Type IV Pilus Assembly Proficiency and Dynamics Influence Pilin Subunit Phospho-Form Macro- and Microheterogeneity in Neisseria gonorrhoeae

Åshild Vik1,2, Jan Haug Anonsen1,2, Finn Erik Aas1,2, Finn Terje Hegge1,2a, Norbert Roos1, Michael Koomey1,2, Marina Aspholm1,2a,b

1 Centre for Molecular Biology and Neuroscience, University of Oslo, Oslo, Norway, 2 Department of Biosciences, University of Oslo, Oslo, Norway

Abstract

The PpIE pilin subunit protein of the gonococcal Type IV pilus (Tfp) colonization factor undergoes multisite, covalent modification with the zwitterionic phospho-form modification phosphoethanolamine (PE). In a mutant lacking the pilin-like PilV protein however, PpIE is modified with a mixture of PE and phosphocholine (PC). Moreover, intrastrain variation of PpIE PC modification levels have been observed in backgrounds that constitutively express PptA (the protein phospho-form transferase A) required for both PE and PC modification. The molecular basis underlying phospho-form microheterogeneity in these instances remains poorly defined. Here, we examined the effects of mutations at numerous loci that disrupt or perturb Tfp assembly and observed that these mutants phenocopy the pilV mutant vis a vis phospho-form modification status. Thus, PC modification appears to be directly or indirectly responsive to the efficacy of pilin subunit interactions. Despite the complexity of contributing factors identified here, the data favor a model in which increased retention in the inner membrane may act as a key signal in altering phospho-form modification. These results also provide an alternative explanation for the variation in PpIE PC levels observed previously and that has been assumed to be due to phase variation of pptA. Moreover, mass spectrometry revealed evidence for mono- and di-methylated forms of PE attached to PpIE in mutants deficient in pilus assembly, directly implicating a methyltransferase-based pathway for PE synthesis in N. gonorrhoeae.

Introduction

In all living organisms, many newly synthesized proteins are post-translationally modified to attain their intended function. These post-translational modifications (PTMs) involve both breakage of covalent bonds (i.e. by proteolytic cleavage) and formation of new bonds by addition of chemical moieties to the protein. Chemical groups added to the protein range from simple phosphorylation and acetylation to more complex modifications such as fucosylation and sialylation. These post-translational modifications of bacterial proteins. Covalent modification of bacterial protein has also been described in many Gram-negative species, phosphorylase (PE) and phosphocholine (PC) [here collectively referred to as phospho-form modifications] are common cell surfaces constituents of pathogenic and commensal bacteria of humans (Fig. 1A). In many Gram-negative species, phosphorylase modifications are found on lipopolysaccharides and lipooligosaccharides where they influence structure, antigenic interactions with innate immune components [1–5]. It has been shown that PC modified constituents expressed on Gram-negative bacterial surfaces have the ability to impact adherence to epithelial cells via interaction with the platelet activating factor (PAF) receptor as well as to act as ligands for C-reactive protein and PC-recognizing antibodies [5–9]. In addition, PE and PC moieties have been identified as direct post-translational modifications of bacterial proteins. Covalent attachment of PE and PC to protein was first demonstrated in Neisseria gonorrhoeae where they were shown to be O-linked to the type IV pilus (Tfp) subunit protein PilE via a serine residue [10]. As seen by reactivity with the TEPC-15 monoclonal antibody directed against the Tfp pilin, pilin proteins of N. gonorrhoeae [11]. However, the general significance of PE and PC phospho-form modifications of neisserial proteins has yet to be determined. Phospho-form modification of bacterial protein has also been described in C. jejuni, where the flagellar rod protein FlgG undergoes PE modification and mutants lacking this PTM display defects in flagellar expression and thus motility and virulence [15,16].
In light of their broad distribution and potential biological significance, increased attention has been directed at characterizing the biosynthesis of phospho-form modifications. The *Escherichia coli* EptB protein catalyzes PE modification of the inner core of LPS, using phosphatidylethanolamine as a donor substrate [17]. Many Gram-negative species express EptB-related proteins implicated in LPS/LOS PE modification including *Salmonella enterica* [18] and *Pseudomonas aeruginosa* [19] from *N. meningitidis*. The neisserial PptA protein (phospho-form transferase Λ) is also structurally related to EptB and is necessary for modification of *N. gonorrhoeae* proteins with both PE and PC [14,20]. In *N. meningitidis*, PptA is implicated in PC modification and high-frequency frame-shifting events within the PilE PC epitope [11]. The structural relatedness between PptA and EptB and other PE transferases utilizing phosphatidylethanolamine as a donor strongly suggests that PptA employs a similar mode of action. It might be assumed that PptA differentially uses both phosphatidylethanolamine and phosphatidylcholine as precursors. However, the latter phospholipid has been only documented once in *N. gonorrhoeae* [21], while a follow up study of gonococcal phospholipids failed to document its presence [22]. Although phosphatidylcholine is found in diverse groups of bacteria, there are no established instances in which its head group serves as a precursor for phosphoform modification of LPS/LOS or proteins.

So far, two pathways for phosphatidylcholine synthesis are established in bacteria: one where endogenous phosphatidylethanolamine undergoes methylation by phospholipid N-methyltransferases and another in which it is synthesized via direct condensation of exogenous choline with CDP-diacylglyceride mediated by phosphatidylcholine synthase [23]. The latter process is responsible for PC modifications of both LPS/LOS and teichoic/lipoteichoic acids and utilizes enzymes encoded by the *lic* genes [4,9,24]. Although present in commensal neisseria species where they are involved in LOS modification, *lic* genes are absent in both *N. gonorrhoeae* and *N. meningitidis* [9]. Thus far, the mechanism by which phosphatidylcholine is produced in the pathogenic neisseria remains unknown and neither phospholipid A-methyltransferases nor phosphatidylcholine synthase orthologs are readily identifiable within neisserial genome sequences.

Another confounding feature of PptA-mediated protein phospho-form modification relates to the variability with which it is observed in gonococi. Under standard laboratory growth conditions gonococcal phospho-form modified proteins carry PE. However, under other growth conditions and in some genetic backgrounds a subset of these modification sites may instead carry PC [10,14,20]. For example, a mixture of PE and PC modified PilE was observed in a mutant carrying a null allele of *pilV* (encoding a Tfp pilin like protein that modifies Tfp-related functions) while PilE from the wild-type background carried only PE. The primary site of phospho-form modification is serine 68 of the mature gonococcal PilE protein, which is in the immediate vicinity of the glycosylation site at serine 65. In addition, there is a second phospho-form modification site at serine 156, in the C-terminus of PilE, that is only partially utilized. However, in the *pilV* null mutant the PE modification at serine 156 is more common [25]. Furthermore, it has been shown that the glycosylated lipoproteins Ngo1043 and Ngo1237 are modified with PE in a wild-type background while PC was detected in a background defective in broad spectrum O-linked protein glycosylation [14]. The phospho-form modification of the lipoproteins was also *pilA* dependent [14]. However, in contrast to what was observed for PilE, PilV status had no effect on phospho-form microheterogeneity of Ngo1043 and Ngo1237 [14]. It is therefore clear that the phospho-form modification status may be modulated by ancillary factors in a protein substrate-specific fashion.

To gain insights into the molecular basis for the PE to PC switch, we sought to identify factors other than PilV involved in the pilus biogenesis processes that modulate PilE phospho-form modification status. Here, we present new data establishing a connection between differential PC modification and the integrity of the Tfp biogenesis pathway. Our data also provide further insight into interplay between O-linked protein glycosylation and phospho-form modification status. Finally, we present data to support a model in which stepwise PE methylation is involved in PC phospho-form modification.

**Experimental Procedures**

**Bacterial strains and culture conditions**

The bacterial strains used in this study were derived from the MS11 background and are described in Table S1. GenBank accession numbers refer to MS11 sequences where appropriate and available. Strains were grown in conventional liquid GC medium or on GC medium plates [26], except that Thiottone E Peptone was replaced by Proteose Peptone No. 3 (Difco). When needed, antibiotics were used in the following concentrations: kanamycin (50 μg/ml), chloramphenicol (30 μg/ml) and erythromycin (8 μg/ml).
Wild-type and altered pilE alleles (E5L, E5V [27], G18 [28], AAM [27], H7T, V9M, A20T, W109S [29], AAM/c6His, E5K/c6His [30]) were ectopically expressed from the iga locus and introduced into the genomes of strain 4/3/1 and KS645 by transformation with genomic DNA and selection for erythromycin resistance. The pilE (EEZ48662.1) mutation was introduced by transformation with plasmid p12-DUS [31] and selection for erythromycin resistant transformants. Null mutants in pilI (EEZ48222.1) (pilI::kan [20]), comP (EEZ40327.1) (comP::cm-m-Tn10 [32]), pilD (EEZ40665.1) (pilD::Trx3erm [26]), pilP (EEZ47066.1) (pilP::m-Tn1 [29]), pilQ (EEZ47065.1) (pilQ::cm-m-Tn1 [26]), pilT (EEZ49064.1) (pilT::m-Tn17 [29]), pilU (pilU::m-Tn3erm [34]), pilV (EEZ40312.1) (pilV::kan [35]), pilC2 (EEZ47021.1) (pilC2::cm [36]), pilH (EEZ47439.1) (pilH::cm) [37], pilI (EEZ47440.1) (pilI::cm), pilJ (EEZ47441.1) (pilJ::cm), pilK (EEZ47442.1) (pilK::cm), pilL (EEZ47443.1) (pilL::cm) [37], pilC (EEZ47055.1) (pilC::kan [10]), and pglE (where the pglE4824 phase-on sequence [38] is identical to the native FA1090 sequence NCO9207/AKW89960) were introduced into various strain backgrounds by transformation as previously described [14,30,39]. Mutants were verified by PCR and/or immunoblotting with appropriate antibodies.

Construction of a strain carrying three copies of pilE

A SacI fragment from pPilE2 containing the entire pilE ORF and 5’ promoter sequences was cloned into p2/16/1 digested with SacI [40,41]. Among a number of clones screened by PCR and sequencing for insertion of the pilE ORF, one turned out to contain two copies of the SacI fragment positioned as a direct tandem repeat. The two linked pilE loci were introduced into the iga locus of the wild-type strain N100 using transformation with the p2/16/1 derivative (KP26) and selection on GC agar plates containing erythromycin. The resulting strain KS647 (3x pilE) thus contained three copies of the pilE locus.

Pili purification

Pili were purified by the ammonium sulfate procedure as previously described [40]. Briefly, cells were resuspended and vortexed in 0.15 mM ethanolamine buffer (pH 10.2). Cells shear depleted of pili were removed by centrifugation at 16,000 g for 5 minutes, and pili were precipitated from the suspension by vortexing in 0.15 mM ethanolamine buffer (pH 10.2). Cells shear depleted of pili were made from the pellet previously described [40]. Briefly, cells were resuspended and vortexed in 0.15 mM ethanolamine buffer (pH 10.2) and diluted one in 1.15 mM ethanolamine buffer (pH 10.2) and diluted relative to the OD/cell number used for the original pilus preparation. Procedures for SDS-PAGE and immunoblotting are described in [26,27]. Pilin was detected using the z-pilin rabbit polyclonal antibody at 1:1000 dilution. These antibodies were raised against the PilE-derived synthetic peptide 31KSAYVTEYLIHNHGPENNTS37 (the peptide is numbered according to the unprocessed full length protein) [27]. PC was detected using the mouse monoclonal TEPC-15 antibody (Sigma) at 1:750 dilution. The N. gonorrhoeae Ac-Gal-z-dNacBac trisaccharide was detected using the rabbit monoclonal npg3 antibody [39].

Sample preparation and infusion MS analysis of intact PilE protein

PilE protein samples were cleaned with a methanol/chloroform procedure as described [1]. The pellet was dried in an inverted tube for 5 minutes before sample was dissolved in 100 μl of 10% MeOH, 70% formic acid, acetonitrile 1:1:3 (v/v/v). Samples were subjected to mass spectrometric analysis or frozen at −80°C. All data were acquired on a LTQ OrbitrapXL mass spectrometer (Thermo Electron, Bremen) operated by Xcalibur 2.0, in the positive ion mode. The LTQ OrbitrapXL mass spectrometer was calibrated (proteomass LTQ/FT-hybrid Cal Mix, Supelco) and tuned using the ion at m/z 1021 prior to PilE analysis. Mass spectrometric analysis of PilE was performed with common parameter settings of: spray voltage at 3200 V, capillary temperature at 275°C, capillary voltage of 30 V, tube lens voltage of 110 V and sheath gas flow of 8 a.u. Sample solutions were infused into the ESI source at a flow rate of 7 μl/min by a syringe. Protein mass spectra were acquired at a resolution of 60000 at m/z 400. Protein masses were determined by deconvolution 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 [25]. All amino acids are numbered according to the unprocessed full length protein.

In-gel protein digestion

Coomassie stained gel slices of purified PilE were washed and destained as previously described [25] except without alkylation and reduction. Digestion steps with trypsin (Sigma) at 37°C over night were performed as described previously [25]. Dried samples were frozen at −80°C and redissolved in 0.1% formic acid prior to liquid chromatographic tandem mass spectrometric (LC-MS2) analyses.

Reverse phase LC- MS2 analysis of proteolytic peptides

Nanoflow LC-MS and MS2 analyses (nano-LC-MS2) of proteolytic peptides were performed using an Agilent 1200 series capillary HPLC system with a corresponding autosampler, column heater, and integrated switching valve coupled via a nanoelectrospray ion source to a LTQ-Orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) as previously described [14] with the following modifications: only a gradient over 60 minutes from 5% to 55% of acetonitrile with 0.1% formic acid prior to liquid chromatographic tandem mass spectrometric (LC-MS2) analyses.
120 at a resolution of 7500 at m/z 400. All Orbitrap analyses were performed with the lock mass option (lock mass set at m/z 445,120024 [42]) for internal calibration. The ion selection threshold was 500 counts and selected fragment ions were dynamically excluded for 180 seconds.

Results

Mutations that disrupt pilus assembly impact on PC modification of PilE

Previous analyses of PilE phospho-form modifications have been made on strains expressing pili on the cell surface. In the course of our studies of mutants failing to express surface localized Tfp, we have occasionally observed alterations in the relative mobility of PilE analogous to that seen in pilE null backgrounds that might be indicative of altered PTMs. Therefore, we examined this possibility in more detail using a panel of congenic strains carrying null alleles of pilD (encoding the prepilin peptidase), pilF (the assembly ATPase [26]), pilQ (the outer membrane channel/secretin [43]), pilP (an inner membrane lipoprotein required for transformation [33]) and pilG (required for transformation [44]). These mutants are all unable to express pili on the cell surface. But, in contrast to the other mutants that fail to assemble pili under any circumstance, the pilQ mutant assembles pili that are retained in the periplasm when the PilI retraction ATPase is absent [45]. PilE from whole cell extracts of all these mutants demonstrated strong PptA-dependent reactivity with the monoclonal PC recognizing antibody TEPC-15, whereas the wild-type background, which expressed normal levels of surface localized pili, showed no reactivity (Fig 1B). PilE carrying an alanine substitution at residue 68 (a pilEs68A background) abolished the TEPC-15 reactivity in all backgrounds suggesting that PC modification occurred on serine 68, consistent with previous results derived from analysis of PC modification in pilE mutants [25]. Together, these results suggest that differential PC modification of PilE can be associated with disruption of Tfp expression.

Impact of effectors of Tfp dynamics and function on PC modification of PilE

To gain further insights into factors affecting PilE phospho-form modification, we next examined pilus-associated factors that impact Tfp dynamics and function but are dispensable for assembly of Tfp [35,37]. Such mutants fall into two classes: one in which Tfp assembly is maintained at high levels while Tfp-associated functions are disrupted, and another in which Tfp assembly is significantly reduced. For the latter class Tfp assembly defects can be suppressed by loss-of-function mutations in PilT, the retraction ATPase, but despite their pilated phenotype, these suppressor mutants are defective in Tfp-associated functions [36,37]. From the first class of mutants examined were those lacking the pilin-like proteins PilV [35], ComP which expression is essential for transformation [32], as well as PilU, a second ATPase influencing Tfp-associated autoagglutination [34]. We confirmed the PptA-dependent PC modification associated with the pilV mutation but observed no TEPSC-15 reactivity in the other two mutants or the wild-type background (Fig 2). We next tested six mutants from the second class including those lacking the Tfp associated adhesin PilC [46] and null mutants individually lacking the pilin-like proteins PilH, PilI, PilJ, PilK and PilE (encoded within the pilH-L locus). These are all required for expression of normal levels of pilination but their individual roles in pilus biogenesis are unknown [37]. In these mutants PilE in whole cell lysates and purified pili showed PptA-dependent PC modification. This reactivity correlated with expression of S-pilin, a soluble smaller pilin that is a cleavage product of the mature pilin protein and that has been correlated with defects in pilus assembly [28,47]. However, PC reactivity was not observed for S-pilin. Conditional repression by pilT in the pilH-L and pilC mutants restored high-level Tfp expression but did not result in any discernible difference in PC reactivity of PilE compared to the pilT wild-type background (Fig. 2). Thus, altered phospho-form modification profiles in the pilC and pilH, l, j, k and l mutants phenocopy that seen in the pilV background.

Influence of pilE missense mutations on phospho-form modifications

Our results suggest that block or delay in pilus assembly leads to PC modification of pilin. However, it is unclear whether the PC modification only relates to dysfunction of the pilus biosynthesis machinery or could also be induced by alteration of the pilin molecule itself. To investigate this, we next sought to find out if mutations within pilE that are known to impede pilus assembly or affect pilus morphology and/or associated functions also lead to PC modification. Various pilE mutants were expressed from the(s)14 locus in a background in which the endogenous pilin locus was under the control of an IPTG derepressible promoter [40]. No IPTG was added to the cultures and thus, the endogenous pilin locus was silent. We tested the pilEs68A, pilEs68N and pilEs68H mutants which carry substitutions at the highly conserved charged residue glutamine +5 (E5) [27,30,48-50], the pilEs68S mutant which encodes a glycine to serine substitution at position +1 relative to the mature protein [28], the pilEs68A pilEs68N pilEs68S triple substitution mutant which produces rare short Tfp and is blocked in S-pilin production [27], and the pilEs68A pilEs68N and pilEs20T mutants which carry alterations in the conserved amino terminal domain of PilE. The E5L, E5V, G-1S, AAMc6His and E5Kc6His mutations have all been shown to preclude Tfp expression and associated functions [28,30,48-51]. The 14T, V9M, and A20T mutants all express Tfp at levels similar to the wild-type strain but exhibit a non-aggregating phenotype and reduced ability to adhere to a human epithelial cell line [29]. Moreover, pilin from these mutants assembles into pili that are morphologically distinct from pili of the wild-type strain [29]. As shown in Fig. 3 all mutants devoid of pilus expression demonstrated strong pptA-dependent PC reactivity of PilE. Furthermore, the pilEs68A mutant that makes few and short pili was also among those with strong PC reactivity. Far more reduced PC reactivity was seen in both whole cells and purified pili from the 3 missense mutants pilEs4T, pilEs7N and pilEs20T. For these missense mutants, the PC reactivity appeared stronger in purified pili but this was probably due to a higher concentration of PilE in extracts of isolated pili. Together, these results suggest a correlation between severity of assembly defect and degree of PC reactivity in the pilE mutants.

Overexpression of pilE is associated with PC modification

Tfp undergo bidirectional remodeling of pilin subunits between an integral inner membrane state and a multimeric, filamentous state. Alteration in this remodeling is a common feature of many of the mutants displaying altered phospho-form modification. We hypothesized that increasing the amount of PilE in a background where other Tfp expression factors were constant might lead to accumulation of pilin in the inner membrane state and in turn lead to PC modification. To address this directly, we constructed strains carrying either one or two identical additional copies of pilE at an ectopic site. Doubling or tripling pilE copy number led to increased levels of PilE in whole cells and increased levels of intracellular pilin in cells depleted of pili by shear fractioning.
Figure 2. Lack of pilus associated proteins leads to PptA-dependent PC modification of PilE. Immunoblot analysis of whole cell lysates of equal numbers of cells and of equal amounts of protein from purified pili. The antibodies used were the PC recognizing TEPC-15 and the PilE peptide specific antibody γ-pilin. Strains used were in A) wild-type (N400), pilT (KS9), pilTind (MW24), pilC (KS787), pilC pilT (KS789), pilV (KS790), pilV pilT (KS791), comP (KS792), comP pilT (KS793), pilT (KS794), pilU (KS795), pilU pilT (KS796), pilU pilTind (KS798) and in B) wild-type (N400), pilTind (KS786), pilH (KS789), pilH pilT (KS800), pilH pilTind (KS801), pilH (KS802), pilH pilT (KS803), pilH pilTind (KS804), pilH pilT (KS805), pilH pilTind (KS807), pilH (KS808), pilI pilT (KS809), pilI pilTind (KS810), pilI pilT (KS811), pilI pilTind (KS812) and pilI pilU pilTind (KS813). The faster migrating protein band below pilin is S-pilin (indicated by an arrow), a proteolytic degradation product of PilE that is a correlate of type IV pilus biogenesis defects and which requires pilT expression [36]. The strains were grown on standard GC plates without inducer such that the pilTind and pilTloci were not expressed. All samples on each blot were run on the same gel and the dotted lines were introduced as guidance facilitating evaluation of the data. Results representative of at least three different experiments are shown. doi:10.1371/journal.pone.0096419.g002
Figure 3. Mutations in pilE that perturb assembly of Tfp lead to PC modification of PilE. Immunoblot of whole cell lysates of equal numbers of cells and of equal amounts of protein from purified pilin. The antibodies used were the TP-E15 antibody and the PilE peptide specific x-pilin antibody. – denotes a null allele and + denotes a wild-type allele of pilA. Strains used were wild-type (N400), pilE (A20T), pilE (4/3/1), pilE (KS130), pilE ppgt (KS813), pilE E5KHis (KS815), pilE E5V (KS816), pilE E5L (KS817), pilE E5KHis (KS818), and pilE E5V (KS819), pilE E5V (KS769), and pilE E5V (KS821), pilE E5V (KS722), and pilE E5V (KS723), pilE (KS724), and pilE E5V (KS774), and pilE E5V (KS775), and pilE E5V (KS776). The strains were grown on standard GC plates without inducer such that pilin was not expressed. All samples on each blot were run on the same gel and the dotted lines were introduced as guidance facilitating evaluation of the data. Results representative of at least three different experiments are shown. doi:10.1371/journal.pone.0096419.g003

status of PilE or that of other proteins of the glycoproteome. Alternatively, the serine 63 mutation may affect the recognition of the adjacent serine 68 as substrate for PC modification. To distinguish between these scenarios the pglC allele was introduced into the 2x pilE S63A pilE E5KHis and 2x pilE S63A pilE E5L backgrounds. The resulting pilE S63A pilE E5KHis and 2x pilE S63A pilE E5L strains, which lack glycan on PilE as well as on other proteins, did not demonstrate increased PC reactivity on PilE compared to the pilE S63A pilE E5KHis and 2x pilE S63A pilE E5L backgrounds. This suggested that the serine 63 mutation affected the recognition of serine 68 as an attachment site for PC modification such that PilE S63A was no longer an optimal substrate for PC modification.

In addition to seeing an effect of pilin glycosylation on PC modification in the pilC versus the pilE E5KHis mutants, we saw that increasing the protein-associated oligosaccharide chain length from a disaccharide to a triasaccharide was associated with diminished PC modification in the 2x pilE pilE E5KHis and 3x pilE pilE E5KHis backgrounds. This effect of glycosylation was also seen by mass spectrometry (MS) analysis of purified pilin, where the 3x pilE pilE E5KHis background demonstrated less PC modification of PilE relative to the 2x pilE pilE E5KHis background. However, the peaks corresponding to the PC moiety and the acetylated trisaccharide on PilE were overlapping, resulting in overestimation of the level of PC modification in the 2x pilE pilE E5KHis background (data not shown).

The effects of pilin glycosylation could have three different causes: Firstly, the triasaccharide moiety could suppress the physiological event that leads to increased PC modification. Secondly, the bulkiness of the longer glycan adjacent to the PE/PC modification site could block accessibility of the PC moiety by the PC reactive monoclonal antibodies. Thirdly, the presence of a longer glycan could alter the properties of PilE making it less optimal as a substrate for PC modification. To discriminate between these possibilities, the pilF mutation was introduced. As shown in Fig. 3B, the presence of triasaccharide on PilE did not suppress PC reactivity in the pilF mutant. This suggests that the triasaccharide modification of pilin did not grossly disturb the recognition of PilE as a substrate for PC modification or the accessibility of the PC epitope for the PC reactive antibodies. We therefore favor the scenario where the presence of glycan on PilE somehow helps suppress the physiological defect that leads to PC modification.

MS analysis confirms altered PilE phospho-form micro- and macroheterogeneity

To confirm and extend the findings made using TP-E15 reactivity as read-out, ‘top-down’ mass spectrometry of PilE was performed using purified Tfp from the pilH pilT, pilI pilT, pilF pilT, pilV pilT, pilK pilT and pilC2 pilT mutants (Fig 6A–G). PC modification increases the mass of PilE by 123.0 Da whereas a PC modification increases the mass of PilE by 165.1 Da [10,14]. The disaccharide Ac-Gal-diNAcBac modification increases the mass of PilE by 123.0 Da whereas a PC modification increases the mass of PilE by 165.1 Da [10,14]. The disaccharide Ac-Gal-diNAcBac modification increases the mass of PilE by 123.0 Da whereas a PC modification increases the mass of PilE by 165.1 Da [10,14]. The disaccharide Ac-Gal-diNAcBac modification increases the mass of PilE by 123.0 Da whereas a PC modification increases the mass of PilE by 165.1 Da [10,14]. The disaccharide Ac-Gal-diNAcBac modification increases the mass of PilE by 123.0 Da whereas a PC modification increases the mass of PilE by 165.1 Da [10,14]. The disaccharide Ac-Gal-diNAcBac modification increases the mass of PilE by 123.0 Da whereas a PC modification increases the mass of PilE by 165.1 Da [10,14]. The disaccharide Ac-Gal-diNAcBac modification increases the mass of PilE by 123.0 Da whereas a PC modification increases the mass of PilE by 165.1 Da [10,14]. The disaccharide Ac-Gal-diNAcBac modification increases the mass of PilE by 123.0 Da whereas a PC modification increases the mass of PilE by 165.1 Da [10,14]. The disaccharide Ac-Gal-diNAcBac modification increases the mass of PilE by 123.0 Da whereas a PC modification increases the mass of PilE by 165.1 Da [10,14]. The disaccharide Ac-Gal-diNAcBac modification increases the mass of PilE by 123.0 Da whereas a PC modification increases the mass of PilE by 165.1 Da [10,14]. The disaccharide Ac-Gal-diNAcBac modification increases the mass of PilE by 123.0 Da whereas a PC modification increases the mass of PilE by 165.1 Da [10,14]. The disaccharide Ac-Gal-diNAcBac modification increases the mass of PilE by 123.0 Da whereas a PC modification increases the mass of PilE by 165.1 Da [10,14]. The disaccharide Ac-Gal-diNAcBac modification increases the mass of PilE by 123.0 Da whereas a PC modification increases the mass of PilE by 165.1 Da [10,14]. The disaccharide Ac-Gal-diNAcBac modification increases the mass of PilE by 123.0 Da whereas a PC modification increases the mass of PilE by 165.1 Da [10,14]. The disaccharide Ac-Gal-diNAcBac modification increases the mass of PilE by 123.0 Da whereas a PC modification increases the mass of PilE by 165.1 Da [10,14]. The disaccharide Ac-Gal-diNAcBac modification increases the mass of PilE by 123.0 Da whereas a PC modification increases the mass of PilE by 165.1 Da [10,14]. The disaccharide Ac-Gal-diNAcBac modification increases the mass of PilE by 123.0 Da whereas a PC modification increases the mass of PilE by 165.1 Da [10,14]. The disaccharide Ac-Gal-diNAcBac modification increases the mass of PilE by 123.0 Da whereas a PC modification increases the mass of PilE by 165.1 Da [10,14].
Phospho-form modified PilE could be subdivided into molecules with (78%) and without (10%) glycosylation. About 13% of PilE carried PC and glycan whereas roughly 2% of PilE carried PC but no glycan (Fig. 7B). In total, 15% of all PilE molecules carried PC that corresponded to a ratio of PC to PE modified PilE of approximately 0.2 (15%/73%).

Together these data show that PilE in all these mutants undergo alterations in phospho-form site occupancy and phospho-form microheterogeneity identical to what is previously reported for pilV null mutants [25]. Furthermore, the simultaneous modification of pilin molecules with both glycan and PC shows that the glycan modification of pilin did not prevent recognition of PilE as a substrate for PC modification.

**Evidence for a PE N-methyltransferase pathway for PC precursor biosynthesis**

In the mass spectra of PilE from the pilus biogenesis mutants, peaks indicative of the addition of one methyl group (theoretical addition of +14.02 Da) and two methyl groups (theoretical addition of +28.04 Da) to PE were observed (Fig. 6). Peaks corresponding to addition of one and two methyl groups to PE were not detected in mass spectra from a ppgA deficient control background (ppgA pilV - data not shown), indicating that the methyl groups were dependent upon the presence of a PE modification.

To determine the sites of methylation, we enzymatically digested PilE from the pglC pilV pilT background (to avoid problems relating to the near identical masses of disaccharide acetylation and three methylation of PE) and subsequently analyzed the tryptic generated peptides by MS. The MS1 spectrum in Fig. 8A shows the quadruply charged precursor at m/z 812.39 corresponding to the mass of the tryptic peptide 52SAVTEYLNHGW-850.16 (giving an observed mass of 3397.62 Da [M+H]+) corresponding to addition of one and two methyl groups to PilE (mass of 843.15, or PE and one methyl group (theoretical addition of 137.03 Da) at m/z 846.65, of PE and two methyl groups (theoretical addition of 151.05 Da) at m/z 850.16 and of PC (theoretical addition of 165.06 Da) at m/z 853.66 to the peptide 32SAVTEYLNHGKW-853.66 as was seen by fragmentation of the precursor ion at m/z 843.15 (calculated and adjusted for charge state). The quadruply charged precursor ion at m/z 843.15 (giving an observed mass of 3396.58 Da [M+H]+) corresponded to the peptide 32SAV-TEYLYNHGKW-PENNTSAAGVASPPTDIK81 (theoretical mass 8245.67 Da) with one PE modification (Fig. 8C). Moreover, the y- and b- ions generated by fragmentation of the precursor ion at m/z 843.15 were consistent with the peptide 32SAVTEYLYNHGKW-PENNTSAAGVASPPTDIK81 (theoretical monoisotopic mass 3246.57 Da) with one PE modification (Fig. 8C). Moreover, the y- and b- ions generated by fragmentation of the precursor ion at m/z 843.15 were consistent with the peptide 32SAVTEYLYNHGKW-PENNTSAAGVASPPTDIK81. In the low mass area in the fragmentation spectrum, the reporter ion at m/z 142.0, corresponding to [promoted] PE, [10,14], could be detected. (Fig. 8C). This demonstrates that the precursor mass at m/z 843.15 is the PE modified version of the peptide 32SAVTEYLYNHGKW-PENNTSAAGVASPPTDIK81. The quadruply charged precursor ion at m/z 846.65 (giving an observed mass of 3383.58 Da [M+H]+) and the quadruply charged precursor ion at m/z 850.16 (giving an observed mass of 3397.62 Da [M+H]+) corresponded to the peptide 32SAVTEYLYNHGKW-PENNTSAAGVASPPTDIK81 with one PE modification and one (Fig. 8D) or two (Fig. 8E) methyl groups, respectively. A similar pattern of y- and b- ions were generated by fragmentation of the precursors ions at m/z 846.65 and at m/z 850.16. As was seen by fragmentation of the precursor ion at m/z 843.15, consistent with the peptide 32SAVTEYLYNHGKW-PENNTSAAGVASPPTDIK81.
No methylation of amino acids was detected by de novo sequencing of the peptides and analysis of b- and y-ions. Also, an abundant reporter ion at m/z 156.0, corresponding to monomethylated PE (mmPE, theoretical mass 156.04 Da) was detected in the fragmentation spectrum of the precursor ion at m/z 846.65 (Fig. 8D) and an abundant reporter ion at m/z 170.1, corresponding to dimethylated PE (dmPE, theoretical mass 170.06 Da) was detected in the fragmentation spectrum of the precursor ion at m/z 850.16 (Fig. 8E). The quadruply charged precursor at m/z 853.66 (giving an observed mass of 3411.62 Da [M+H]+) corresponded to the peptide SAVTEYLYNHWK-PENNTSAGVASPTTDK31 with one PC modification. Moreover, in the low mass area of this spectrum, an abundant reporter ion at m/z 184.1, corresponding to PC was detected [10, 14] (Fig. 8F). The MS analysis therefore confirmed that the phospho-form modified peptide SAVTEYLYNHWK-PENNTSAGVASPTTDK31 was modified with PE, mmPE, dmPE and PC (Fig. 8G).

Our discovery of mmPE and dmPE on a PilE phospho-form modified peptide led us to also investigate Ngo1043 for evidence of mmPE and dmPE. This was done by analyzing trypsin generated peptides from Ngo1043-His purified from a liquid medium induces PC on pilin [20]. Which of the phospho-form moieties have the greatest functional significance in vivo is not currently clear.

Discussion

Since the discovery of phospho-form post-translational modifications in N. gonorrhoeae, the question regarding how PC modification is regulated has remained unanswered. Here, we characterized a defined set of mutants in which PilE undergoes PptA-dependent PC modification and that have perturbations in Tfp expression as their common denominator. These include effectors of pilus homeostasis in which Tfp dynamics are perturbed as well as bona fide assembly factors in which Tfp biogenesis is abolished. Analyses of PilE missense mutants also revealed a strong correlation between assembly competence and phospho-form microheterogeneity. Finally, both altered O-linked protein glycosylation and increased PilE expression were found to increase phospho-form microheterogeneity.

Our data showed that about 15% of the pilin molecules became PC modified in a Tfp effector mutant whereas the majority carried only PE. Based on this, one could argue that the major effect on phospho-form microheterogeneity could be that more of the pilins carried 2 PE moieties instead of one. However, it is worth noting that the ratio of PC to PE modified pilin also is environmentally influenced. For instance, it has been shown that growth in defined liquid medium induces PC on pilin [20]. Which of the phospho-form moieties have the greatest functional significance in vivo is therefore not currently clear.

How does perturbing the integrity of Tfp assembly and dynamics lead to differential phospho-form modification of PilE? As mutants of both classes show reduced or abolished piliation, they are likely to have increased intracellular concentration of the protein substrate and increased retention time of the protein substrate in the inner membrane relative to what is seen in the wild-type background. This could result in changes in membrane sublocalization and increased interaction with the enzymatic
machinery responsible for PC modification, providing an explanation for our observations. This scenario could also underlie the altered phospho-form modification associated with increased PilE expression. For example, elevating the exact concentrations of PilE in the inner membrane relative to other Tfp-related proteins might create situations where the latter are limiting. Overexpressing PilE may therefore induce PC modification in much the same way as the Tfp homeostasis mutants.

Anonsen and colleagues previously reported that loss of O-linked protein glycosylation was associated with a switch from PE modification alone to both PE and PC in two membrane-associated glycoproteins, Ngo1043 and Ngo1237 [14]. Neither of these proteins have any known relation to pilus function or biogenesis. Another important finding from that work is that the loss of PilV, which induces PC modification of PilE, did not impact phospho-form microheterogeneity in either Ngo1043 or Ngo1237. Thus, it seems the signals provoking phospho-form microheterogeneity are intrinsic to the protein substrates themselves and result in specific or localized responses rather than global alterations in which all protein substrates are targeted. These findings argue against any model where increased PC modification is a result of increased abundance of the modifying enzyme(s) or the PC donor substrate.

Our data suggested that the glycosylation status of PilE caused increased PC modification of pilin and also in this case did the effect seem to be intrinsic to the protein itself. We have previously shown that glycosylation of PilE can impact on Tfp dynamics by effects mediated at the level of pilin subunit-subunit interactions and bidirectional remodeling of pilin between its membrane-associated and assembled states [31]. It is therefore possible that glycosylation status can induce phospho-form microheterogeneity through its effects on Tfp assembly. Alternatively, glycosylation may directly affect the efficiency with which pilin is PC modified. It is for instance possible that the glycan directly affects the pilin interaction with the enzyme machinery or the membrane sublocalization of pilin such that it co-localizes better with the PC modification machinery and the PC donor. These models could also explain the interplay between glycosylation and phospho-form modification in Ngo1043 and Ngo1237. Another possibility that has been suggested is that the glycan may sterically hinder the interaction between the PC modifying machinery and its target protein [7]. In gonococcal pilin, the glycosylation site and PC modification site are relatively close, at S63 and S68, respectively. Although we cannot completely rule out that steric hindrance has some effect, our MS data clearly show that pilin can carry both PC and glycan on the same protein molecule. In fact, the ratios of glycosylated to non-glycosylated pilin carrying PE and PC in Tfp effector mutants were very similar, suggesting that steric hindrance as a model fails to fully explain our results.

Other models that in themselves easily could explain our data regarding modification of PilE are difficult to generalize. For instance, all mutants characterized here phenocopy pilV null mutants with regard to PC modification and phospho-form hypermodification. Thus, an alternative explanation for our data could not be that the mutations utilized here are epistatic to pilV and therefore preclude PilV function. It has previously been observed that hyper-modification occurs in the absence of PilV while hypomodification occurs when PilV is overexpressed [7]. In this model PilV would inhibit PptA-dependent modification. Perhaps then, PilV might physically occlude PilE from interacting with PptA or otherwise reduce accessibility of PilE to PptA. However, it is likely that pilV mutants could share a common defect in the extension-retraction pathway with the other pilus related mutants. In fact, we observed here for the first time that a pilV null mutant expresses S-pilin, a soluble truncated form lacking the N-terminal 39 residues, and that this altered processing (but not PC modification) was suppressed in conjunction with a pilT null mutation. This phenotype is also observed with mutations in other effectors of pilus homeostasis, suggesting that pilV mutants may induce PC modification in the same way as they do.

It is important to note that an earlier study reported what was described as phase variation of the PilE PC epitope as seen by its variable detection in a panel of intrastrain pilin antigenic variants in N. gonorrhoeae [12]. Based on the subsequent identification of PptA as the responsible phospho-form transferase [25], and of its gene being potentially subject to high frequency, slipped strand-based frameshifting [11], it has been generally assumed that such variation was due to pptA phase variation. However, gonococcal strain MS11 that was used in that earlier study and that is the parent to that used by us here carries a non-phase variable pptA allele. Based on our findings here and fact that different PilE antigenic variants can vary in their intrinsic assembly proclivities, we suggest that structural PilE diversity arising via gene conversion and associated variation in assembly proclivity provides a heretofore unrecognized source for so-called phase variation of the PC epitope.

Another interesting aspect of protein targeted phospho-form micro-heterogeneity yet to be resolved relates to the source of the PC moiety. PptA is a member of the PE transferase protein family that recognizes the phosphoplipid head group of phosphatidylycerolamine as a donor. Accepting that phosphatidylycerolamine is
between m/z 800-900 of the precursor masses corresponding to the quadruply charged peptide 52SAVTEYYLNHGKWPENNTSAGVASPPTDIK81 modified with one dmPE. The reporter ion for dmPE at m/z 843.65 (M+H)\(^+\) (observed monoisotopic mass of 3370.56 [M+H]) confirming that peptide 52SAVTEYLHGKWPENNTSAGVASPPTDIK81 was modified with one PE. The reporter ion for PE at m/z 142.0 could be detected in the low mass area. D) MS2 HCD spectrum of the precursor peptide at m/z 847.15 (M+H)\(^+\) (observed monoisotopic mass of 3384.58 [M+H]) confirming that peptide 52SAVTEYLHGKWPENNTSAGVASPPTDIK81 was modified with one mmPE. The reporter ion for mmPE at m/z 156.0 could be detected in the low mass area. E) MS2 HCD spectrum of the precursor peptide at m/z 850.65 (M+4H\(^+\)) (observed monoisotopic mass of 3398.59 [M+H\(^+\)]) confirming that peptide 52SAVTEYLHGKWPENNTSAGVASPPTDIK81 was modified with one dmPE. The reporter ion for dmPE at m/z 170.1 could be detected in the low mass area. F) MS2 HCD spectrum of the precursor peptide at m/z 854.16 (M+4H\(^+\)) (observed monoisotopic mass of 3421.61 [M+H\(^+\)]) confirming that peptide 52SAVTEYLHGKWPENNTSAGVASPPTDIK81 was modified with one PE. The reporter ion for PE at m/z 184.1 could be detected in the low mass area. G) The structure of PE, mmPE, dmPE and PC together with their respective reporter ion m/z.

do1:10.1371/journal.pone.0096419.g008

Supporting Information

Figure S1 Identification of methylated PE on Ngo1043. (DOCX)

Table S1 Strains used in this study. (DOCX)

Author Contributions

Conceived and designed the experiments: AV JHA FEA FTH NR MK. Performed the experiments: AV JHA FEA FTH NR MA. Analyzed the data: AV JHA FEA FTH NR MK. Contributed reagents/materials/analysis tools: NR MK. Wrote the paper: AV JHA MK.

References

1. Lewis LA, Choudhury B, Bahlhazar JT, Martin LE, Ram S, et al. (2009) Phosphothanolamine substitution of lipid A and resistance of Neisseria gonorrhoeae to cationic antimicrobial peptides and complement-mediated killing by normal human serum. Infect Immun 77: 1112–1120.

2. Mackinnon FG, Cox AD, Plesôt JG, Tang CM, Makepeace K, et al. (2002) Identification of a gene (bptC) required for the addition of phosphothanolamine to the lipopolysaccharide inner core of Neisseria meningitidis and its role in mediating susceptibility to bactericidal killing and opsonophagocytosis. Mol Microbiol 43: 931–943.

3. Lee H, Hsu FF, Turk J, Groisman EA (2004) The PmrA-regulated pmrC gene mediates phosphoethanolamine modification of lipid A and polymyxin resistance in Salmonella enterica. J Bacteriol 186: 4124–4133.

4. Weiser JN, Shchepetov M, Chong ST (1997) Decoration of lipopolysaccharide with phosphorylcholine: a phase-variable characteristic of Haemophilus influenzae. Mol Microbiol 23: 9–14.

5. Weiser JN, Shchepetov M, Chong ST (1997) Decoration of lipopolysaccharide with phosphorylcholine: a phase-variable characteristic of Haemophilus influenzae. J Bacteriol 186: 4124–4133.

6. Swobod KE, Buscher BA, Ver Steeg B, Preston A, Nichol WA, et al. (2000) Non-typeable Haemophilus influenzae adhere to and invade human bronchial epithelial cells via an interaction of lipooligosaccharide with the PAF receptor. Mol Microbiol 37: 13–27.

7. Sermo L, Virji M (2002) Genetic and functional analysis of the phosphorylcholine epitope on the lipooligosaccharide of Neisseria meningitidis. Molecular microbiology 43: 437–448.

8. Hogge JT, Hitchens PG, Aas FE, Kristianen H, Lovoll G, et al. (2004) Unique modifications with phosphocholine and phosphorylcholine define alternate antigenic forms of Neisseria gonorrhoeae type IV pili. Proceedings of the National Academy of Sciences of the United States of America 101: 10798–10803.

9. Warren MJ, Jennings MP (2003) Identification and characterization of pta: a gene involved in the phase-variable expression of phosphorylcholine on pili of Neisseria meningitidis. Infect Immun 71: 6892–6898.

10. Weiser JN, Goldberg JB, Pan N, Wilson L, Virji M (1998) The phosphorylcholine epitope undergoes phase variation on a 43-kilodalton protein in Pseudomonas aeruginosa and on pili of Neisseria meningitidis and Neisseria gonorrhoeae. Infect Immun 66: 4263–4267.

11. McFadden DA, Slanetz CE, Wilson JC, Schulz BL, Jennings MP (2013) Substrate recognition of a structure motif for phosphorylcholine post-translational modification in Neisseria meningitidis. Biochim Biophys Res Commun 431: 808–814.

12. Ansonen JH, Egger-Jacobsen W, Aas FE, Borud B, Kooeney M, et al. (2012) Novel protein substrates of the phospho-form modification system in Neisseria gonorrhoeae and their connection to O-linked protein glycosylation. Infect Immun 80: 22–30.

the precursor to PptA-mediated modifications, the question of how and when PC is generated remains perplexing. We previously suggested one scenario in which PptA might directly utilize phosphatidylcholine as a head group donor and an alternative one in which PptA would transfer PE onto PptA, that would then be transformed in situ into PC by PE methyltransferase(s) [20]. The phosphatidylcholine donor model is problematic due to the inability to identify phosphatidylcholine in N. gonorrhoeae and the lack of identifiable phosphatidylcholine synthase genes within gonococcal genome sequences. Moreover, PE (not PC) modification was seen when PptA and PilE were co-expressed in P. aeruginosa, a species known to make phosphatidylcholine [20]. The second model involving methylation of PE already attached to pilin is also problematic. Specifically, the active sites of the orthologous phosphatidylethanolamine - utilizing phosphoethanolamine transferases PmrC and Cg0256 (modifying lipid A and lipid A as well as the FlgG protein respectively) are predicted to be periplasmically exposed [3,16]. Accordingly, PE - modified PilE must a priori be localized to the periplasm. However, the methyl donor S-adenosylmethionine required for subsequent modification is unlikely to be available at this site. Thus barring retrograde re-localization of PilE to the cytoplasm (for which there is no evidence), this model is problematic as well. Whatever the case, our MS-based results clearly establish for the first time the presence of mono- and di-methylated forms of PE linked to protein. These findings do not differentiate between the scenarios but they directly implicate a methyltransferase-mediated biosynthetic pathway and corroborate data showing that PC is generated by de novo synthesis [20].

In conclusion, the results presented here provide insight into features affecting protein phospho-form modification and suggest that PC modification may act as a molecular marker for altered membrane protein sublocalization. Understanding how, why and when bacteria use these modifications may reveal their physiological relevance to processes operating in vivo.
15. Reynolds CM, Kalb SR, Cotter RJ, Raetz CR (2005) A phosphoethanolamine
16. Cullen TW, Trent MS (2010) A link between the assembly of flagella and
17. Reynolds CM, Kalb SR, Cotter RJ, Raetz CR (2005) A phosphoethanolamine
18. Cullen TW, Madsen JA, Ivanov PL, Brodbelt JS, Trent MS (2012)
19. Cox AD, Wright JC, LiJ, Hood DW, Moxon ER, et al. (2003) Phosphorylation
20. Naessan CL, Egge-Jacobsen W, Heiniger RW, Wolfgang MC, Aas FE, et al. (2008) The
21. Geiger O, Lopez-Lara IM, Sohlenkamp C (2013) Phosphatidylcholine
22. Rahman MM, Kolli VS, Khler DH, Lovold C, Hitchen PG, et al. (2007) New functional identity for the DNA
23. Koomey M, Bergstrom S, Blake M, Swanson J (1991) Pilin expression and
24. Campbell HA, Kent C (2001) The CTP:phosphocholine cytidylyltransferase
25. Aas FE, Egge-Jacobsen W, Winther-Larsen HC, Lovold C, Hitchen PG, et al.
26. Freitag NE, Seifert HS, Koomey M (1995) The product of the pilQ gene is essential for the biogenesis of type IV pili in Neisseria gonorrhoeae. Mol Microbiol 16: 451–464.
27. Rahman M, Lauer P, Park HS, Brossay L, Hebert J, et al. (1998) PilT mutations lead to simultaneous defects in competence for natural transformation and twitching motility in pilated Neisseria gonorrhoeae. Molecular microbiology 29: 321–330.
28. Rahman M, Kallstrom H, Normark S, Jonsson AB (1997) PilC of pathogenic Neisseria is associated with the bacterial cell surface. Mol Microbiol 25: 11–25.
29. Aas FE, Egge-Jacobsen W, Winther-Larsen HC, Lovold C, Hitchen PG, et al. (2007) Substitutions in the N-terminal alpha helical spine of Neisseria gonorrhoeae pilin affect Type IV pilus assembly, dynamics and associated functions. Mol Microbiol 63: 69–85.
30. Vik A, Aas FE, Juvela J, Koomey M, Egge-Jacobsen W, et al. (2010) Control of pilus dynamics in Neisseria gonorrhoeae: Components and dynamics of fiber formation define a ubiquitous biogenesis pathway for bacterial pili. EMBO J 19: 6408–6418.
31. Cullen TW, Trent MS (2010) A link between the assembly of flagella and lipooligosaccharide of the Gram-negative bacterium Campylobacter jejuni. Proc Natl Acad Sci U S A 107: 5160–5165.
32. Reynolds CM, Kalb SR, Cotter RJ, Raetz CR (2005) A phosphoethanolamine transferase specific for the outer 3-deoxy-D-manno-octulosonic acid residue of Escherichia coli lipopolysaccharide. Identification of the epbG gene and Ca2+ hypersensitivity of an epbG deletion mutant. J Biol Chem 280: 21202–21211.
33. Salmond OH, Frye SA, Tonjum T (2007) New functional identity for the DNA
34. Ambur OH, Frye SA, Tonjum T (2007) Structure and antigenic analyses of glycan diversity in the O-linked modification of flagellar rod protein FlgG by a conserved set of pilin-like molecules controls type IV pilus dynamics and origin-gene-associated functions in Neisseria gonorrhoeae. Molecular microbiology 56: 903–917.
35. Close Oj, Rodriguez RL (1982) Construction and characterization of the chloramphenicol-resistance gene cartridge: a new approach to the transcriptomal mapping of extrachromosomal elements. Gene 20: 305–316.
36. Olen IV, de Godoy LM, Li G, Macek B, Mortensen P, et al. (2003) PmrA-regulated genes. FEMS Immunol Med Microbiol 43: 249–258.
37. Cullen TW, Madsen JA, Ivanov PL, Brodbelt JS, Trent MS (2012)
38. Aas FE, Vik A, Vedele J, Koomey M, Egge-Jacobsen W (2007) Neisseria gonorrhoeae O-linked pilin glycosylation: functional analyses define both the biosynthetic pathway and glycan structure. Mol Microbiol 63: 627–634.
39. Borud B, Aas FE, Vik A, Winther-Larsen HC, Egge-Jacobsen W, et al. (2010) Genetic, structural, and antigenic analyses of glycan diversity in the O-linked protein glycosylation systems of human Neisseria species. J Bacteriol 192: 2016–2029.
40. Aas FE, Vik A, Vedele J, Koomey M, Egge-Jacobsen W (2007) Neisseria gonorrhoeae PilV, a type IV pilus-associated protein essential to human epithelial cell adherence. Proc Natl Acad Sci U S A 98: 15276–15281.
41. Close Oj, Rodriguez RL (1982) Construction and characterization of the chloramphenicol-resistance gene cartridge: a new approach to the transcriptomal mapping of extrachromosomal elements. Gene 20: 305–316.
42. Close Oj, Rodriguez RL (1982) Construction and characterization of the chloramphenicol-resistance gene cartridge: a new approach to the transcriptomal mapping of extrachromosomal elements. Gene 20: 305–316.
43. Close Oj, Rodriguez RL (1982) Construction and characterization of the chloramphenicol-resistance gene cartridge: a new approach to the transcriptomal mapping of extrachromosomal elements. Gene 20: 305–316.
44. Close Oj, Rodriguez RL (1982) Construction and characterization of the chloramphenicol-resistance gene cartridge: a new approach to the transcriptomal mapping of extrachromosomal elements. Gene 20: 305–316.
45. Wolfgang M, Laer P, Park HS, Brossay L, Hebert J, et al. (1998) PilT mutations lead to simultaneous defects in competence for natural transformation and twitching motility in pilated Neisseria gonorrhoeae. Molecular microbiology 29: 321–330.
46. Rahman M, Kallstrom H, Normark S, Jonsson AB (1997) PilC of pathogenic Neisseria is associated with the bacterial cell surface. Mol Microbiol 25: 11–25.
47. Close Oj, Rodriguez RL (1982) Construction and characterization of the chloramphenicol-resistance gene cartridge: a new approach to the transcriptomal mapping of extrachromosomal elements. Gene 20: 305–316.
48. Close Oj, Rodriguez RL (1982) Construction and characterization of the chloramphenicol-resistance gene cartridge: a new approach to the transcriptomal mapping of extrachromosomal elements. Gene 20: 305–316.
49. Close Oj, Rodriguez RL (1982) Construction and characterization of the chloramphenicol-resistance gene cartridge: a new approach to the transcriptomal mapping of extrachromosomal elements. Gene 20: 305–316.
50. Horiuchi T, Komano T (1998) Mutational analysis of plasmid R64 thin pilus prepilin peptidase. J Bacteriol 180: 4613–4620.
51. Aas FE, Vik A, Vedele J, Koomey M, Egge-Jacobsen W, et al. (2010) Genetic, structural, and antigenic analyses of glycan diversity in the O-linked protein glycosylation systems of human Neisseria species. J Bacteriol 192: 2016–2029.
52. Vik A, Aas FE, Anonsen JH, Bägbrough S, Schneider A, et al. (2009) Broad spectrum O-linked protein glycosylation in the human pathogens Neisseria gonorrhoeae. Proc Natl Acad Sci U S A 106: 4447–4452.
53. Marceau M, Forest K, Beretti JL, Tainer J, Nassif X (1998) Consequences of the loss of O-linked glycosylation of meningococcal type IV pilin on piliation and associated epithelial cell adherence and multicellular behavior by the PilU effectors of PilE phosphoform modification.