Biochemical and Biophysical Research Communications 431 (2013) 808–814

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Substrate recognition of a structure motif for phosphorylcholine post-translational modification in *Neisseria meningitidis*

Freda E.-C. Jen [a], Christopher E. Jones [b], Jennifer C. Wilson [a], Benjamin L. Schulz [c], Michael P. Jennings [a,*]

[a] Institute for Glycomics, Griffith University, Gold Coast Campus, QLD 4222, Australia
[b] School of Science and Health, The University of Western Sydney, Locked Bag 1797, Penrith, NSW 2751, Australia
[c] School of Chemistry and Molecular Biosciences, The University of Queensland, Brisbane, QLD 4072, Australia

ARTICLE INFO

Article history:
Received 17 December 2012
Available online 27 December 2012

Keywords:
Bacteria
Phosphorylcholine
Neisseria
Pili

ABSTRACT

Neisseria meningitidis is a human pathogen that can cause life threatening meningitis and sepsis. Pili of *Neisseria* are one of the major virulence factors in host–pathogen interaction. Pilin of *N. meningitidis* is post-translationally modified by a glycan and two phosphorylcholines (ChoP). ChoP modifications have been found to have an important role in bacterial colonisation and invasion. Unlike *N. gonorrhoeae*, ChoP modifications on pili seem to be restricted to the C-terminus of pilin protein in *N. meningitidis*. In this study, we investigate the substrate recognition of phosphorylcholine transferase. We found that a single sequence of D–A–S after the disulphide bond of pilin protein is able to form a motif for ChoP modifications and the charge residue in this motif and the local structure are essential for the substrate recognition.

1. Introduction

Bacterial post-translational modifications (PTM), such as glycosylation, phosphorylation, methylation etc., have a variety of important functions in biology. The PTM of a protein can be varied from strain to strain due to the transferase and the target sequence. Studying the protein PTM target sequence for the transferase recognition can be important as it can be applied to protein identification and manipulation in both *in vivo* and *in vitro* contexts [1]. One of the well-characterised PTM systems is glycosylation. In *Neisseria*, O-linked glycosylation happens when PglL adds sugar on the Ser of N-T-S(glycan)-A-G motif of pilin protein and the local sequence motif in other membrane proteins which mimics PilE glycosylation site is also found to be glycosylated in the same system (Schulz et al., submitted) [2].

Phosphorylcholine (ChoP) is a PTM commonly found on the surface of the host cells such as platelet activating factor (PAF) and sphingomyelin. Yet several bacterial isolates (such as oral *Actinomycetes*, *Fusobacterium nucleatum*, and *lactococcus* spp.) from human mouth and respiratory tract were found to be ChoP positive in an immunoassay [3]. The ChoP epitope has been discovered on some important bacterial surface exposed virulence factors. In *Haemophilus influenzae* [4] and commensal *Neisseria* [5], ChoP attaches to glycans such as lipopolysaccharide (LPS) and in *Streptococcus pneumoniae*, ChoP attaches to cell wall-associated teichoic and lipoteichoic acid [6]. In addition, ChoP can also attach to a protein such as pilin in *Neisseria meningitidis* and *N. gonorrhoeae* [7] and elongation factor Tu in *Pseudomonas aeruginosa* [8]. Previously, we have identified the sites of ChoP modification on pilin in *N. meningitidis* and showed its importance in binding to human PAF receptor (Jen et al., submitted). The aim of this study is to understand the ChoP PTM system in *N. meningitidis*. Here, we present the identification and characterisation of the peptide sequence and structure motif specificity for ChoP modification on pilin.

2. Materials and methods

2.1. Bacterial strains and media

Meningococcal strains used in this study were *N. meningitidis* C311#3 [9], a ChoP deficient mutant C311#3*pptA::kan* [9], and the pilin deficient mutant C311#3*pilE::kan* [10]. Meningococcal strains were grown on Brain Heart Infusion agar (BHI; Oxoid) at 37 °C with 5% CO_2 for 16–18 h. BHI plates were made with 1% agar and supplemented with 10% Levinthals Base [11]. All recombinant plasmids were replicated in *Escherichia coli* DH5α and grown on Luria–Bertani (LB) plates supplemented with tetracycline (5 μg/ml).

2.2. DNA manipulation and recombination

Most DNA manipulation and recombination methods were previously described in [12]. The site-directed mutagenesis was done

Abbreviations: ChoP, phosphorylcholine; PAF, platelet activating factor; CRP, C reactive protein.

* Corresponding author. Fax: +61 7 5552 8098.
E-mail address: m.jennings@griffith.edu.au (M.P. Jennings).

0006-291X/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2012.12.088

by inverse PCR of plasmid pGemTetMB*pilElpxC* using KOD Polymerase (Merck) as described in [13]. Primers used to amplify PCR products are shown in Supplementary Table S1. Inverse PCR products were treated with T4 Polynucleotide Kinase (NEB) and then self-ligated using T4 DNA liagse (NEB). Ligated plasmids were electroporated into DH5α and transformants were selected by 5 μg/ml tetracycline.

2.3. Western immunoblot analysis

Pilin from *N. meningitidis* strains were isolated as previously described [14]. Outer Membrane Complex (OMC) was isolated as described in [15]. Isolated samples were analysed by western immunoblotting using the anti-pilin polyclonal anti-serum [14], the mAb TEPC-15 (specific for ChoP; [7]) and mAb anti-AniA [16]. Samples were analysed in 4–12% Bis-Tris gel and MOPS running buffer. Molecular weight marker was BenchMark™ Pre-Stained Protein Ladder from Invitrogen.

2.4. NMR spectroscopy

NMR spectra were acquired on Bruker spectrometers at 750 MHz (5 mm PATXI triple resonance, z-gradient probe), 600 MHz (TCl CryoProbe) and 500 MHz (5 mm selective excitation, inverse detection, z-gradient probe). One-dimensional spectra were routinely collected over a spectral width of 10 ppm, with 64 K complex data points. For structure determination ^{1}H-^{1}H COSY (2048[f2] × 512[f1]), TOCSY (MLEV τ_m = 80 ms, 4096[f2] × 700[f1]) and ROESY (τ_m = 300 ms, 400 ms, 4096[f2] × 700[f1]) spectra were collected over 10 ppm spectral widths in both dimensions. Two-dimensional spectra were acquired at 278 K and 290 K. States-TPPI was used for quadrature detection. Spectra were zero-filled once in f1, and a π/2 shifted, squared sine-bell window function applied to both dimensions prior to Fourier transformation. All spectra were acquired in 90% H_2O (20 mM phosphate buffer, pH 6.8)/10% D_2O. Water suppression was achieved using a WATERGATE sequence [17]. One-dimensional variable-temperature experiments were conducted over the range 278–298 K in aqueous solutions using the 500 MHz spectrometer. Spectra were referenced to the high-field 4,4-dimethyl-4-silapentane sodium sulphate peak at 0 ppm. The details of NMR structure calculations are described in the Supplementary material.

3. Results and discussion

3.1. ChoP in N. meningitidis is synthesised in a novel pathway

In bacteria, the two common biosynthetic pathways of ChoP are the *lic1* pathway [18–20] and *pmtA* pathway [21]. However, there has been no observation of *lic* or *pmt* genes in pathogenic *Neisseria* [5] suggesting that there may be other genes involved in choline uptake or other pathways involved in ChoP biosynthesis. To determine whether the source of phosphorylcholine for post-translational modification and attachment to pilin was exogenous or endogenous, *N. meningitidis* C311#3 was grown in choline-free chemically defined media [22]. Our results showed that choline was not essential for the growth of *N. meningitidis* C311#3 and did not affect the post-translational modification of pilin by ChoP (see Supplementary Fig. S1). Therefore the ChoP moiety could be endogenously synthesised in *N. meningitidis*. A similar observation has been reported in *N. gonorrhoeae* [23].

3.2. Location of ChoP modification and modified protein

PptA, pilin phosphorylcholine transferase A, is the only known protein involved in phosphorylcholine modification of pilin in *N. menigitidis* currently [9]. Based on the Signal IP and TMHMM analysis of PptA, PptA is predicted to be an integral membrane protein containing four helices in the inner membrane. About 70% of PptA C-terminus is located in the periplasmic space and this part of protein shows homology to sulfatase (pfam00884). This predicted PptA structure has also shows homology to other known proteins involved in bacterial phosphoethanolamine post-translational modification, such as Lpt3 and Lpt6 of *Neisseria* and some *E. coli* phosphoethanolamine transferases (data not shown). Based on this predicted result, we hypothesis that ChoP post-translational modification pathway occurs in the periplasm.

Pilin and some other glycoproteins which in *Neisseria* are under a general pathway in the periplasm [15,12] (see Fig. 4(A)). To determine whether ChoP modification can also be found in other protein in *Neisseria*, an outer membrane complex (OMC) enriched sample and cell lysate of *N. meningitidis* C311#3 were analysed by western immunobloting with the TEPC-15 monoclonal antibody. However, none of the other proteins in *N. meningitidis* were found to be ChoP positive (data not shown). As the result shows, pilin in *N. meningitidis* seems to be the only detectable substrate of ChoP transferase. We then further investigated the importance of sequence specificity of pilin for ChoP post-translational modification.

3.3. A single sequence of DAS in C-terminal of pilin is sufficient for ChoP modification

We have previously shown that ChoP modifications of *N. meningitidis* C311#3 pilin are on Ser157 and Ser160 of the C-terminal peptide ^{153}CRDASDAS^{160} (Jen et al., submitted). To identify the substrate sequences important for recognition by the ChoP transferase, the site-direct mutagenesis of pilin was performed to generate the following mutants, C311#3S157A (^{153}CRDAADAS^{160}), C311#3S160A (^{153}CRDASDAA^{160}), C311#3ExDAS (^{153}CRDAADAADAS^{163}), C311#3DAS(^{153}CRDAS^{157}). In these set of mutants, we set out to determine if ChoP modification on pilin requires the repeat sequence of the DAS peptide. As shown in Fig. 1(A,B), the western analysis of the pilin from these mutants shows that pilin from all these mutants are ChoP positive indicating a single DAS peptide in the C-terminus of pilin is sufficient to act as a substrate of ChoP modification by PptA.

3.4. A charged residue is essential for ChoP modification

The peptide composition of the ChoP substrate, DAS, contains a negatively charged residue, aspartic acid. To identify the significance of the negative charge residue in the ChoP substrate recognition, the residue Asp was mutated to Ala, Glu and Lys. C311#3 mutants of C311#3AAS, C311#3EAS and C311#3KAS were made. Reversal of the Asp charge to a neutral residue of Ala changes the phenotype of pilin to be ChoP negative as shown in the western immunoblots analysis in Fig. 1(C) and the substitution of Asp to Glu and Lys maintains the ChoP modification on pilin.

3.5. Cys is required for pilin formation

There is no available pilin structure for *N. meningitidis*. Based on the structural studies, the closely related type IV pilin of *N. gonorrhoeae*, the pilin structure [24] contains a disulphide-bond in the C-terminal region as show in purple in Fig. 2, which is close to ChoP modification sites. In the crystal structure, pilin of *N. gonorrhoeae* only shows one disulphide-bond linking the end of the C-terminus region to the last β sheet of the whole pilin protein. It has been reported that a single mutation in the C-terminal disulphide-bonded Cys residue of type IV pilin in *P. aeruginosa* influences pilus assembly [25]. To investigate if

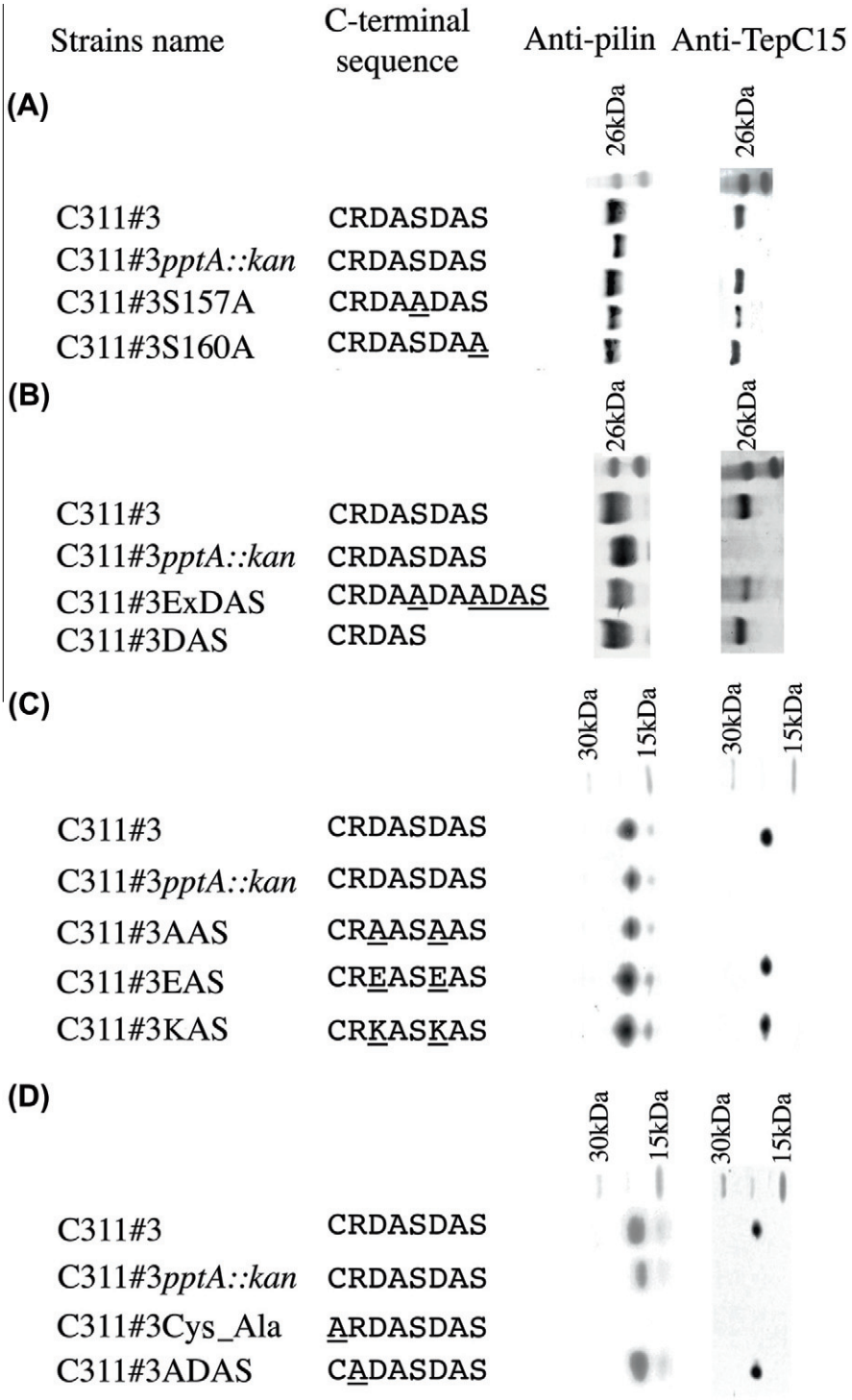

Fig. 1. Analysis of pilin from *N. meningitidis* C311#3 and its site-directed mutagensis mutants Pilin was isolated from C311#3, C311#3pptA::kan and site-directed mutagenesis mutants as previously described [10]. Western immunoblot with the polyclonal antiserum anti-pilin shows presence of pilin in samples and mAb TEPC-15 shows presence of ChoP in the samples.

the disulphide bond of C311#3 pilin is important for pilin formation and the ChoP modification in *N. meningitidis*, Cys120 and Cys153 were mutated to Ala120 and Ala153. Cell lysate of C311#3Cys_Ala mutant was analysed by western immunoblots. The western immunoblots with the polyclonal anti-pilin and monoclonal TEPC-15 antibody in Fig. 1(D) showed that there was no pilin production and therefore ChoP modification could not be assessed.

3.6. Pilin C-terminal sequence variation in N. meningitidis

To determine whether the C-terminal peptide of pilin is surface exposed for substrate recognition, molecular modelling was performed. As shown in Fig. 2(A), Cys120 and Cys153 forms a disulphide bond which constrains the end 7 amino acids of pilin peptide close to the main structure. *In vivo*, pilin exists as polymer, pili. As shown in Fig. 2(B), in the formation of pili, the pilin

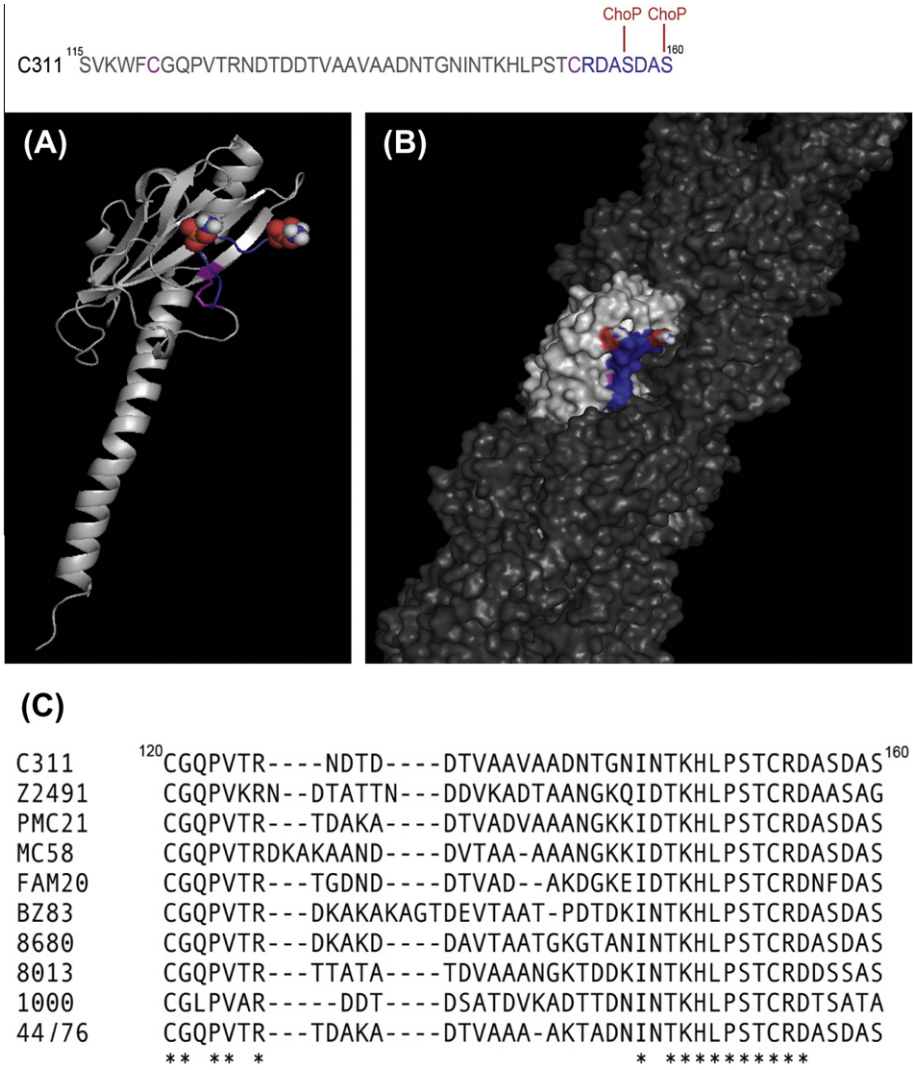

Fig. 2. Comparison of *N. meningitidis* pilin C-terminal sequence. (A) Single subunit of pilin protein. Two cysteines residues forming disuphide bond is indicated in purple. The C-terminal peptide after Cysteine is coloured in blue. B) Modelling of C-terminal peptide of pilin is surface expose in pilus fibre. (C) The C-terminal sequence alignment of various *N. meningitidis* strains. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

C-terminal peptide is on the surface suggesting the accessibility of the substrate. The C-terminus of C311#3 pilin sequence was then aligned with other *N. meningitidis* strains in Fig. 2(C). The alignment result shows the two cysteines forming the disulphide bond are conserved in all the *N. meningitidis* strains and the Arg154 and Asp155 of C311#3 pilin are also present in other *N. meningitidis* strains suggesting the importance of these residues. The Asp residue has been shown to be important in substrate recognition for ChoP modification. Arg154 was also mutated to generate strain C311#3ADAS. The western immunoblot analysis in Fig. 1 (D) shows that changing Arg154 to Ala did not affect on ChoP post-tranlsational modification.

3.7. Structural analysis of the ChoP modified peptide

The C-terminal pilin ChoP modification site is constrained by the disulphide bond involving Cys 153 which appears important for pilin formation, however, we wanted to determine if the last seven amino acids were conformationally constrained. To explore this we synthesised a heptapeptide corresponding to the ChoP modification site (RDASDAS) of pilin and examined it in aqueous solutions using NMR.

The 400 ms ROESY spectrum (Supplementary Fig. S2) shows that the RDASDAS amide peaks are not only well dispersed, but there is all the expected sequential ROE peaks as well as several longer-range ROEs. Several lines of evidence suggest this peptide adopts a β-turn conformation in aqueous solution. Firstly, characteristic ROEs are observed between NH Ala 3 and NH Ser 4, Hα Ala3 and NH Asp 5 and NH Ser 4 and NH Asp 5 (Fig. 3(A), Supplementary Fig. S2); secondly, Ala 3 has a small $^{3}J_{HN-H\alpha}$ of ~4.5 Hz and all others are >6.5 Hz and thirdly, the temperature coefficient of the Asp 5 amide proton (−2.6 ppb/K) is markedly reduced compared to all other amide protons (Table 1). The lack of temperature dependent movement of the Asp 5 amide proton compared to the other amides is highlighted in Fig. 3(B), and it suggests that the amide-proton is either solvent-shielded, which is unlikely in a small peptide such as this, or the proton is involved in hydrogen-bonding. We used ROE-derived restraints to solve the structure of RDASDAS in the absence of an explicit hydrogen bond, and used these initial structures to determine that the carbonyl oxygen of Asp 2 is the likely partner in the hydrogen-bond involving Asp 5. From our data and initial structures there appears to be no involvement of the side-chain of Asp 2 in the hydrogen-bond as would occur in a ST-turn [26]. The 20 lowest energy structures representing

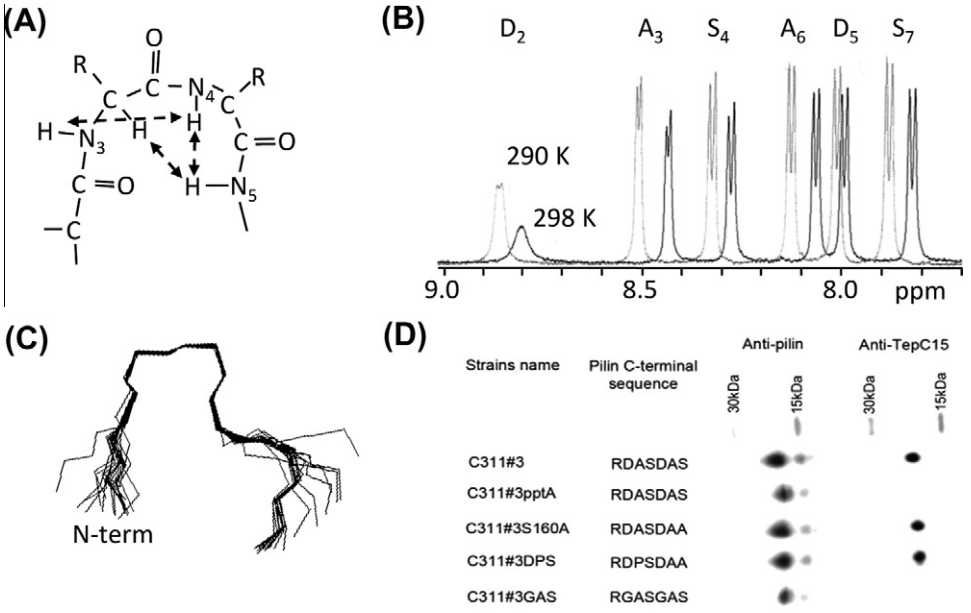

Fig. 3. Structural analysis of the pilin ChoP modification peptide – RDASDAS. (A) Schematic representation of the structure of RDASDAS showing ROE peaks characteristic of a turn (arrows) observed in the 400 ms ROESY spectrum. (B) One-dimensional spectra of RDASDAS at 298 K (black trace) and 290 K (grey trace). Note small $^{3}J_{HN-H\alpha}$ for A3 and the lack of a large temperature dependent change in the chemical shift of D5 HN. (C) Overlay of the 20 lowest energy backbone conformers of RDASDAS. (D) Western analysis of pilin formation and ChoP modification of mutants of the ChoP modification peptide. Only mutant RGASGAS fails to undergo ChoP modification.

Table 1
Table showing HN–Hα coupling constants ($^{3}J_{HN-H\alpha}$) and temperature coefficients (over temperature range 278–298 K) for the wild-type pilin ChoP modification peptide (RDASDAS) and for the mutant peptides – RDASDAA, RGASGAS and RDPSDAA. Bold highlights the small coupling constant for A3 in RDASDAS and the small temperature coefficient of D5 in RDASDAS, RDASDAA and RDPSDAA which is not apparent in RGASGAS. All the bold values have $P < 0.05$.

Wild-type			Mutants					
RDASDAS			RDASDAA		RGASGAS		RDPSDAA	
	$^{3}J_{NHH\alpha}$ (Hz)	Δppm/K		Δppm/K		Δppm/K		Δppm/K
D	6.63 ± 0.27	-7.3×10^{-3}	**D**	-6.6×10^{-3}	**G**	-7.5×10^{-3}	**D**	-6.2×10^{-3}
A	**4.58 ± 0.17**	-10.2×10^{-3}	**A**	-9.6×10^{-3}	**A**	-8.8×10^{-3}	**P**	
S	6.95 ± 0.10	-6.5×10^{-3}	**S**	-6.5×10^{-3}	**S**	-8.8×10^{-3}	**S**	-6.1×10^{-3}
D	6.90 ± 0.10	$\mathbf{-2.6 \times 10^{-3}}$	**D**	$\mathbf{-2.9 \times 10^{-3}}$	**G**	-6.4×10^{-3}	**D**	$\mathbf{-3.8 \times 10^{-3}}$
A	6.72 ± 0.10	-8.8×10^{-3}	**A**	-7.5×10^{-3}	**A**	-6.0×10^{-3}	**A**	-8.4×10^{-3}
S	7.47 ± 0.17	-8.1×10^{-3}	**A**	-9.1×10^{-3}	**S**	-8.5×10^{-3}	**A**	-9.8×10^{-3}

the solution structure of RDASDAS are shown overlaid in Fig. 3 (C) (heavy atom RMSD = 0.58 Å over residues 2–5) and the energy and structural statistics are provided in the (Supplementary Table S1). RDASDAS forms a type-I β-turn in aqueous solutions, and although it is quite surprising that a short peptide forms such a well defined structure in water it is not unprecedented [27].

To determine if mutations can alter the conformation of this peptide and potentially the ChoP modification ability we prepared several mutants peptides: RDASDAA, RGASGAS and RDPSDAA and the corresponding mutants in *N. meningitidis* C311#3 pilin. Fig. 3(D) shows that of the three mutants only RGASGAS was unable to undergo ChoP modification. We next analysed the temperature dependence of the amide protons for each of the three mutant peptides. Table 1 shows that both RDASDAA and RDPSDAA maintained the small temperature coefficient for the amide of Asp 5 suggesting that the hydrogen-bond is still prevalent and the turn structure is maintained. In contrast, for RGASGAS the temperature-coefficient is similar for all amide protons. Temperature coefficients of between −6 and −10 ppb/K are thought to occur for extended chain conformations, suggesting that RGASGAS does not adopt the same turn structure as RDASDAS. It is likely that the lack of a bulky side-chain allows RGASGAS to access a wider conformational landscape compared to the more sterically hindered RDASDAS and this prevents the formation of a stabilizing hydrogen-bond. Given that we have shown that abolishing the charged residue (Asp) also correlates with a loss of ChoP modification it is likely that the modification efficiency is an interplay between both charge and conformational factors. The lack of an amino-acid that is sterically unhindered yet is charged means it is difficult to precisely determine the importance of the turn-conformation of the pilin ChoP modification peptide. Nevertheless, given the importance of structural recognition of protein acceptors in *Neisseria* O-glycosylation it is tempting to speculate that the turn observed for the ChoP modification site in pilin is functionally strategic.

3.8. ChoP modification peptide on AniA::pilin C-terminus fusion protein

As mentioned previously, glycosylation biosynthesis (as shown in Fig. 4(A)) has been well described [28,14,29] in distinct compartments. The glycan biosynthesis occurs in a PglB-dependent fashion with transfer of the basal residue to an undecaprenol carrier. Further sugar groups are then added by phase variable glycosyltransferases PglA and PglE until the trisaccharide is complete. PglF 'flips' the undecaprenol-linked trisaccharide to the periplasm, where PglL transfers the glycan to pilin [30]. As described previously, 70% of

PptA, ChoP transferase, is predicted to be located in the periplasm compartment which is similar to PglL as shown in Fig. 4(A). AniA, a nitrite reductase, was found to be post-translationally modified by the same glycan as pilin in *N. meningitidis* [15]. To determine whether ChoP can be added to AniA if the C-terminal sequence of pilin (CRDASDAS) is present, the peptide CRDASDAS was added to the C-terminus of AniA. The AniA mutant of C311#3, C311#3AniADASDAS, was made. AniA is glycosylated at the C-terminus of the protein [15]. To eliminate the factor that glycan modification on the C-terminus of AniA may affect the recognition site of ChoP modification, the glycotransferase gene, *pglL*, was knocked out resulting in strain C311#3AniADASDAS/*pglL::kan*. In *N. meningitidis*, AniA glycosylation becomes more efficient in the absence of pilin [15]. To increase the possibility of ChoP modification in the C-terminal of AniA, the pilin gene, *pilE*, was knocked out to give strain C311AniADASDAS/*pilE::kan*. The western analysis of the OMC enrich prep from C311#3AniADAS, C311#3AniADASDAS/*pglL::kan* and C311#3AniADASDAS/*pilE::kan* showed that none of the AniA::DASDAS expressed by these strains was modified with ChoP in the presence of pilin C-terminal peptide as shown in Fig. 4(B).

Expression of ChoP is subject to phase variable expression (random ON/OFF switching) in *H. influenzae*, *S. pneumoniae*, commensal *Neisseria* [4], *N. meningitidis* [9] and *P. aeruginosa* [7]. Studies found that ChoP can increase the adherence of pneumococci and nontypable *H. influenzae* (NTHi) [31,32] and aids in the colonisation of the nasopharynx by *H. influenzae*. Further studies showed that ChoP can bind to the PAF receptor in NTHi [32] and the commensal *N. lactamica* [5] and its binding to PAF increases the adhesion and invasion to the respiratory epithelium. In our recent study, it has also been demonstrated that *N. meningitidis* pili can adhere PAF receptor of human respiratory epithelium (Jen et al., submitted). On the contrary, in *S. pneumoniae* [33], *H. influenzae* [34] and *N. meningitidis* [35], ChoP binds to C-reactive protein (CRP) in the nasopharynx and CRP binding activities complement by the

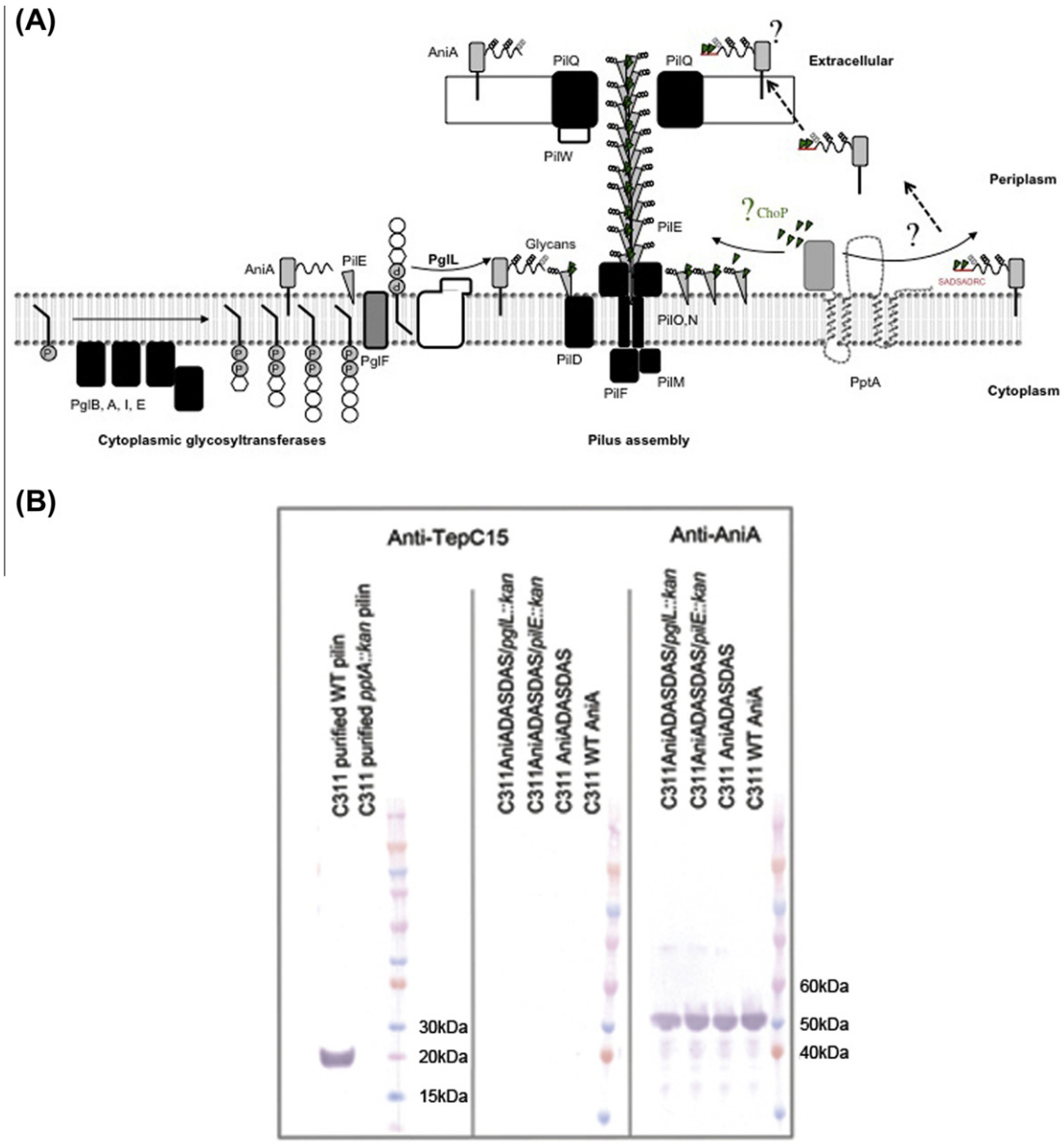

Fig. 4. Pilin ChoP modification peptide on AniA. (A) Pilin glycosylation based on wzy-dependent O-antigen biosynthesis and predicted pilin-linked ChoP post-translational modification biosynthesis pathway. On the left hand side of pilus assembly shows the biosynthesis of pilin glycosylation [30]. On the right hand side of pilus assemly shows the proposed ChoP modification pathway. (B) Western immunoblot of samples from C311#3 WT and various mutants detected with anti-TEPC15 or anti-AniA sera.

classical pathway and acts as an opsonin, resulting in bacterial killing. PptA is currently the only known protein involved in ChoP modification in *Neisseria* and it is responsible for the phase variable expression of ChoP in *Neisseria*. The predicted localisation of PptA suggests that pilin is modified with ChoP in the periplasm. We established the sequence requirement for ChoP modification on pilin and we also applied the sequence to another protein (AniA) which has the same glycosylation system as pilin in *N. meningitidis*. However, it seems that not only the sequence but also the substrate protein location are required for ChoP modification. This may also indicate that the locations of post-translational modification of glycan and ChoP happen in different compartment in the cell. Pilin of *N. meningitidis* can still be modified with ChoP when there is no choline in the growing media, which suggests that there is an unknown de novo biosynthetic pathway of ChoP.

Acknowledgments

This work was supported by NHMRC program grant 565526 and by a Helen C. Levitt Endowed Annual Visiting Professorship to M.P.J., NHMRC CDF APP1031542 to B.L.S.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.12.088.

References

[1] T.L. Foley, M.D. Burkart, Site-specific protein modification: advances and applications, Curr. Opin. Chem. Biol. 11 (2007) 12–19.
[2] A. Vik, F.E. Aas, J.H. Anonsen, S. Bilsborough, A. Schneider, W. Egge-Jacobsen, M. Koomey, Broad spectrum O-linked protein glycosylation in the human pathogen *Neisseria gonorrhoeae*, Proc. Natl. Acad. Sci. USA 106 (2009) 4447–4452.
[3] S.H. Gillespie, S. Ainscough, A. Dickens, J. Lewin, Phosphorylcholine-containing antigens in bacteria from the mouth and respiratory tract, J. Med. Microbiol. 44 (1996) 35–40.
[4] J.N. Weiser, M. Shchepetov, S.T. Chong, Decoration of lipopolysaccharide with phosphorylcholine: a phase-variable characteristic of *Haemophilus influenzae*, Infect. Immun. 65 (1997) 943–950.
[5] L. Serino, M. Virji, Genetic and functional analysis of the phosphorylcholine moiety of commensal *Neisseria* lipopolysaccharide, Mol. Microbiol. 43 (2002) 437–448.
[6] W. Fischer, T. Behr, R. Hartmann, J. Peter-Katalinic, H. Egge, Teichoic acid and lipoteichoic acid of *Streptococcus pneumoniae* possess identical chain structures. A reinvestigation of teichoid acid (C polysaccharide), Eur. J. Biochem. 215 (1993) 851–857.
[7] J.N. Weiser, J.B. Goldberg, N. Pan, L. Wilson, M. Virji, 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 (1998) 4263–4267.
[8] M. Barbier, A. Oliver, J. Rao, S.L. Hanna, J.B. Goldberg, S. Alberti, Novel phosphorylcholine-containing protein of *Pseudomonas aeruginosa* chronic infection isolates interacts with airway epithelial cells, J. Infect. Dis. 197 (2008) 465–473.
[9] M.J. Warren, M.P. Jennings, Identification and characterisation of pptA: a gene involved in the phase-variable expression of phosphorylcholine on pili of *Neisseria meningitidis*, Infect. Immun. 71 (2003) 6892–6898.
[10] M. Virji, J.R. Saunders, G. Sims, K. Makepeace, D. Maskell, D.J. Ferguson, Pilus-facilitated adherence of *Neisseria meningitidis* to human epithelial and endothelial cells: modulation of adherence phenotype occurs concurrently with changes in primary amino acid sequence and the glycosylation status ofpilin, Mol.Microbiol. 10 (1993) 1013–1028.
[11] H.E. Alexander, The *Haemophilus* group, in: Bacterial and Mycotic Infections of Man, Pitman Medical Publishing Co, London, 1965.
[12] J. Sambrook, D.W. Russell, Molecular Cloning: A Laboratory Manual, 3rd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 2001.
[13] O. Erster, M. Liscovitch, A modified inverse PCR procedure for insertion, deletion, or replacement of a DNA fragment in a target sequence and its application in the ligand interaction scan method for generation of ligand-regulated proteins, Methods Mol. Biol. 634 (2010) 157–174.
[14] P.M. Power, L.F. Roddam, M. Dieckelmann, Y.N. Srikhanta, Y.C. Tan, A.W. Berrington, M.P. Jennings, Genetic characterisation of pilin glycosylation in *Neisseria meningitidis*, Microbiology 146 (Pt 4) (2000) 967–979.
[15] S.C. Ku, B.L. Schulz, P.M. Power, M.P. Jennings, The pilin O-glycosylation pathway of pathogenic *Neisseria* is a general system that glycosylates AniA, an outer membrane nitrite reductase, Biochem. Biophys. Res. Commun. 378 (2009) 84–89.
[16] J.A. Cardinale, V.L. Clark, Expression of AniA, the major anaerobically induced outer membrane protein of *Neisseria gonorrhoeae*, provides protection against killing by normal human sera, Infect. Immun. 68 (2000) 4368–4369.
[17] M. Liu, Improved WATERGATE pulse sequences for solvent suppression in NMR spectroscopy, J. Magn. Reson. 132 (1998) 125–129.
[18] J.L. Mosser, A. Tomasz, Choline-containing teichoic acid as a structural component of pneumococcal cell wall and its role in sensitivity to lysis by an autolytic enzyme, J. Biol. chem. 245 (1970) 287–298.
[19] X. Fan, C.D. Pericone, E. Lysenko, H. Goldfine, J.N. Weiser, Multiple mechanisms for choline transport and utilization in *Haemophilus influenzae*, Mol. Microbiol. 50 (2003) 537–548.
[20] L. Serino, M. Virji, Phosphorylcholine decoration of lipopolysaccharide differentiates commensal *Neisseriae* from pathogenic strains: identification of licA-type genes in commensal *Neisseriae*, Mol. Microbiol. 35 (2000) 1550–1559.
[21] F. Martinez-Morales, M. Schobert, I.M. Lopez-Lara, O. Geiger, Pathways for phosphatidylcholine biosynthesis in bacteria, Microbiology 149 (2003) 3461–3471.
[22] S.A. Morse, L. Bartenstein, Purine metabolism in *Neisseria gonorrhoeae*: the requirement for hypoxanthine, Can. J. Microbiol. 26 (1980) 13–20.
[23] C.L. Naessan, W. Egge-Jacobsen, R.W. Heiniger, M.C. Wolfgang, F.E. Aas, A. Rohr, H.C. Winther-Larsen, M. Koomey, Genetic and functional analyses of PptA, a phospho-form transferase targeting type IV pili in *Neisseria gonorrhoeae*, J. Bacteriol. 190 (2008) 387–400.
[24] L. Craig, N. Volkmann, A.S. Arvai, M.E. Pique, M. Yeager, E.H. Egelman, J.A. Tainer, Type IV pilus structure by cryo-electron microscopy and crystallography: implications for pilus assembly and functions, Mol. Cell 23 (2006) 651–662.
[25] H. Harvey, M. Habash, F. Aidoo, L.L. Burrows, Single-residue changes in the C-terminal disulphide-bonded loop of the *Pseudomonas aeruginosa* type IV pilin influence pilus assembly and twitching motility, J. Bacteriol. 191 (2009) 6513–6524.
[26] W.J. Duddy, J.W. Nissink, F.H. Allen, E.J. Milner-White, Mimicry by asx- and ST-turns of the four main types of beta-turn in proteins, Protein Sci. 13 (2004) 3051–3055.
[27] B. Song, P. Kibler, A. Malde, K. Kodukula, A.K. Galande, Design of short linear peptides that show hydrogen bonding constraints in water, J. Am. Chem. Soc. 132 (2010) 4508–4509.
[28] M.P. Jennings, M. Virji, D. Evans, V. Foster, Y.N. Srikhanta, L. Steeghs, P. van der Ley, E.R. Moxon, Identification of a novel gene involved in pilin glycosylation in *Neisseria meningitidis*, Mol. Microbiol. 29 (1998) 975–984.
[29] P.M. Power, M.P. Jennings, The genetics of glycosylation in Gram-negative bacteria, FEMS Microbiol. Lett. 218 (2003) 211–222.
[30] P.M. Power, K.L. Seib, M.P. Jennings, Pilin glycosylation in *Neisseria meningitidis* occurs by a similar pathway to wzy-dependent O-antigen biosynthesis in Escherichia coli, Biochem. Biophys. Res. Commun. 347 (2006) 904–908.
[31] D.R. Cundell, N.P. Gerard, C. Gerard, I. Idanpaan-Heikkila, E.I. Tuomanen, *Streptococcus pneumoniae* anchor to activated human cells by the receptor for platelet-activating factor, Nature 377 (1995) 435–438.
[32] W.E. Swords, B.A. Buscher, K. Ver Steegli, A. Preston, W.A. Nichols, J.N. Weiser, B.W. Gibson, M.A. Apicella, Non-typeable *Haemophilus influenzae* adhere to and invade human bronchial epithelial cells via an interaction of lipooligosaccharide with the PAF receptor, Mol.Microbiol. 37 (2000) 13–27.
[33] A.J. Szalai, D.E. Briles, J.E. Volanakis, Role of complement in C-reactive-protein-mediated protection of mice from *Streptococcus pneumoniae*, Infect. Immun. 64 (1996) 4850–4853.
[34] J.N. Weiser, N. Pan, K.L. McGowan, D. Musher, A. Martin, J. Richards, Phosphorylcholine on the lipopolysaccharide of *Haemophilus influenzae* contributes to persistence in the respiratory tract and sensitivity to serum killing mediated by C-reactive protein, J. Exp. Med. 187 (1998) 631–640.
[35] R. Casey, J. Newcombe, J. McFadden, K.B. Bodman-Smith, The acute-phase reactant C-reactive protein binds to phosphorylcholine-expressing *Neisseria meningitidis* and increases uptake by human phagocytes, Infect. Immun. 76 (2008) 1298–1304.