). However, studies that focused on the features and functions of *C. hutchinsonii* CTDs are very limited. Our study reports that CTD_{CHU_2708} as the signal for the T9SS is *N*-glycosylated in the periplasm before being transported across the outer membrane. A previous study reported that the extensive mutation of potential glycosylation sites in the CTD of RgpB did not yield any carbohydrate modification sites and had no effect on the secretion or cell surface A-LPS modification of the virulence factor RgpB in *P. gingivalis* (46). No GFP-related proteins were detected in the periplasm when GFP fused with CTD of HBP35 was expressed in *P. gingivalis* (8). Moreover, attachment of the CTD of *F. johnsoniae* RemA to the superfolder GFP had no obvious modification (9). Therefore, whether there is *N*-glycosylation of CTDs in other *Bacteroidetes* species requires further exploration.

The contributions of *N*-linked glycan to proteins are usually associated with protein folding, stability, intracellular trafficking, subcellular targeting, and other properties in eukaryotic cells (47). But many of the roles assumed by protein *N*-glycosylation in eukaryotes are not applicable to *Bacteria* (48). A previous study reported that one *N*-glycosylation site of the T4SS component VirB10 is important for the stability of the T4SS in *C. jejuni* (49). A recent study showed that *N*-linked glycans take part in enhancing the thermostability of the multidrug efflux pump CmeABC in *C. jejuni* (50), but the role of *N*-linked glycans in bacteria is still not fully understood. Our study found that the *N*-glycosylation of the T9SS recognition signal CTD_{CHU_2708} plays an important role in the secretion and localization of the recombinant cargo protein. It was reported that glycosylation sites are often located in flexible parts of folded proteins in the bacterial system (51). Our study showed that mutation of N296 in CTD_{CHU_2708} to glutamine resulted in a significant reduction of the glycosylated protein in the periplasmic space of $N^{296}Q$ (Fig. 3B). In the predicted three-dimensional structure of CTD_{CHU_2708}, the N296 site is in the amorphous region of a typical sandwich-like fold of the immunoglobulin superfamily domain (Fig. S3). The mutation of N296 might affect the conformation of the CTD, leading to changes in the accessibility of glycosyltransferase, thereby reducing the glycosylation rate of the GFP-CTD_{CHU_2708} fusion protein. The decreased secretion of GFP-related proteins in the $N^{296}Q$ mutant also indicated that glycosylation of CTD_{CHU_2708} is important for GFP-CTD_{CHU_2708} recombinant protein secretion. No modified GFP was detected on the outer membrane of the $N^{296}Q$ mutant strain, indicating that the *N*-glycosylation of the CTD may be linked with the anchoring of T9SS substrates to the cell surface. Glycans of proteins play a role as labels in binding and recognition by other proteins in eukaryotes (52). Our results suggested that glycosylation of the CTD might be involved in the recognition of cargo proteins by the T9SS. Moreover, the mutation of N276 of CTD_{CHU_2708} to glutamine caused not only the loss of the glycosylated GFP but also the disappearance of the GFP-CTD_{CHU_2708} fusion protein in the periplasm (Fig. 3B). A similar phenomenon occurred when N276 was converted to alanine (Fig. S2C). The weak band of GFP-CTD_{CHU_2708} fusion protein in the periplasm of

TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Description[a]	Source or reference
E. coli strains		
BL21	Strain with vector pUT-miniTn*5*-gfp-tet used for GFP gene cloning	TaKaRa
DH5α	Strain used for plasmid replication	TaKaRa
C. hutchinsonii strains		
ATCC 33406	Wild type	ATCC
WT^{GFP} strain	WT containing $promoter_{CHU\_2708}$-$signal\ peptide_{CHU\_2708}$-GFP	This study
$WT^{GFP\text{-}CTD}$ strain	WT containing $promoter_{CHU\_2708}$-$signal\ peptide_{CHU\_2708}$-GFP-$CTD_{CHU\_2708}$	This study
CHU_2708^{His} strain	WT with His-tagged CHU_2708	This study
$N^{273}Q$ mutant	Site-directed mutation of N273 of $WT^{GFP\text{-}CTD}$	This study
$N^{276}Q$ mutant	Site-directed mutation of N276 of $WT^{GFP\text{-}CTD}$	This study
$T^{278}A$ mutant	Site-directed mutation of T278 of $WT^{GFP\text{-}CTD}$	This study
$N^{276}A$ mutant	Site-directed mutation of N276 of $WT^{GFP\text{-}CTD}$	This study
$N^{296}Q$ mutant	Site-directed mutation of N296 of $WT^{GFP\text{-}CTD}$	This study
$C3842^{GFP\text{-}CTD}$ strain	C3842 containing $promoter_{CHU\_2708}$-$signal\ peptide_{CHU\_2708}$-GFP-$CTD_{CHU\_2708}$	This study
Δ3842 strain	*chu_3842* deleted	This study
C3842 strain	Complementation of Δ3842	This study
Plasmids		
pTSK	Gene deletion template plasmid with *ermF* flanked by two FRT sites; Ap^r Em^r	17
pTSK3328	Plasmid for deletion of *chu_3328*; Ap^r Em^r	20
pCFXSK3328	Plasmid for deletion of *chu_3328*; Ap^r Cfx^r	59
pCHF	Plasmid for complementation of Δ 3842; Ap^r Cm^r	17

[a]Ap^r, ampicillin resistance; Em^r, erythromycin resistance; Cfx^r, cefoxitin resistance; Cm^r, chloramphenicol resistance.

the $N^{276}A$ was detected. Increasing the amount of proteins detected by Western blotting, a weak band of GFP-CTD_{CHU_2708} fusion protein could also be found in the periplasm of the $N^{276}Q$ mutant strain. These results showed that site N276 was also very important for the stabilization of GFP recombinant proteins. Proteases of the HtrA family play a central role in the degradation of aberrant proteins (53). Previously, we found that CHU_0052 is DegQ of *C. hutchinsonii*, which participates in the degradation or refolding of periplasmic misfolded proteins (28). The mutation of site N276 in CTD_{CHU_2708} caused a significant increase of the transcription level of *degQ*, suggesting that there is high folding stress in the periplasmic space of the $N^{276}Q$ strain. Whether the instability of GFP-CTD_{CHU_2708} recombinant protein was caused by direct change of N276 or by lack of glycosylation of N276 deserves further study.

Our study not only expanded the scope of the *N*-glycosylation system in bacteria, but also enriched the function of *N*-glycosylation in bacterial proteins. The *N*-glycosylation of CTD is important for the secretion and localization of the *C. hutchinsonii* T9SS cargo protein. Moreover, the *N*-glycosylation of *C. hutchinsonii* affects stress resistance, cell motility, and cellulose degradation. The wide range of phenotypic characteristics of *C. hutchinsonii N*-glycosylation also enriched our understanding of the bacterial *N*-glycosylation system. Our further study will focus on identifying more glycoproteins and analyzing the glycostructure of them in *C. hutchinsonii*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and general growth conditions. Bacterial strains and plasmids are listed in Table 1. *C. hutchinsonii* strain ATCC 33406 and mutants were cultured in PY6 medium at 30°C with shaking at 160 rpm (17). Unless otherwise specified, solid medium with 1% agar was used. Filter paper-covered solid Stanier medium (14) was used to detect cell cellulose degradation. The soft-agar medium was made up of PY2 medium (54). The *Escherichia coli* DH5α strain was grown in Luria-Bertani medium at 37°C. Antibiotic concentrations were as follows: ampicillin, 100 μg/mL; erythromycin, 30 μg/mL; cefoxitin, 15 μg/mL; and chloramphenicol, 15 μg/mL. The primers are listed in Table 2.

Generation of expression strains. The basic expression fragment contained the promoter and N-terminal signal peptide (SP) of CHU_2708 followed by GFP ($promoter_{CHU\_2708}$-SP_{CHU_2708}-GFP). On this basis, CTD_{CHU_2708} with different operations was linked to the C terminus of GFP ($promoter_{CHU\_2708}$-SP_{CHU_2708}-GFP-CTD). The fragment spanning the promoter and N-terminal signal peptide of CHU_2708 was obtained with primers P2708F (engineered SalI site) and PS2708R. The primers GFPF and GFPR (engineered SacI site) were used to amplify a 720-bp GFP region with a stop codon from the vector pUT-miniTn*5*-gfp-tet (Table 1). Using primers P2708F and GFPR, a fragment containing the promoter, signal

TABLE 2 Sequences of primers used in this study

Primer	Sequence[a]
3328H1F	TTAGCATGCTCTGTTGAGCAGGTTCTACTGGG
3328H2R	ATAGGATCCTCTATAATTGGCTGACCGACACG
P2708F	TGTTGTCGACCAAATCGCAGCGAAGAAATAAT
PS2708R	CTTCTCCTTTGCTTACCATTGAATTCCAAAGTACAA
GFPF	ATTGTACTTTGGAATTCAATGGTAAGCAAAGGAGAAG
GFPR	GGGGGAGCTCTTATTTGTATAGTTCATCCATGCCA
GFPR2708	AAAGTTACTGATCCAGCTTTGTATAGTTCATCCAT
CTDF2708	ATGGATGAACTATACAAAGCTGGATCAGTAACTTT
CTDR2708	CCCCGAGCTCTTACTTAACAACTACTAATTCAGTTTTAG
2708H1F	AGCTTCTAGAATCATGGTTCTGGAGTTCTCAAAAC
HISR	TTGGTGATGGTGATGGTGATGTGCGCTAGCAGTCATTACA
HISF	GCACATCACCATCACCATCACCAAAATCAAATTGTACTTT
SHISF	CATCACCATCACCATCACCAAAATCAAATTGTACTTTGGAA
2708H1R	CCCCGAGCTCCTTAACAACTACTAATTCAGTTTTA
2708H2F	GCGAGGGTACCTTAACCGCTCTATAATAGTATTAA
2708H2R	ACCAGGATCCTAAAGAGTAATCATGGTACGAAGGT
EMF	CAAGTTGTCGGTTGTGATT
2708UF	GAGTAGGGATGGATATTTATTGCCCGTTAT
2708UR	AGGAATCGGTACAGACTTTCATAGCGGT
273NQR	CGTTTGAATTTTGTTTCAAAGAAACTTCAGCTGTGAAA
273NQF	CTTTCACAGCTGAAGTTTCTTTGAAACAAAATTCAAAC
276NQR	TGATTGTTACTTGTGAATTGTTTTTCAAAGAAACTTCA
276NQF	TGAAGTTTCTTTGAAAAACAATTCACAAGTAACAATCA
276NAR	GATTGTTACAGCTGAATTGTTTTTCAAAGAAACTTCAG
276NAF	CTGAAGTTTCTTTGAAAAACAATTCAGCTGTAACAATC
278TAR	TCAGATAAGATGATAGCTACGTTTGAATTGTTTTTCAAA
278TAF	TTTGAAAAACAATTCAAACGTAGCTATCATCTTATCTGA
296NQR	AGCAGAACCTTGAGCAGCAGATTTAGTAGCAATTTGCT
296NQF	AGCAAATTGCTACTAAATCTGCTGCTCAAGGTTCTGCT
Q0052F	GGTGCGTATCGGTGAGTGG
Q0052R	TATGCTCCCGTAGGACTTG
3842H1F	GAAAGAGCTCAAGCAACAGATGACAACTGGATGAA
3842H1R	GGTGGTCGACATATTGGGCTTGATGGTATACTGCA
3842H2F	TGAGCCATGGGGCAATAACGTACAGTGTTTACTCA
3842H2R	AATGGGATCCATAAGGTTCTTGCCTGCTGACTGGA
3842UF	ACATGCCAAAACACAGATTATTAAAGGCTC
3842UR	GAAGTGTCTGCTCAAAGTAAATCGAATC
C3842F	ACGGGAGCTCCATAGACATAAATCAATGCGGCGGC
C3842R	CGCGGTCGACTCAGTGAATGTCATTTGTATTCGT
YF	GTTCTCTCCTGAAATACGCT
YR1	AACCCATTGTCAAACGTGTT
YR2	GCAACAAAAAATCACTATGAAACGGCTACT

[a]Restriction sites are underlined.

peptide, and GFP was obtained by overlapping PCR. The fragment was ligated to pTSK3328 with SalI and SacI sites, producing pTSK3328-PSGFP. Then primers 3328H1F and 3328H2R were used to amplify the expression cassette. The PCR product was transformed into the competent cells of *C. hutchinsonii* by electroporation. The pseudogene *chu_3328* of *C. hutchinsonii* was replaced with the expression cassette by the linear DNA double-crossover method (55). Transformants were grown on PY6 solid medium with erythromycin. The PCR products from two sets of primers (YF/YR1 and YF/YR2) were sequenced to screen the correct expression strains. Primers GFPF and GFPR2708 were used to amplify GFP without a stop codon. A fragment of the CTD was amplified with primers CTDF2708 and CTDR2708 (engineered SacI site). By overlap PCR, the complete fragment containing GFP and all necessary elements for T9SS secretion was obtained. Then the fragment was ligated to pTSK3328 or pCFXSK3328. The expression cassette was expressed in the *C. hutchinsonii* wild type or mutant strains for further study. The linear DNA double-crossover deletion method and overlap PCR were used to construct *C. hutchinsonii* with His-tagged CHU_2708.

Site-directed mutagenesis of CTD_{CHU_2708}. The amino acids of CTD_{CHU_2708} were individually mutated to glutamine or alanine by overlap PCR. Primer CTDF2708 and mutagenic primers with the desired mutation points (273NQR, 276NQR, 276NAR, 278TAR, and 296NQR) were used to amplify the first half fragment of the CTD. The second half fragment of the CTD was obtained by primers with the mutation points (273NQF, 276NQF, 276NAF, 278TAF, and 296NQF) and CTDR2708. Then the CTD fragments containing mutation sites were obtained by overlap PCR. The complete fragment containing the promoter, N-terminal signal peptide, and site-directed mutation CTD was digested and ligated into plasmid

pTSK3328. The obtained expression cassettes were expressed in *C. hutchinsonii*. The strains were designated the $N^{273}Q$, $N^{276}Q$, $N^{276}A$, $T^{278}A$, and $N^{296}Q$ mutants, respectively.

Cell fractionation procedures. The extracellular and outer membrane proteins were extracted according to the method of Zhao et al. (55). The mid-exponential-phase cells of *C. hutchinsonii* were harvested by centrifugation (5,000 × *g*, 4°C, 10 min). Cells in the supernatants were further removed with a 0.22-μm-pore-size polyvinylidene difluoride (PVDF) filter. Then the collected supernatants were precipitated with 10% (wt/vol) trichloroacetic acid for at least 1 h to obtain extracellular proteins. The washed cell pellets were resuspended in piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES) buffer (containing 0. 5 M NaCl) with shaking at 150 rpm (4°C, 30 min). The supernatant without cell debris was ultracentrifuged (100,000 × *g*, 4°C, 30 min), and the outer membrane proteins were in the sediment. The periplasmic space proteins were obtained as described by Soares et al. (56). The collected cells were incubated in sucrose buffer (20% sucrose, 1 mM EDTA, and 0. 3 mM Tris [pH 8. 0]) at 4°C for 15 min. Then the cells were pelleted (5,000 × *g*, 4°C, 10 min) and then incubated in double-distilled water (4°C, 15 min). The cell pellet was removed by ultrafiltration (25,000 × *g*, 4°C, 10 min), and the resulting supernatant was the periplasmic proteins.

Western blot analysis. The Western blot operation was performed according to the method of Wang et al. (17). Equal biomasses of extracellular, outer membrane, and periplasmic proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and then proteins on the gel were transferred onto a 0.45-μm-pore-size PVDF membrane by semidry electrophoresis. The obtained PVDF membrane was blocked in TBS (10 mM Tris, 150 mM NaCl [pH 7.4]) containing 5% nonfat powdered milk overnight. The anti-GFP rabbit monoclonal antibody was supplemented at a dilution of 1:5,000. After 4 h of incubation, the PVDF membrane was washed 5 times with TBST (TBS containing 0.1% Tween 20) for 8 min each time. The PVDF membrane was probed with anti-rabbit IgG polyclonal secondary antibody at a 1:10,000 dilution for 2 h. After washing the membrane with TBST, development was carried out as per Sparkjade ECL super (Shandong Sparkjade Biotechnology Co., Ltd.) instructions. For the detection of His-tagged proteins, anti-His mouse monoclonal antibody (MAb) was used at a 1:6,000 dilution.

Purification of GFP-related proteins. The GFP-related proteins in the periplasmic space of the *C. hutchinsonii* strain were purified by anti-GFP tag nanobody-conjugated agarose beads (Engibody, Shanghai, China) following the manufacturer's instructions.

Protein preparation for liquid chromatography-tandem mass spectrometry analysis. The proteins in the SDS-PAGE gel were reduced with dithiothreitol (DTT) and then alkylated with iodoacetamide. Trypsin was used to further digest the proteins. The resulting product was acidified with trifluoroacetic acid (TFA) and then analyzed by liquid chromatography-tandem mass spectrometry (57).

Detection of glycosylation. The purified GFP-related proteins on the SDS-PAGE gel were stained by periodate-Schiff chemicals (58). The purified proteins were also transferred to a PVDF membrane by semidry electrophoresis and then probed with ConA lectin per manufacturer's instructions.

Deglycosylation of protein. Proteins were processed using a deglycosylation kit (Rhinozyme, Suzhou, China) per the manufacturer's instructions. The kit utilizes peptide-*N*-glycosidase F, which is specially designed for *N*-glycosylation. Then processed proteins were separated, blotted, and probed with anti-GFP rabbit MAb to detect the deglycosylation of GFP-related proteins.

Real-time quantitative PCR analysis. The cells of the $WT^{GFP\text{-}CTD}$ and $N^{276}Q$ strains were harvested when the optical density at 600 nm (OD_{600}) was 0.7. Then the total RNA was extracted referring to the method by Guan et al. (59). Quantitative PCR was performed with the SYBR green Premix Pro *Taq* HS qPCR kit (AG, Hunan, China) on a LightCycler 480 system. The relative quantitation/comparative threshold cycle ($\Delta\Delta C_T$) method was used to analyze data from three biological repeats of *C. hutchinsonii* (60). The 16S rRNA gene served as an endogenous control.

Construction of the *chu_3842* deletion mutant. The 3842H1 fragment, of about 2.5 kbp, containing the region upstream of *chu_3842* and the first 291 bp of *chu_3842* was amplified using primers 3842H1F and 3842H1R. 3842H1 was ligated to the pTSK plasmid through the SacI and SalI sites, generating pTSK-3842H1. The 3842H2 fragment, of approximately 2.5 kbp, was amplified using primers 3842H2F and 3842H2R. The fragment included the last 478 bp of *chu_3842* and the region downstream of *chu_3842*. 3842H2 was cloned into pTSK-3842H1 through the NcoI and BamHI sites, producing pTSK-3842H1H2. Other procedures were as previously described. The *chu_3842* deletion mutant was complemented using the complementation plasmid pCH. The fragment including *chu_3842* and its promoter was obtained with primers C3842F and C3842R. Then it was ligated into pCHF by the SacI and SalI sites, generating pCHF-3842. Plasmid pCHF-3842 was electroporated into the Δ3842 mutant. Chloramphenicol was used to select the transformants.

Phenotypic assay of the Δ3842 mutant. The periplasmic proteins of *C. hutchinsonii* strains were enriched using ConA lectin affinity chromatography per the manufacturer's instructions. The disk diffusion test of susceptibility of cells to chemicals was performed as described by Bai et al. (61). The agents were sodium dodecyl sulfate (10%), dithiothreitol (2 mM), and cumene hydroperoxide (2 mM). For the quantitative detection of susceptibility, cells of the WT and Δ3842 mutant in the same growth phase were treated with different chemicals, and the absorbance at 600 nm was monitored. The filter paper degradation and colony spreading assay were conducted as previously described by Guan et al. (59). Cellulase activity was detected as described by Wang et al. (21). Significance of the data was calculated using Student's *t* test. The protein concentration was quantified as described by Bradford (62). The data are the averages and standard deviations from three independent experiments.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.3 MB.

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