Coordinated evolution of glycan structure, oligosaccharyltransferase protein targeting specificity and Tfp pilin subunit glycosylation within the genus *Neisseria*

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Type IV pili (Tfp) are essential colonization factors in the pathogenic *Neisseria* species. PilE, the major Tfp subunit in these species is grouped into two pilin classes (class I and class II). A key difference between the two pilin classes is that only class I pilins undergo high-frequency antigenic variation at the *pilE* expression locus. However, both class I and II pilin subunits are subject to *O*-linked glycosylation with antigenically variable glycoforms. Comparative analyses in the genus have shown that although broad-spectrum, *O*-linked protein glycosylation is conserved in the human-restricted *Neisseria*, PilE glycosylation is not observed in commensal species. Our previous work suggests that PilE glycosylation involves both the intrinsic structure of different pilins and the targeting specificities of the PglL/PglO oligosaccharyltransferase (*O*-OTase). To further validate this, we expressed (via allelic replacement) the OTase and the *pilE* allele from *N. gonorrhoeae* in the commensal species *N. elongata* and showed that these two factors are sufficient for pilin glycosylation. Here, the exogenous PilE was fully glycosylated with a tetrasaccharide glycan expressed by *N. elongata*, reinforcing previous observations that *O*-OTases can utilize diverse glycan donors. In addition, we analyzed the proteomes of isogenic *N. gonorrhoeae* strains expressing exogenous neisserial OTases using MS/FAIMS and discovered unique glycoproteome profiles.

Bioinformatic analyses showed that despite sequence diversity of PilE subunits in *Neisseria*, PilE of pathogenic species carry a conserved serine (Ser) glycan attachment site at position 63 that is absent in those from commensal species. To better understand the structural determinants of PilE constraining glycosylation in commensal species, we expressed commensal PilEs carrying a Ser63 substitution in *N. gonorrhoeae*. The results varied with Ser63 leading to fully, partially and no glycosylation phenotypes suggesting that Ser63 is necessary but not sufficient and acts in a context dependent fashion. Together, the findings suggest coordinated pathways of substrate and OTase evolution govern PilE glycosylation within the genus *Neisseria*.