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Insights into type IV pilus biogenesis and dynamics from genetic analysis of a C-terminally tagged pilin: a role for *O*-linked glycosylation

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Summary

Type IV pili are surface organelles essential for pathogenicity of many Gram-negative bacteria. In *Neisseria gonorrhoeae*, the major subunit of type IV pili, PilE, is a target of its general *O*-linked glycosylation system. This system modifies a diverse set of periplasmic and extracellular gonococcal proteins with a variable set of glycans. Here we show that expression of a particular hexa-histidine-tagged PilE was associated with growth arrest. By studying intra- and extragenic suppressors, we found that this phenotype was dependent on pilus assembly and retraction. Based on these results, we developed a sensitive tool to identify factors with subtle effects on pilus dynamics. Using this approach, we found that glycan chain length has differential effects on the growth arrest that appears to be mediated at the level of pilin subunit–subunit interactions and bidirectional remodelling of pilin between its membrane-associated and assembled states. Gonococcal pilin glycosylation thus plays both an intracellular role in pilus dynamics and potential extracellular roles mediated through type IV pili. In addition to demonstrating the effect of glycosylation on pilus dynamics, the study provides a new way of identifying factors with less dramatic effects on processes involved in type IV pilus biogenesis.

Introduction

Type IV pili (Tfp) are proteinaceous surface structures expressed by many bacterial species. Tfp are involved in the expression of many phenotypes including motility, multicellular behaviour, sensitivities to bacteriophage, DNA uptake in natural genetic transformation, and adherence to host tissue and inanimate surfaces (Mattick, 2002). A cardinal feature of Tfp is that they are dynamic structures (Merz *et al.*, 2000; Skerker and Berg, 2001), where levels of steady-state polymer reflect the net equilibrium achieved through organelle extension and retraction events. Cycles of Tfp extension and retraction are integral to organelle function (Wolfgang *et al.*, 1998a; Merz and So, 2000; Mattick, 2002). Forward and reverse genetic screens in a number of Tfp systems have identified a highly conserved set of components required for Tfp expression and associated activities. Similar components are found in the type II secretion, archaeal flagella and archaeal bindosome systems as well as transformation competence systems (Koomey, 2009). The mechanisms by which most of these shared ancillary components function remain poorly understood. The dynamic nature of Tfp is made possible by virtue of a unique biogenesis pathway in which pilus formation occurs at the cytoplasmic membrane and the intact organelle is extruded across the outer membrane (Wolfgang *et al.*, 2000). Current models invoke that Tfp extension and retraction reflect pilin subunit polymerization and depolymerization events mediated by the cytoplasmic PilF and PilT ATPases (Wolfgang *et al.*, 1998b; 2000; Merz and Forest, 2002). Bidirectional remodelling of the pilin subunits between a monomeric integral membrane form and a polymeric, assembled form is accommodated in part by conserved structural features including an N-terminally localized alpha-helical domain. This highly conserved domain functions at many steps in pilus biogenesis from inner membrane insertion, processing by pre-pilin peptidase, and polymerization into and stabilization of the pilus. The importance of this and other domains to the latter steps has been inferred from crystallographic as well as EM structural data and validated by molecular modelling approaches and mutagenesis (Craig *et al.*, 2004). The role of particular residues in polymerization and protomer contacts has also been revealed by studies of intermolecular complementation in which mutant pilins incapable of self-assembly are incorporated into pili in the presence of a wild-type pilin (Aas *et al.*, 2007b).

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Tfp pilin subunits within the species *Neisseria gonorrhoeae* and *Neisseria meningitidis* are unique in that they undergo extensive, antigenic variation by a process of gene conversion involving a single expression locus (*pilE*) and multiple variant silent loci (*pilS*) encoding truncated alleles (Zhang *et al*., 1992). This variability has been assumed to be a major factor in the failure of pilus-based gonococcal vaccines. This system also generates pilins that can differ dramatically in their pilus-forming capabilities (leading to what has been termed pilus phase variation) as well as their ability to support adherence to human tissue (even when Tfp are expressed at high levels) (Swanson *et al*., 1986). Thus, the *in vivo* pilin repertoire might be influenced by altered antigenicity, assembly proclivity and adhesion promoting activities. Neisserial pilins also undergo post-translational modifications with *O*-linked glycans at serine 63 (Parge *et al*., 1995). This glycan is added by the general *O*-linked protein glycosylation (*pgl*) system of *N. gonorrhoeae*. The glycosylation process initiates with the production of a lipid-linked monosaccharide on the cytoplasmic face of the inner membrane by the action of the PglB, C and D proteins. The lipid-linked monosaccharide may be further elongated by the glycosyltransferases PglA, PglH and PglE after which the PglF flippase flips it into the periplasm where the sugar is transferred *en bloc* onto protein by the PglO oligosaccharyltransferase (Aas *et al*., 2007a; Børud *et al*., 2011). Serine 63 is in a variable part of pilin and thus, variants lacking serine at this site may not be glycosylated. Moreover, the structure of the gonococcal oligosaccharide can undergo intrastrain variation due to the phase-variable expression of the *pglA*, *pglE* and *pglH* genes encoding glycosyltransferases with differing specificities in the synthesis of di- and trisaccharides (Børud *et al*., 2011). The phenotypic consequences of glycan variation have yet to be fully elucidated. Marceau and colleagues reported that the ablation of meningococcal pilin glycosylation (by virtue of a serine to alanine substitution at residue 63) was associated with modest increases in piliation levels and adherence to human epithelial cells (Marceau *et al*., 1998). It remains unclear however as to whether the phenotypic alterations observed were due directly to the lack of glycosylation or rather the alteration in PilE primary structure. Oligosaccharide alterations can also impact on the antigenicity and immunogenicity of the PilE glycan (Børud *et al*., 2010) as well as on their abilities to contribute to the activation of complement receptor 3 during interactions with primary human cervical epithelial cells (Jennings *et al*., 2011).

Genetic screens and selections for mutants with altered Tfp expression and related functions have relied primarily on phenotypic changes involving alterations in colony morphology, autoagglutinability and resistance to bacteriophage infection. As these phenotypic shifts generally require dramatic alterations in piliation levels, mutants with subtle changes in pilation phenotype are likely to have escaped detection. Given the potential discovery bias, the identification of additional components and regulators that might contribute to Tfp expression and dynamics remains an important issue.

Table 1. Differential effects of C-terminal 6His-tag in PilE.

	Recovery	
Amino acids replaced by 6His[a]	*wt*[b]	*pilC*[c]
6	+	+
5	–	+
4	–	+
4 (AAM)[d]	+	+

a. PilE with no. of the most C-terminal amino acids replaced by a 6His tag.
b. *wt* denotes a $pilE_{ind}$ background (strain 4/3/1).
c. *pilC* denotes a *pilC*, $pilE_{ind}$ background (*pilC1,2* alleles in strain 4/3/1). PilC-depleted mutants demonstrate defects in pilus homeostasis.
d. Denoted as $PilE_{AAMc6His}$ in this article.

In this work, we established a refined phenotypic assay that relates to the integrity of the Tfp assembly and retraction pathways and used this methodology to examine the potential contributions of gonococcal pilin glycosylation to these events. The results are discussed in the context of gonococcal pilin antigentic variation, pilus assembly, glycoform variation and the broad-spectrum, *O*-linked glycosylation system operating in *N. gonorrhoeae*.

Results

Phenotypes associated with hexa-histidine-tagged PilE constructs

C-terminal, hexa-histidine tagging of the pseudopilin PulG has been exploited in studies of the pullulanase type II protein secretion system (Vignon *et al*., 2003). To examine the utility of this strategy in studies of gonococcal type IV pilus biology, three derivatives of PilE carrying hexa-histidine tails were constructed using a *pilE* allele previously characterized in strain N400 (Wolfgang *et al*., 2000). One involved the replacement of the six native terminal residues with hexa-histidine ($PilE_{-6+6His}$), another removal of the last five residues and addition of hexa-histidine ($PilE_{-5+6His}$), and a third, removal of the last four residues and addition of hexa-histidine ($PilE_{-4+6His}$). Alleles encoding these variant PilE forms were constructed in a vector facilitating their expression from an ectopic site within a background in which expression of *pilE* from the endogenous locus was conditionally repressed. Surprisingly, these alleles gave differing outcomes following transformation into gonococci (Table 1). The $pilE_{-6+6His}$ allele gave high-level recovery of transformants that homogeneously

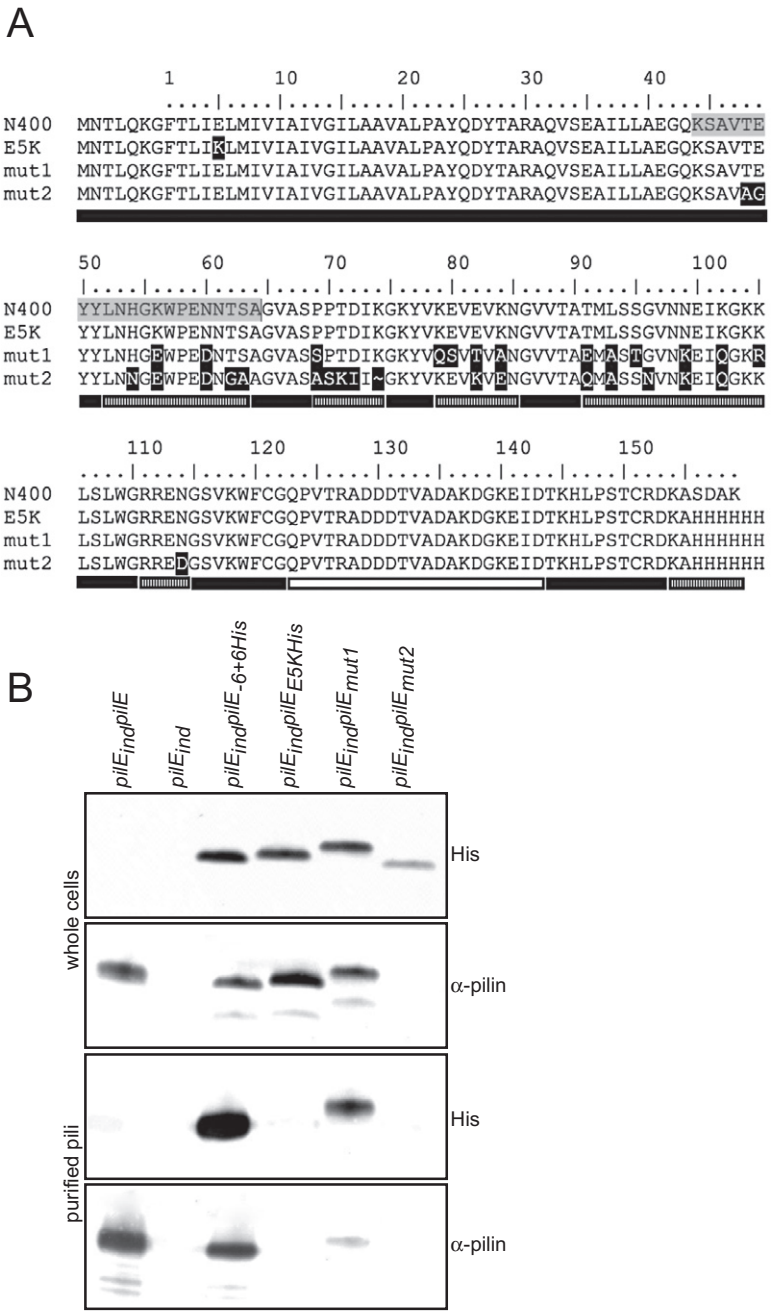

Fig. 1. Mutations in the pilin subunit suppress the synthetic growth defect associated with the hexa-histidine-tag in PilE$_{-4+6His}$.
A. Polypeptide sequences derived from sequencing the *pilE*$_{-4+6His}$ locus of three different mutants suppressing the growth defect. The mutations in the suppressors (denoted E5K, mut1 and mut2) are marked in black. The polypeptides are numbered according to the mature protein sequence, i.e. from the pre-pilin cleavage site. The conserved regions of PilE are shown as solid black bars, the semi-variable regions as striped bars, and the hypervariable region as a white bar (Swanson and Koomey, 1989). The grey area of the N400 sequence shows the sequence of the peptide to which the α-pilin antibodies were raised.
B. Immunoblots of purified pili showed that a strain expressing *pilE*$_{-6+6His}$ produced pili at levels comparable to wild-type *pilE* whereas *pilE*$_{-4+6His}$ suppressor mutants *pilE*$_{E5Kc6His}$, *mut1* and *mut2* produced less or no pili. Strains used were *pilE*$_{ind}$ (4/3/1), *pilE*$_{ind}$ *pilE* (KS130), *pilE*$_{ind}$ *pilE*$_{-6+6His}$ (KS735), *pilE*$_{ind}$ *pilE*$_{E5Kc6His}$ (KS731), *pilE*$_{ind}$ *pilE*$_{mut1c6His}$ (KS732) and *pilE*$_{ind}$ *pilE*$_{mut2c6His}$ (KS733). Cells were grown on GC plates without IPTG and the amount of material loaded was normalized to cell number. The antibodies used were the tetra-His antibody and the α-pilin antibody. Note that mut1 and mut2 contain alterations in the peptide sequence to which the α-pilin antibodies were raised. In the case of mut2, these alterations made the pilin unrecognizable to the α-pilin antibodies.

expressed the corresponding pilin as type IV pili at levels close to that seen for the unadulterated pilin. The other two alleles were associated with an overall reduction in the recovery of transformants and when examined, the majority of transformants expressed PilE that failed to react with poly-histidine recognizing antibodies and thus, had lost the hexa-histidine tag. The rarer class of transformants for the *pilE*$^{-4+6His}$ and *pilE*$^{-5+6His}$ alleles retained expression of hexa-histidine-tagged PilE. To analyse the basis for suppression of the toxic effect of the hexa-histidine tag, three *pilE*$_{-4+6His}$ transformants were selected for further characterization. Sequence analysis showed that one carried a single base change resulting in a substitution of lysine for glutamate at position +5 (*pilE*$_{E5Kc6His}$, denoted E5K in Fig. 1A). This strain failed to express pili (Fig. 1B) and pilus-associated phenotypes, which is consistent with what was previously reported for *N. gonorrhoeae* and *Pseudomonas aeruginosa* strains carrying substitutions at the same site (Pasloske *et al*., 1989; Aas *et al*., 2007b). In two other transformants, *pilE* alleles with new variable domains had been created by intragenic recombination without perturbing the hexa-histidine extension (denoted mut1 and mut2

in Fig. 1). These strains had reduced levels of purifiable pili compared with a wild-type strain (Fig. 1B). Together these data suggested that PilE$_{-4+6His}$ pilin impacts negatively on cell growth and that this effect is influenced by its ability to assemble into pili.

A derivative of PilE$_{-4+6His}$ acts as a dominant-negative pilin

To test if the PilE$_{-4+6His}$ associated phenotypes were related to assembly proficiency, we first examined the effect of a three-residue substitution spanning residues 38–40 (AlaAlaMet in place of LeuLeuAla) previously shown to inhibit pilus expression at the level of subunit–subunit interactions (Aas *et al*., 2007b). Alteration of these three residues in the context of the –4+6His tag allowed the stable introduction of the allele in a *pilE* inducible background. Here, pili were not detectable even though the protein was expressed at levels equivalent to that seen for wild-type pilin (Fig. S1A and B). We denoted the allele encoding the –4 + 6 His-tagged pilin with the AAM substitution as *pilE$_{AAMc6His}$*.

Defective piliation in strains expressing PilE missense mutants appears to result primarily from defective subunit–subunit interactions and in some instances, these defects can be suppressed by coexpression of a wild-type subunit protein (Aas *et al*., 2007b). Therefore, we examined if the presence of wild-type PilE might alter the behaviour associated with the poly-histidine-tagged pilins by transforming the altered *pilE* alleles into a strain expressing wild-type PilE from the native locus. Although stable transformants coexpressing wild-type and the PilE$_{-6+6His}$ form were readily recovered, none were found for those transformed with the *pilE$_{-5+6His}$*, *pilE$_{-4+6His}$* and *pilE$_{AAMc6His}$* alleles.

The ability to recover transformants for the *pilE$_{AAMc6His}$* allele in the absence but not the presence of wild-type PilE expression suggested that interactions between the two pilin forms might be refractory to cell growth or viability. To examine this possibility directly, we exploited the de-repressibility of wild-type *pilE* in the background ectopically expressing the PilE$_{AAMc6His}$ form. In these experiments, de-repression of the wild-type allele was achieved by inclusion of IPTG as a gratuitous inducer. As shown in Fig. 2A, growth of this strain was inhibited around a filter disk impregnated with IPTG. This indicated that the defective growth phenotype was indeed dependent on wild-type PilE expression.

Null mutations in genes involved in Tfp biogenesis and dynamics suppress the pilin-associated growth arrest

To determine if the growth arrest phenotype was dependent on the integrity of the pilus assembly pathway, null mutations in *pilC2*, *pilF, pilQ* and *pilV* were introduced into the background and the resulting strains analysed for growth arrest. The products of the first three genes impact on Tfp expression at distinct levels with PilF being essential for Tfp expression while PilC2 and PilQ are required for Tfp expression only in the presence of the PilT retraction ATPase (Wolfgang *et al*., 1998a; 2000). Although PilQ and PilC2 thus act as pilus homeostasis effectors, they are functionally distinct as PilQ is required for Tfp to cross the outer membrane while PilC2 is not (Wolfgang *et al*., 2000). PilV is a pilin-like molecule that also acts as a pilus homeostasis effector, but its role is partially masked by functional redundancy imparted by ComP, a related pilin-like molecule (Aas *et al*., 2002). Despite the varying nature of their roles, IPTG-dependent growth inhibition was suppressed in all four cases (Fig. 2B and data not shown). We also examined the influence of the PilT pilus retraction ATPase and found that a *pilT* null mutation also suppressed IPTG-dependent growth inhibition (Fig. 2B). In contrast to the other backgrounds in which growth inhibition was suppressed, this strain retained pilus expression and the PilE$_{AAMc6His}$ pilin was incorporated into pili in this background (Fig. S1B). That this incorporation was only observed in the presence of wild-type PilE and absence of PilT was consistent with a model in which retraction of PilE$_{AAMc6His}$ confers growth arrest. Since wild-

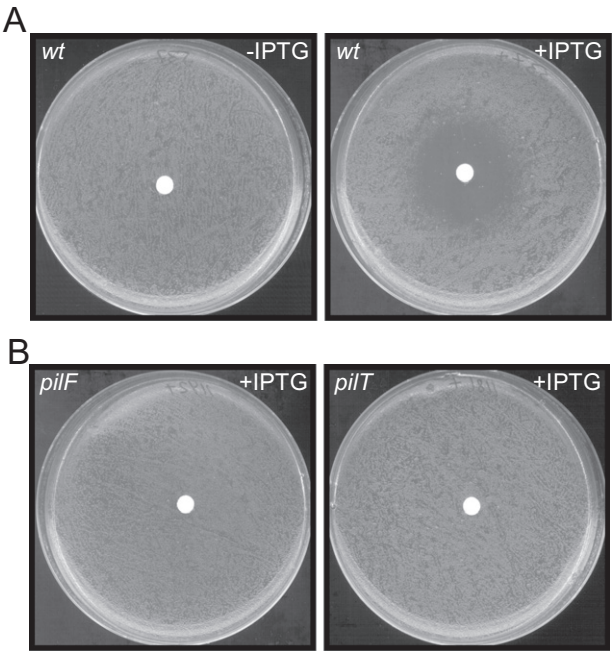

Fig. 2. Coexpression of PilE$_{AAMc6His}$ and PilE causes growth arrest.
A. Induction of PilE from an IPTG-inducible promoter in a strain that constitutively expressed PilE$_{AAMc6His}$ (KS525) caused growth arrest.
B. The growth arrest depended on pilus biogenesis and retraction. When the same assay was performed in strain backgrounds where *pilF* (KS723) or *pilT* (KS529) were deleted, no growth arrest was observed.

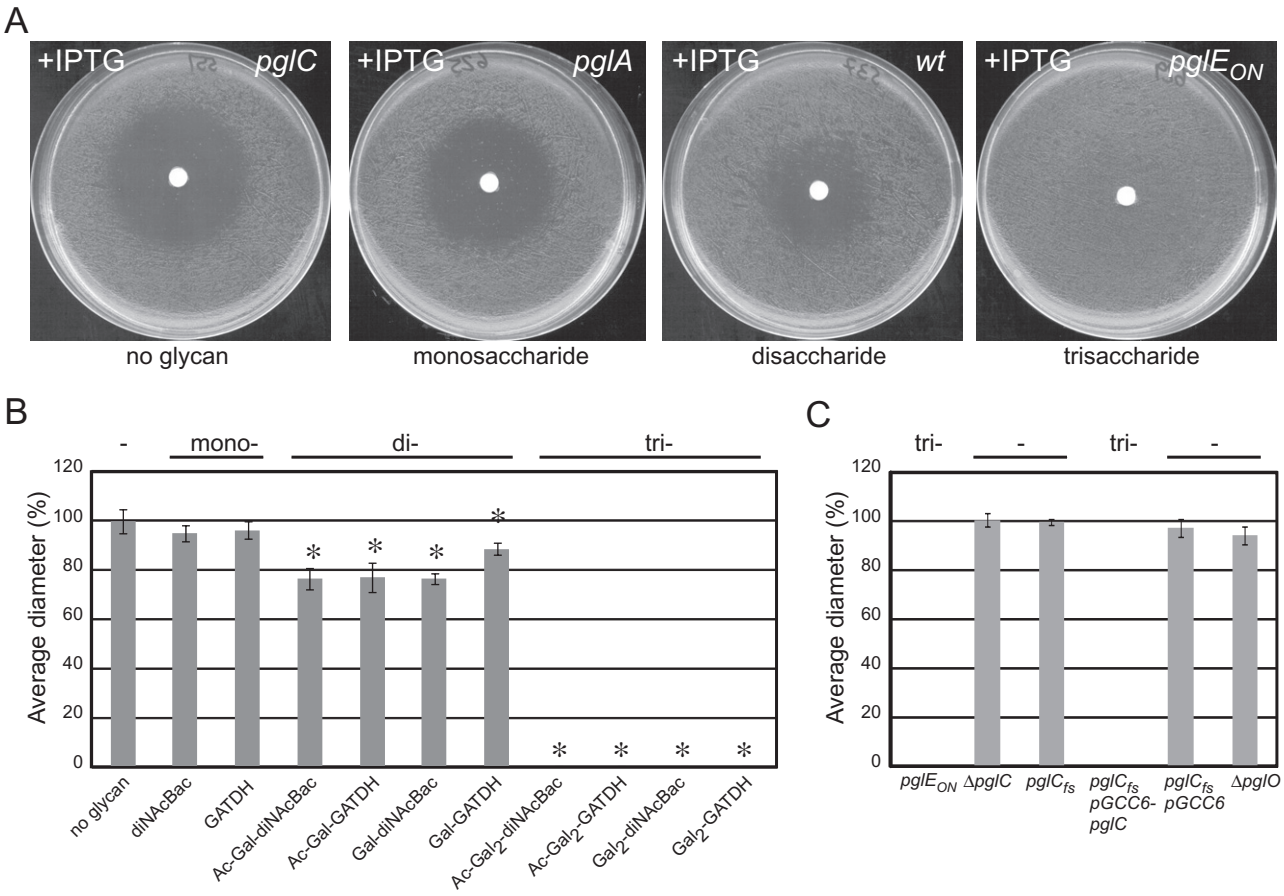

Fig. 3. Protein glycosylation status affects the synthetic growth arrest phenotype.
A. Using strains that either did not glycosylate proteins (*pglC*, KS531), or produced glycoproteins with diNAcBac (*pglA*, KS534), Ac-Gal-diNAcBac (*wt*, KS525) or Ac-Gal$_2$-diNAcBac (*pglE$_{ON}$*, KS538) we showed that producing longer glycans led to smaller zones of growth arrest.
B. Quantification of the diameter of the growth arrest zones. Bar graph shows averages ± standard deviations of the diameter of the zones of growth inhibition for at least three isogenic strains of each genotype. Averages are normalized to that of the non-glycosylating (*pglC*) strain background. Strains used produced no glycan (*pglC*, KS530–3), diNAcBac (*pglA*, KS534–7), GATDH (*pglB2 pglA*, KS541–4), Ac-Gal-diNAcBac (*wt*, KS524–7), Ac-Gal-GATDH (*pglB2*, KS545–8), Gal-diNAcBac (*pgll*, KS549–52), Gal-GATDH (*pglB2 pgll*, KS553–6), Ac-Gal$_2$-diNAcBac (*pglE$_{ON}$*, KS538–40), Ac-Gal$_2$-GATDH (*pglB2 pglE$_{ON}$*, KS557–60), Gal$_2$-diNAcBac (*pglE$_{ON}$ pgll*, KS561–4) and Gal$_2$-GATDH (*pglB2 pglE$_{ON}$ pgll*, KS565–8). Strains denoted as *pglB2* carried the *pglB2* allele of *pglB* and hence expressed GATDH glycans instead of diNAcBac. Bars marked * denote a significantly different average from that of the *pglC* background ($P < 0.05$).
C. Expressing the *pglC* gene from an ectopic locus complemented the *pglC* mutation in a *pglE$_{ON}$ pglC* background. The diameter of the zone of growth inhibition of a strain with a *pglO* mutation that removes the oligosaccharyltransferase activity was similar to that of a *pglC* mutant. Strains used were *pglE$_{ON}$* (KS538–40), Δ*pglC* (KS569–71), *pglC$_{fs}$* (KS572–4), *pglC$_{fs}$ pGCC6-pglC* (KS575–7), *pglC$_{fs}$ pGCC6* (KS578–80) and Δ*pglO* (KS581–3). All strains used in (C) carried the *pglE$_{ON}$* allele, contained the IPTG-inducible allele of PilE and constitutively expressed PilE$_{AAMc6His}$ from the ectopic (*iga*) locus.
The abbreviations: -, mono-, di- and tri- denote strains producing no glycan, monosaccharide, disaccharide or trisaccharide respectively.

type pilin was required for assembly of PilE$_{AAMc6His}$, the growth arrest conferred by retraction of PilE$_{AAMc6His}$ was only observed when wild-type pilin was expressed.

Protein glycoform status influences pilin-associated growth inhibition

Given the knowledge that PilE is the most abundant gonococcal glycoprotein (Vik *et al.*, 2009) in combination with the availability of a sensitized assay to test phenotypes associated with pilus biogenesis and dynamics, we then decided to assess the potential effects of various glycoforms utilized in *O*-linked protein glycosylation (Fig. 3). The wild-type background that constitutively expressed *pilE$_{AAMc6His}$* and had an IPTG-inducible allele of *pilE* (*pilE$_{ind}$*) expresses glycoproteins with the disaccharide *N*,*N*′-diacetylbacillosamine-*O*-acetyl galactose (Ac-Gal-diNAcBac) (Aas *et al.*, 2007a; Hartley *et al.*, 2011). We made strains carrying *pilE$_{ind}$* and the constitutive *pilE$_{AAMc6His}$* allele in conjunction with mutations that disrupt or alter protein glycosylation at various steps in the pathway. These included null alleles of *pglC* (whose

product is required for formation of the undecaprenyl pyrophosphate-linked glycan donor), *pglA* (thus producing glycoproteins with diNAcBac monosaccharide) and *pglI* (producing non-*O*-acetylated glycans), as well as a phase-on version of *pglE* ($pglE_{ON}$, producing glycoproteins with the trisaccharide Ac-Gal_2-diNAcBac) and a replacement of the native *pglB* gene with the *pglB2* allele from *N. meningitidis* strain 8013 [producing glyceramido-acetamido trideoxyhexose (GATDH)-based rather than diNAcBac-based glycans] (Chamot-Rooke *et al.*, 2007; Børud *et al.*, 2010). Using these strains, we tested the propensity for growth inhibition upon induction of $pilE_{ind}$ by measuring the zone of growth inhibition. As shown in Fig. 3A, alterations in protein glycosylation status had significant effects on the size of the growth arrest zone. These differences were quantified by determining the diameters and normalizing them to the average diameter of the non-glycosylating *pglC* strain (Fig. 3B). This strain and the monosaccharide-producing *pglA* strains exhibited the largest zones although they were not significantly different from each other. The disaccharide-producing wild-type strain formed a significantly smaller zone while, the trisaccharide-producing $pglE_{ON}$ strain formed no growth arrest zone at all. Thus, there appeared to be an inverse correlation between glycan chain length and the degree of growth inhibition. However, we observed that neither the nature of the first sugar (diNAcBac versus GATDH) nor the absence of the acetyl group had an effect on the growth inhibition, although the strain expressing the unacetylated GATDH-based disaccharide (*pglB2 pglI*) was slightly more susceptible to growth arrest than the wild-type, *pglB2* and *pglI* strains. As seen in Fig. 3C, the mutation in *pglC* could be complemented by expressing wild-type *pglC* from an ectopic locus, demonstrating that it was indeed the absence of PglC that conferred the increased propensity for growth arrest. Furthermore, strains harbouring a mutation in *pglC* gave a growth arrest phenotype indistinguishable from a strain with a mutation in the oligosaccharyl transferase gene *pglO*. Therefore, for the purposes of this phenotype, there was no difference between strains that failed to produce undecaprenyl pyrophosphate-linked glycan donors and strains that did not transfer the undecaprenyl pyrophosphate-linked glycans to proteins. This implied that it was protein-attached glycans that were important for the growth arrest phenotype. These findings were further corroborated by experiments assessing the recoverability of defined *pilE* alleles in different glycosylation backgrounds following transformation (Table 2). Taken together, the results showed that protein glycosylation quantitatively affected the growth arrest phenotype and that the glycan chain length was a factor such that the larger the glycan, the less susceptible the strain was to growth inhibition.

Table 2. Effects of protein and pilin glycosylation on the recoverability of defined *pilE* alleles following transformation.

	Alleles introduced		
Strain background	$pilE_{AAMc6His}$	$pilE_{AAMS63Ac6His}$	$pilE_{-4+6His}$
N400 $pglE_{ON}$	+	+	+
N400 pglC	–	–	–
N400 $pilE_{S63A}$	–	–	–
$pilE_{ind}$	+	–	–
$pilE_{ind}$ $pglE_{ON}$	+	+	–
$pilE_{S63A\ ind}$ $pglE_{ON}$	–	–	–

Pilin glycosylation status influences growth inhibition phenotype

Given that the growth inhibition phenotype was influenced by protein glycosylation status we then decided to test whether the phenotype was related directly to the glycosylation status of pilin. Since the strain contained two pilin loci, $pilE_{AAMc6His}$ and $pilE_{ind}$, point mutations that resulted in alanine substitutions in place of serine at reside 63 (the sole glycan attachment site) where made in both loci. To increase the dynamic range of the assay, we chose to test the effect of the *pilE* point mutations in a $pglE_{ON}$ trisaccharide-expressing background. We then assayed IPTG-dependent growth inhibition in strains bearing various combinations of these mutations. As can be seen in Fig. 4A, the S63A mutation in $pilE_{AAMc6His}$ resulted in a growth arrest indistinguishable from a *pglC* mutant, whereas the S63A mutation in $pilE_{ind}$ caused an intermediate phenotype. The latter mutant was significantly different from both the $pglE_{ON}$ and the *pglC* strains. Therefore, the glycosylation status of $PilE_{AAMc6His}$ pilin had a stronger influence on the growth arrest phenotype than the glycosylation status of the wild-type pilin. This difference could be due to the lower level of expression of the wild-type pilin expressed from its de-repressed *lac* promoter relative to $PilE_{AAMc6His}$ constitutively expressed from the PilE promoter or to a *bona fide* stronger effect of the glycan on $PilE_{AAMc6His}$. That both $PilE_{AAMc6His}$ and PilE were glycosylated and that the S63A mutations abolished glycosylation of both pilins is shown in Fig. 4B. These findings were also supported by experiments assessing the recoverability of defined *pilE* alleles in different glycosylation backgrounds following transformation (Table 2). Together these data demonstrated that the effect of glycosylation on the growth arrest phenotype could be genetically mapped to *pilE* and that it was indeed the glycosylation of pilin that conferred the effect.

Glycosylation impacts on the relative incorporation of $PilE_{AAMc6His}$ into Tfp

Because the growth arrest associated with coexpression of PilE and $PilE_{AAMc6His}$ was suppressed by defects in pilus

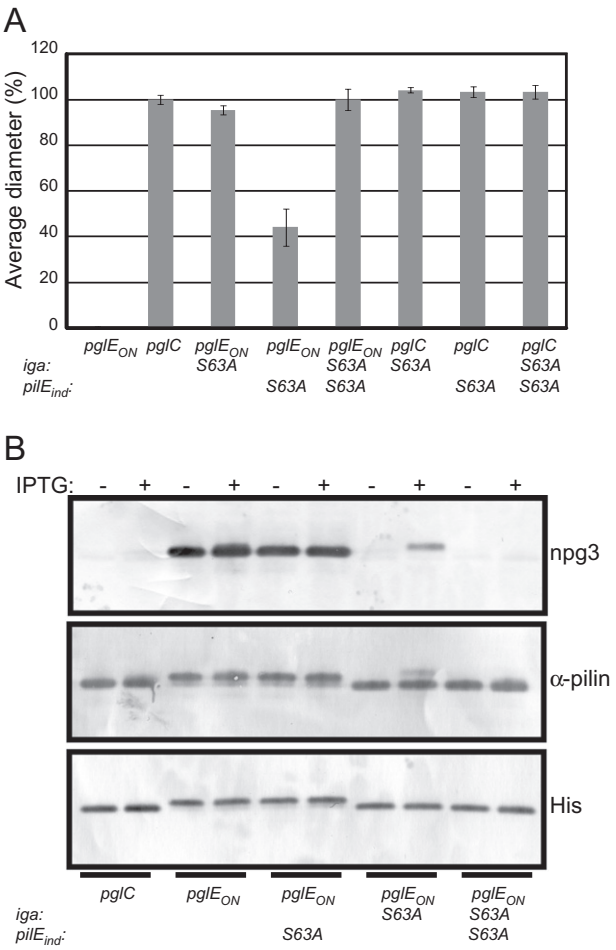

Fig. 4. The effect of glycosylation status on the synthetic growth arrest is due to glycosylation of PilE and $PilE_{AAMc6His}$.
A. The bar graph shows average diameters of zones of growth arrest normalized to that of the *pglC* strain. Each bar represents the average ± standard deviations of at least three isogenic strains assayed at the same time. Strains used were $pglE_{ON}$ (KS538–40), *pglC* (KS530–2), $pglE_{ON}$ *iga::S63A* (i.e. $iga::pilE_{AAMS63Ac6His}$) (KS584–6), $pglE_{ON}$ $pilE_{ind}$*::S63A* (i.e. IPTG-inducible $pilE_{S63A}$) (KS588–90), $pglE_{ON}$ $pilE_{ind}$*::S63A iga::S63A* (KS591–3), *pglC iga:S63A* (KS594–6), *pglC* $pilE_{ind}$*::S63A iga::S63A* (KS597–9) and *pglC* $pilE_{ind}$*::S63A iga::S63A* (KS600–2). The average for the $pglE_{ON}$ $pilE_{ind:S63A}$ strain was significantly different from both the $pglE_{ON}$ strain and the *pglC* strain ($P < 0.05$).
B. Immunoblot of whole-cell lysates showed that both $PilE_{ind}$ and $PilE_{AAMc6His}$ were glycosylated and that the S63A mutations in $PilE_{ind}$ and $PilE_{AAMc6His}$ removed the glycans. Strains used were *pglC* (KS530–2), $pglE_{ON}$ (KS538–40), $pglE_{ON}$ $pilE_{ind}$*::S63A* (KS588–90), $pglE_{ON}$ *iga::S63A* (KS584–6) and $pglE_{ON}$ $pilE_{ind}$*::S63A iga::S63A* (KS591–3). The antibodies used were the trisaccharide-specific npg3 antibody, the pilin-specific α-pilin antibody and the poly-histidine-specific tetra-His antibody.

assembly and retraction and also strongly influenced by the glycosylation status of pilin, we wondered whether glycosylation status influenced the amount of $PilE_{AAMc6His}$ in assembled pili. Since the trisaccharide expressing $pglE_{ON}$ strain survived coexpression of $PilE_{AAMc6His}$ and PilE, we could study pilin incorporation by immunoelectron microscopy in this strain background. As mentioned earlier, a strain expressing only $PilE_{AAMc6His}$ was non-piliated while strains coexpressing PilE and $PilE_{AAMc6His}$ produced pili (Fig. 5A). Using primary antibodies specific for the poly-histidine tag, we demonstrated that $PilE_{AAMc6His}$ was incorporated into pili (Fig. 5B). Furthermore, by affinity separation of $PilE_{AAMc6His}$ from PilE, we could show that both variants of pilin were found in their glycosylated forms in pili (Fig. 5C). This demonstrated that trisaccharide modification of pilin did not grossly disturb pilus assembly, nor did it block incorporation of $PilE_{AAMc6His}$ into pili. This was consistent with the previous finding that $PilE_{AAMc6His}$ could be found in purified mixed pili in the absence of the retraction ATPase PilT (Fig. S1B). To further test whether pilin glycosylation affected the relative levels of $PilE_{AAMc6His}$ in pili, we purified pili from strains carrying either the *pglC* or the $pglE_{ON}$ allele and constitutively expressing both PilE and $PilE_{AAMc6His}$ in the absence of PilT. Electrospray ionization mass spectrometry (ESI-MS) analysis of purified pili from these strains showed a statistically significant difference ($P < 0.01$) in the ratio of $PilE_{AAMc6His}$ to PilE recovered (Fig. 5D and Fig. S2). The data therefore suggest that glycosylation status could affect the relative capacity of $PilE_{AAMc6His}$ to be incorporated into pili. The decreased ratio of $PilE_{AAMc6His}$ found in the $pglE_{ON}$ strain could explain the suppression of growth arrest observed in this strain, since less $PilE_{AAMc6His}$ would be available for retraction.

Discussion

Tfp play important roles as bacterial colonization factors and are often requisites for disease pathogenesis and symbiosis. As such, a thorough knowledge of the structure–function relationships associated with these organelles could provide novel means of preventing and controlling infection. In contrast to the dramatic consequences of defective *O*-linked glycosylation seen in autotransporter and some flagellar systems (Benz and Schmidt, 2001; Logan, 2006), functional correlates of *O*-linked glycosylation of Tfp pilin subunits reported to date are remarkably few and until now, our own comparative studies of gonococcal glycosylation variants and null mutants have revealed no significant alterations in piliation levels or Tfp-associated phenotypes (data not shown).

Fusion proteins have been powerful tools in studies of membrane trafficking and secretion pathways in eubacteria. However, only in a few instances has fusion technology targeting pilins and pseudopilins of Tfp and related systems been exploited (Vignon *et al.*, 2003). The limited number of such reports appear in part to reflect a lack of functionality or other incompatibilities associated with pilin-based fusions, a situation that is likely exacerbated by the functional requirement for pilin to exist in

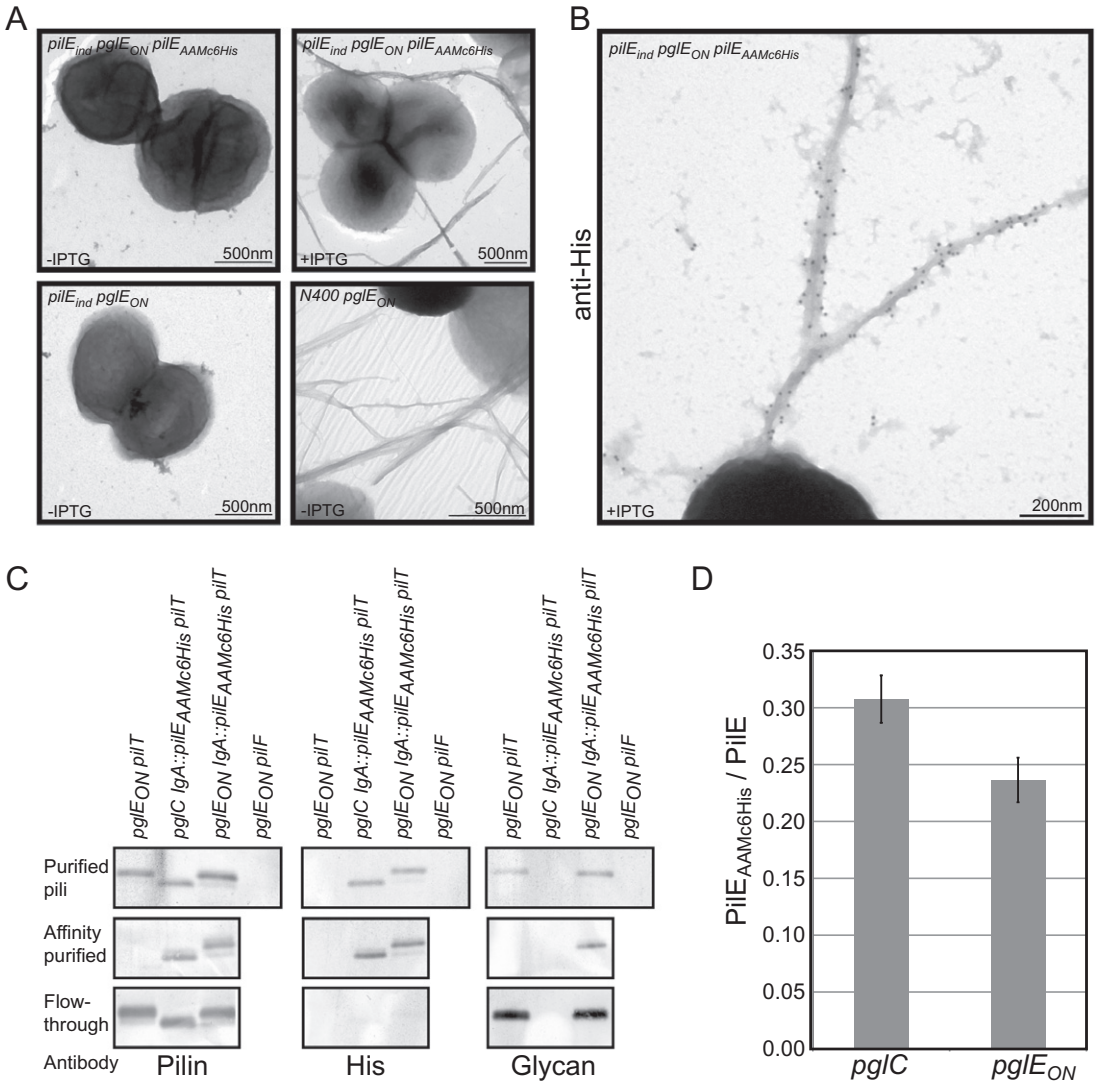

Fig. 5. Glycosylated PilE and $PilE_{AAMc6His}$ are found in assembled pili.
A. Transmission electron microscopy (TEM) images of uranyl acetate negatively stained cells grown on plates with or without IPTG and therefore expressing either no PilE [*pilE*$_{ind}$ *pglE*$_{ON}$ (KS127)], constitutively expressing PilE only [*N400 pglE*$_{ON}$ (KS142)], constitutively expressing $PilE_{AAMc6His}$ only [*pilE*$_{ind}$ *pglE*$_{ON}$ *pilE*$_{AAMc6His}$ (KS538)], or constitutively expressing $PilE_{AAMc6His}$ and expressing PilE from an IPTG-inducible promoter [*pilE*$_{ind}$ *pglE*$_{ON}$ *pilE*$_{AAMc6His}$ (KS538) grown on plates with 0.25 mM IPTG].
B. TEM images of uranyl acetate stained cells immunogold labelled with tetra-His antibodies. The strain used was *pilE*$_{ind}$ *pglE*$_{ON}$ *pilE*$_{AAMc6His}$ (KS538) grown on plates with 0.25 mM IPTG.
C. Immunoblots of purified pili and affinity-purified fractions thereof. The 'affinity purified' fraction was eluted from Ni-NTA beads and represents $PilE_{AAMc6His}$ whereas the 'flow-through' fraction failed to bind to the same beads and represents pilE lacking poly-histidine. Strains used were *pglE*$_{ON}$ *pilT* (KS676), *pglC pilE*$_{AAMc6His}$ *pilT* (KS716), *pglE*$_{ON}$ *pilE*$_{AAMc6His}$ *pilT* (KS725) and *pglE*$_{ON}$ *pilF* (KS671). All strains constitutively expressed wild-type pilE. Pilin was detected using the α-pilin antibody, poly-histidine was detected using the tetra-His antibody, and glycan was detected using the npg3 antibody.
D. Ratio of $PilE_{AAMc6His}$ to PilE in purified pili from *pglC* (KS716–21) and *pglE*$_{ON}$ (KS725–30) backgrounds analysed by ESI-MS. The strains constitutively coexpressed both PilE and $PilE_{AAMc6His}$ in the absence of PilT. Bar graph shows average ± standard deviations based on analysis of five biological replicates for each genotype.

both integral membrane and polymerized forms. Here, we first observed that expression of a structurally modified pilin ($PilE_{-4+6His}$) led to a synthetic defect as seen by the inability to establish stable recombinants. In addition to the seemingly unprecedented nature of this finding, this was remarkable since this phenotype was not imparted by a similarly modified pilin differing only by the absence of two amino acid residues at the site of the C-terminal poly-histidine extension. This finding was nonetheless interesting as ultrastructural and modelling studies of *N. gonorrhoeae* Tfp suggest that the C-terminal domain of PilE contributes to subunit interactivity along the length of

the pilus fibre (Craig *et al*., 2006). Intragenic suppressors failing to express the C-terminal tag confirmed the importance of the extension to the phenotype while those retaining tag expression had compensatory alterations in the PilE ORF. Most notable among the latter was that having a single substitution at a highly conserved glutamate at position 5 (E_5K). Although missense mutations at this highly conserved site abolish self-assembly, the altered pilin can be incorporated into pilus filaments when coexpressed with a wild-type subunit (Aas *et al*., 2007b). We confirmed that the E_5K pilin alone was not assembled but could be found in pili when coexpressed with wild-type PilE (data not shown). Together, these findings suggested that the defective growth phenotype was related to the assembly competence of the altered pilin and that the $PilE_{-4+6His}$ protein might be a useful tool for studying Tfp biogenesis.

To overcome the defective growth phenotype associated with the $PilE_{-4+6His}$ construct, a second site modification designed to reduce assembly proficiency was introduced. The stable growth of the non-piliated strain expressing this derivative ($PilE_{AAMc6His}$) corroborated the association between assembly and growth perturbation. The dominant-negative nature of the $PilE_{AAMc6His}$ protein when wild-type pilin was conditionally expressed then provided a background in which to examine the role of other factors. Consistent with the conditional nature of the growth defect and the implication that pilus dynamics might be involved, the growth defect was suppressed by the introduction of null alleles for *pilC2*, *pilF*, *pilQ, pilV* as well as *pilT*. In the latter case, the modified pilin was integrated into pilus filaments. To better understand these findings it is important to recognize that Tfp are protein polymerization motors that convert chemical energy into mechanical energy and force via subunit multimerization. Pilin polymerization and depolymerization are driven to chemical non-equilibrium via nucleotide hydrolysis mediated by the assembly and retraction ATPases (PilF and PilT in *N. gonorrhoeae* respectively). The mechanisms by which energy is transduced from these sources to drive bidirectional remodelling of pilin between its integral membrane and polymeric forms are poorly understood. In addition, a number of gonococcal components termed effectors of pilus homeostasis have been identified including PilCs 1 and 2, PilQ, PilV and other pilin-like proteins PilH-L. When mutations blocking synthesis of these components are introduced into otherwise wild-type backgrounds, pilus expression is abolished or dramatically reduced. However when these mutations are introduced into a *pilT* background, piliation is still observed (Wolfgang *et al*., 1998a; 2000; Winther-Larsen *et al*., 2005). Thus, such effectors are dispensable for pilin polymerization but impact on the system by shifting pilus dynamics in favour of assembly versus disassembly. That mutations affecting both pilus assembly and retraction blocked the growth arrest observed in this study is consistent with a model where retraction of a sufficient amount of Tfp bearing C-terminally extended pilin caused growth arrest. However, the precise mechanism behind the growth arrest caused by retraction of the extended pilin remains obscure. One explanation for the toxic phenotype might be that it reflects a form of hybrid protein jamming (Bowers *et al*., 2003) with the growth arrest resulting from direct disruption of vital, inner membrane-associated processes. Given the PilT-dependence of the phenotype and that all other suppressors mutations are epistatic to *pilT*, an alternative explanation could be that jamming hyperactivates PilT that in turn leads to diminished levels of ATP and thus, growth inhibition. Further studies are needed to resolve these issues.

By utilizing the dominant-negative nature of the $PilE_{AAMc6His}$ protein, we observed a strong correlation between glycan chain length and ability to suppress the conditional growth deficiency. Clear differences were seen with the trisaccharide glycoform being associated with the highest degree of suppression, followed by the disaccharide glycoform and then the monosaccharide form. In fact, the suppression demonstrated by the pilus assembly and retraction mutants was phenocopied by trisaccharide glycoform expression. Importantly, the analysis demonstrated that the glycosylation effect was caused by the glycosylation status of pilin *per se* (and not other proteins), and in particular $PilE_{AAMc6His.}$ We could further show that glycosylation status had a subtle but statistically significant effect on the ratio of $PilE_{AAMc6His}$ in pili. We speculate that the differential effects of PilE glycosylation relate to varied effects on the efficacy of pilin protomer interactions in the cytoplasmic membrane. Given the variable influence of oligosaccharide chain length or bulkiness, the site of action could be at the levels of pilin–pilin or pilin–lipid interactions, the rates of pilin folding or even on the rates of lateral diffusion of pilin within the membrane (Helms, 2002).

Regardless of the precise mechanisms behind the hybrid pilin-associated growth inhibition and its suppression, our findings clearly demonstrate that pilin glycosylation status impacts differentially on these phenotypes and thus, pilus dynamics. These findings are unanticipated as protein glycoform variation has until now been viewed as being driven primarily by selection at the level of host adaptive and innate immune systems as well as potential cell surface receptors (Børud *et al*., 2010; Jennings *et al*., 2011). Our observations indicate that glycoform variation also affects the intrinsic processes of pilus dynamics and likely subunit–subunit interactions, that is, events going on at the intracellular level. That glycosylation could affect intracellular events is also in line with the observation that the *pgl* system, in addition to PilE, glycosylates many

other proteins, several of which are not predicted to be surface-exposed (Vik *et al*., 2009). The idea is furthermore in concordance with recent findings made in *P. aeruginosa* strains expressing type IV pilin subunits that undergo *O*-linked glycosylation with homopolymers of d-arabinofuranose (Harvey *et al*., 2011). There, mutants defective in either the synthesis of the glycan precursor or its transfer to pilin showed clear defects in levels of pilus expression. The importance of *O*-linked glycosylation to Tfp biogenesis and associated processes is thus likely to be more prevalent than previously recognized. It is also worth noting that PilE in pathogenic *Neisseria* strains is subject to extensive antigenic variation due to combinatorial diversification involving gene conversion-like events. As such, glycoform variation may represent a means to fine-tune glycan-mediated effects to the vast array of structural variants arising *in vivo*.

In conclusion, this work provides a new perspective on the functional correlates of bacterial protein glycosylation and glycoform diversification. Moreover, the establishment of assays such as that detailed here opens up the possibility for unbiased, forward genetic approaches that may give critical insights into the complexities of Tfp biogenesis and dynamics.

Experimental procedures

Bacterial strains and culture conditions

The bacterial strains are described in Table S1 and were derived from the MS11 background. NGO numbers refer to Gene IDs from the stdgen database (http://stdgen.northwestern.edu/) and are based on FA1090 sequences. GenBank accession numbers used in this manuscript refer to MS11 sequences where appropriate and available.

Strains were grown on conventional GC medium plates (Freitag *et al*., 1995), except that Thiotone E Peptone was replaced by Meat Peptone (Merck).

Whole-cell lysates for immunoblotting were made from cultures grown in liquid GC medium containing 10 mM fresh sodium carbonate. Cultures were inoculated at OD 0.1 and grown for 2 h with shaking, whereupon IPTG was added to 0.25 mM final concentration and the cultures allowed to grow for an additional 2 h. Whole-cell lysates were subsequently made from equal numbers of cells as assessed by optical densities (ODs).

Strains carrying the $pilE_{S63Aind}$ allele were constructed by non-selective transformation (Gunn and Stein, 1996) of strain 4/3/1 with a PCR product containing the $pilE_{S63A}$ mutation made by PCR-based splice overlap extension (SOE) using primers av2540, av2541, av2542 and av2543 (for primer sequences see Table S2). Pilin mutants were screened for by loss of reactivity against the trisaccharide-recognizing antibody npg3 (Børud *et al*., 2010) on immunoblots and verified by sequencing.

Mutations in *pilQ* (*NGO0094/EEZ47065*) [*pilQ::mTncm21* (Drake *et al*., 1997)], *pilT* (*NGO0346/EEZ49064*) [*pilT::mTncm17* (Park *et al*., 2001)], *pilF* (*NGO1673/EEZ48666*) [*pilF::cm* (Aas *et al*., 2007b)], *pilV* (*NGO1441/EEZ48312*) [*pilV::kan* (Winther-Larsen *et al*., 2001)], *pilC2* (*EEZ47021*) [*pilC2::cm* (Wolfgang *et al*., 1998b)], *pglC* (*NGO0084/EEZ47055*) [*pglC::kan* (Hegge *et al*., 2004) and $pglC_{fs}$ (Anonsen *et al*., 2012)], *pglA* (*NGO1765/EEZ48757*) [*pglA::kan* (Aas *et al*., 2007a)], *pglI* [*NGO0065/AAW88829* (FA1090 protein sequence identical to MS11)] [*pglI::kan* (Aas *et al*., 2007a)], *pglO* (*NGO0178/EEZ47149*) [*pglO::kan* (Aas *et al*., 2007a)], *pglB* (*NGO0085/EEZ47056*) [*pglB2* (Børud *et al., 2010*)] and *pglE* [where the $pglE_{ON}$ phase-on sequence (Aas *et al*., 2007a) is identical to the native FA1090 sequence *NGO0207*/AAW88960] were introduced into various strain backgrounds by transformation as previously described (Aas *et al*., 2007a; Børud *et al*., 2010; Anonsen *et al*., 2012). Mutants were verified by PCR and/or immunoblotting with appropriate antibodies.

Plasmid construction

The C-terminal hexa-histidine-tag was introduced into *pilE* by SOEing. Plasmids ppilE2 (Wolfgang *et al*., 2000) and pPilE2-AAM (Aas *et al*., 2007b) were used as templates for PCR to generate the various $pilE_{+6His}$ and $pilE_{AAMc6His}$ alleles. To generate *pilE* and $pilE_{AAM}$ alleles where the four last amino acids were exchanged with hexa-histidine, primers pilE5′ in combination with pilE-4 + 6HisR and pilE6HisF in combination with pilE3′ were used to generate overlapping PCR fragments. To generate a *pilE* allele where the five last amino acids in the C-terminus was changed to hexa-histidine, primers pilE6HisF in combination with p2/16/1 3′ and pilE-5 + 6HisR in combination with p2/16/1 5′ were used. To generate a *pilE* allele where the six last amino acids were exchanged with hexa-histidine, primers pilE6HisF in combination with P2/16/1 3′ and pilE-6 + 6HisR in combination with p2/16/1 5′ were used. Each pair of overlapping PCR fragments containing the hexa-histidine-tag was SOEd together by using the flanking primers p2/16/1 5′ and p2/16/1 3′. The SacI fragments from the PCR fragments were cloned into the plasmid ppilE2 digested with SacI, such that the hexa-histidine-tagged pilE variants could be expressed ectopically from the *iga* locus.

KP14 (p2/16/1 $pilE_{AAMS63Ac6His}$) was made from p2/16/1 $pilE_{AAMc6His}$ by replacing the Bsu36I–BglI fragment containing the N-terminal end of *pilE* with a PCR product of the same region containing the S63A mutation made by SOEing using primers av2540, av2541, av2542 and av2543. Transformation with KP14 was selected for on erythromycin (8 μg ml^{-1}) and gave rise to the strains carrying the $iga::pilE_{AAMS63Ac6His}$ locus.

KP19 (pGCC6-pglC) was made by subcloning the *pglC* open reading frame PCR-amplified from strain N400 using primers av2111 and av2112 into the restriction sites PacI and NsiI of pGCC6 (Mehr and Seifert, 1998; Mehr *et al*., 2000).

Characterization of suppressor mutants

Suppressor mutants of the growth arrest phenotype of the $pilE_{-4+6His}$ mutation arose during transformation of strain 4/3/1 with the ppilE2$_{-4+6His}$ construct. Suppressor mutants were isolated from transformation plates, colony purified and analysed for PilE$_{-4+6His}$ expression using mouse monoclonal tetra-His antibodies (Qiagen, Hilden, Germany). The $pilE_{-4+6His}$ allele in the *iga* locus was amplified by using the

primer pair p2/16/1 5′ and p2/16/1 3′ and the PCR fragments were sequenced using primers pilEseqL and pilEseqR. To confirm that the suppressing effect was conferred by the *pilE*$_{-4+6His}$ allele itself the *pilE*$_{-4+6His}$ allele was backcrossed into the 4/3/1 background under selection for Erm resistance.

Pilin-related growth arrest assay

Growth arrest assays were performed by spreading approximately 10^6 cells per plate on 1- to 2-week-old GC agar plates. Four microlitres of 2 M IPTG was added to a sterile Whatman disc after it was placed at the centre of the plate. The plates were incubated at 37°C for 27 h with 5% CO_2 and then scanned on a CanoScan 5600F and the zones of growth arrest measured from the stored images. All bar graphs show averages and standard deviations from measurements on at least three isogenic strains analysed in the same experiment. *P*-values were calculated using a two-tailed, two-sample unequal variance Student's *t*-test.

SDS-PAGE, immunoblotting, immunogold labelling and TEM

Procedures for SDS-PAGE and immunoblotting, transmission electron microscopy and immunogold labelling of pili have been described previously (Freitag *et al.*, 1995; Aas *et al.*, 2007b). For purposes of immunoblotting pilin was detected using the α-pilin rabbit polyclonal antibody (Aas *et al.*, 2007b) at 1:1000 dilution. These antibodies were raised against the PilE-derived synthetic peptide ^{44}KSAVTEYYLNHGKWPENNTSA^{64}. Poly-histidine tags were detected using the mouse monoclonal tetra-His antibody at 1:1000 dilution and glycans were detected using the npg3 monoclonal rabbit antibody specific for the gonococcal trisaccharide at 1:10000 dilution (Børud *et al.*, 2010). For immunogold labelling the tetra-His antibody was used at 1:100 dilution and 10 nm Protein-A Gold (Cell Microscopy Center, Utrecht, the Netherlands) was used at 1:60 dilution.

Pilus purification

Pili were purified by the ammonium sulphate procedure as previously described (Wolfgang *et al.*, 2000) from equal ODs of cells grown overnight on GC plates.

Affinity separation of PilE$_{AAMc6His}$ and PilE from purified pili

PilE$_{AAMc6His}$ was separated from PilE by using magnetic Ni-NTA beads (Qiagen). 1.25 µg of purified pili were diluted in 50 µl of lysis buffer [100 mM NaH_2PO_4, 10 mM Tris, 8 M urea, 0.05% Tween 20, 100 mM NaCl, 15 mM imidazole, 1 × Complete Mini EDTA-free protease inhibitor cocktail (Roche Applied Science, Indianapolis, USA), 1 mM phenylmethylsulphonyl fluoride (PMSF), 1% L-lauroylsarcosine sodium salt, pH 8.0]. This sample was incubated for 30 min with washed Ni-NTA beads equivalent to 50 µl of bead suspension. The Ni-NTA beads were extracted using a magnet, wash buffer (100 mM NaH_2PO_4, 10 mM Tris, 8 M urea, 0.05% Tween 20, 100 mM NaCl, 15 mM imidazole, 1% L-lauroylsarcosine sodium salt, pH 6.3) was added and the sample containing the beads set aside. Another aliquot of washed Ni-NTA beads (equivalent to 50 µl of bead suspension) was then added to the supernatant, incubated, extracted and pooled with the previous sample of beads. The supernatant, containing only pilin lacking the poly-histidine tag (wild-type PilE), was diluted with 1 volume (50 µl) of SDS sample loading buffer (labelled 'flow-through' in Fig. 5), bringing the total volume to 100 µl. The pooled beads were washed five times in wash buffer and poly-histidine-tagged pilin eluted in 100 µl of SDS sample loading buffer (labelled 'affinity purified' in Fig. 5). In this way both the affinity-purified and flow-trough samples were diluted to the same extent.

Sample preparation and infusion MS analysis of intact PilE protein

Purified pili were precipitated using a methanol/chloroform procedure as described (Wessel and Flugge, 1984). The pellet was dried for 5 min before the sample was dissolved in 50 µl of 10% MeOH, 70% formic acid and acetonitrile at 1:1:3 (v/v/v). All data were acquired on a LTQ XL Orbitrap (Thermo Electron, Bremen, Germany) operated by Xcalibur 2.0 in the positive ion mode. The MS analysis was performed after calibration and tuning with typical settings for spray voltage at 3200 V, capillary temperature at 275°C, capillary voltage at 30 V, tube lens voltage at 110 V and sheath gas flow at 1 a.u. Sample solutions were infused into the ESI source at a flow rate of 5 µl min^{-1}. Protein mass spectra were acquired at a resolution of 60 000 at *m/z* 400. Protein mass deconvolution was done using the integrated Xcalibur 2.0 extract algorithm. Masses of unmodified and modified proteins were determined from calculated theoretical masses, mass differences and previous work (Aas *et al.*, 2006). Relative quantities of PilE and PilE$_{AAMc6His}$ were calculated from deconvoluted protein masses. Because PilE and PilE$_{AAMc6His}$ was coexpressed in the same strain and purified in the same sample, a ratio of PilE$_{AAMc6His}$ to PilE protein amount could be directly calculated without the need for an internal standard.

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