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The pilin *O*-glycosylation pathway of pathogenic *Neisseria* is a general system that glycosylates AniA, an outer membrane nitrite reductase

S.C. Ku [1], B.L. Schulz [1], P.M. Power [2], M.P. Jennings *

School of Molecular and Microbial Sciences, The University of Queensland, St. Lucia, Brisbane, Qld 4072, Australia

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ABSTRACT

O-Glycosylation is emerging as a common posttranslational modification of surface exposed proteins in bacterial mucosal pathogens. In pathogenic *Neisseria* an *O*-glycosylation pathway modifies a single abundant protein, pilin, the subunit protein that forms pili. Here, we identify an additional outer membrane glycoprotein in pathogenic *Neisseria*, the nitrite reductase AniA, that is glycosylated in its C-terminal repeat region by the pilin glycosylation pathway. To our knowledge, this is the first report of a general *O*-glycosylation pathway in a prokaryote. We also show that AniA displays polymorphisms in residues that map to the surface of the protein. A frame-shift mutation abolishes AniA expression in 34% of *Neisseria meningitidis* strains surveyed, however, all *Neisseria gonorrhoeae* strains examined are predicted to express AniA, implying a crucial role for AniA in gonococcal biology.

There are now many reports of glycosylation of proteins in bacteria, particularly in bacterial pathogens. In most of these reports a specific glycoprotein has been identified using structural, immunological or genetic approaches, and in some cases key genes involved in the glycosylation process have been identified (reviewed in [1,2]). Recently there have been key advances in understanding the physiology and molecular mechanisms of glycosylation in the two best characterised bacterial systems; *N*-glycosylation in *Campylobacterjejuni* [3–5] and *O*-glycosylation in pathogenic *Neisseria*. Pilin of pathogenic *Neisseria* was one of the first examples of an *O*-glycosylated glycoprotein in a bacterial pathogen [6]. A series of studies have identified the genes encoding glycosyltransferases required for the biosynthesis of the pilin glycan [7–9]; and of alternate structures than can be synthesised in different strains [1,10,11] (Fig. 1A). Recent studies in *Neisseria meningitidis* have proposed that the addition of *O*-linked glycan to pilin is performed by an *O*-oligosaccharyltransferase, PglL, which is homologous to *O*-antigen ligases that catalyse addition of *O*-antigen to lipopolysaccahride [12]. This is supported by subsequent work in *Neisseria gonorrhoeae* [13], and by the transfer of the *N. meningitidis* and *Pseudomonas aeruginosa* pilin *O*-glycosylation systems into *Escherichia coli* [14]. In this report we demonstrate that the pilin *O*-glycosylation system in pathogenic *Neisseria* is a general pathway for protein *O*-glycosylation.

Materials and methods

Bacterial strains and culture conditions. Neisseria meningitidis and *Neisseria gonorrhoeae* strains are listed in are Supplementary Tables 1 and 2 see [15]), and were grown on brain heart infusion medium (BHI) supplemented with Levinthal's base (and IsoVitaleX For *N. gonorrhoeae*).

Immunological analysis. Western blotting was performed essentially as previously described [8]. Primary antibodies used were rabbit anti-trisaccharide sera [8], rabbit anti-pilin sera [8], mouse anti-AniA monoclonal antibodies (mAb) [16], or mouse anti-FLAG mAb (Sigma-Aldrich). Secondary antibodies used were anti-rabbit IgG and anti-mouse IgG (Sigma-Aldrich). Whole cell ELISA was performed as described [17], with mouse anti-AniA mAb and HRP-conjugated anti-mouse IgG (Dako). Colony-immunoblotting was performed as previously described [18], using mouse anti-AniA mAb and anti-mouse IgG.

Enrichment of novel glycoproteins from outer membrane subcellular fractions. Outer membrane proteins were enriched essentially as described [19]. Solubilised proteins were enriched by anion exchange Q Sepharose™ Fast Flow (GE Healthcare) column.

In vivo nitrite utilisation assays. Cells were grown to OD 0.5 A_{600}, washed once in PBS containing 27.8 mM glucose, then resuspended to an OD of 1 in 10 ml of the same solution. After equilibration at 37 °C for 30 min, $NaNO_2$ was added to the cultures to a final

* Corresponding author. Fax: +61 733654620.
E-mail address: jennings@uq.edu.au (M.P. Jennings).
[1] These authors contributed equally to this work.
[2] Current address: Molecular Infectious Diaeases Group, Department of Paediatrics, University of Oxford,Oxford, United Kingdom OX3 9DU.

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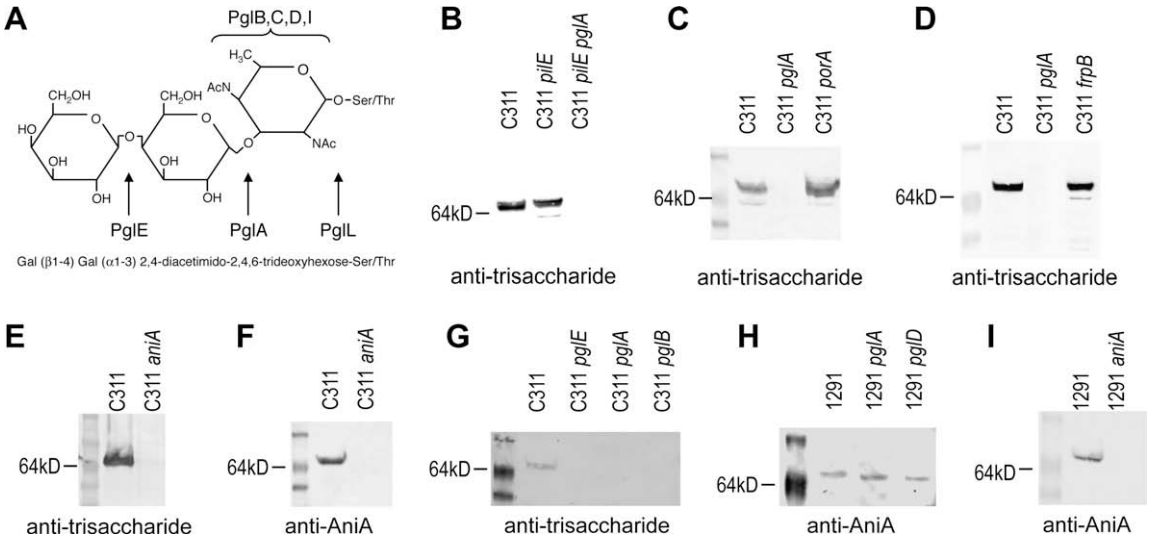

Fig. 1. Identification of AniA as a glycoprotein in *N. meningitidis* and *N. gonorrhoeae*. (A) The *N. meningitidis* trisaccharide and biosynthetic enzymes. Outer membrane proteins reactive with (B–E) anti-trisaccharide sera or (F) anti-AniA mAb. (G) Glycosylation of AniA in glycosylation pathway mutant strains. (H) *N. gonorrhoeae* strain 1291, glycosylation pathway mutant strains 1291*pglA* and 1291*pglD*; (I) strains 1291 and 1291*aniA* (anti-AniA mAb).

concentration of 1 mM. Nitrite utilisation was assayed colorimetrically with the Griess reagent [20] essentially as described [21].

Nucleotide sequence analysis of aniA from clinical isolates. The *aniA* gene was amplified and sequenced using primers aniA_upstm and aniA_downstm or primers aniA_F and aniA_R (Table 1). Multiple sequence alignment was performed with ClustalW (http://www.ebi.ac.uk/Tools/clustalw2/index.html), and a phylogenetic tree built with WorkBench (http://workbench.sdsc.edu/).

Construction of porA, frpB and aniA mutant strain in C311. The plasmid pIP52 (Dr. Ian Peak, unpublished) was used to inactivate *porA* in strain C311. The *frpB* gene was amplified using frpB14R and frpB11F, cloned into pGEM-T Easy (Promega), generating pGEM-*frpB*. The *aniA* gene in two fragments, using primers aniA_F and aniA5′XhoI_R and primers aniA_R and aniA5′XhoI_F, introducing a *XhoI* restriction site and cloned into pGEM-T Easy, generating pGEM-*aniA*(*XhoI*). pGEM-*aniA*(*XhoI*) and pGEM-*frpB* were digested with *XhoI* and ligated together with the isolated *SalI* digested fragment from pUC4Kan (Amersham Biosciences) containing the kanaymcin cassette. The *porA*, *frpB* and *aniA* mutant constructs were linearised and transformed into *N. meningitidis* strain C311 and *N. gonorrhoeae* strain 1291 as described previously [22]. The pUC4kan kanamycin cassette has no promoter or terminator that is active in *Neisseria* and does not affect transcription nor have a polar effect on expression of adjacent genes [22,23].

Construction and purification of FLAG-tagged AniA from N. meningitidis strain C311. Sequence encoding a FLAG-tag (N-Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys-C) was fused to the C-terminus of aniA of C311. Vector pT7blueaniA::flagtetMB was constructed by overlap PCR using the primers aniA_F, NMB1624_R, 3′flag_XhoI_ds and NMB1624_F_XhoI. The 3′flag_XhoI_ds primer encoded a FLAG-tag extension followed by a stop codon to allow incorporation of the tag in frame with aniA. The aniA::flag construct with NMB1624 (downstream of aniA) was initially cloned into pT7blue (Novagen) to create pT7blueaniA::flag. A tetracycline antibiotic resistant cassette (TetMB) was digested with SalI and cloned into XhoI linearised pT7blueaniA::flag to create pT7blueaniA::flag tetMB, which was transformed to the chromosome of C311 by homologous recombination as described above. Cells expressing AniA-FLAG were grown on BHI agar containing 1 μg/mL tetracycline and 1 mM of sodium nitrite at 37 °C for 24 h. AniA-FLAG protein was purified according to the manufacturer's instructions.

Mass spectrometry. Protein was reduced/alkylated with dithiothreitol/iodoacetamide. AniA-FLAG was precipitated by addition of 4 volumes of 1:1 acetone:methanol, incubation at −20 °C

Table 1
Primers used in this study.

Primer name	Sequence (5′–3′)
frpB14R	CGCATCGTGTTGAGCCACATGAAAG
frpB11F	TTGCGGCAGGTTTTGCCCACGC
aniA_F	ATGAAACGCCAAGCCTTAG
NMB1624_R	TTATCGGCTTGTGCAACGGAAGCCC
3′flag_XhoI_ds	AATAACCGGACATACTTCATCTCGAGTCACTTGTCGTCATCGTCCTTGTAGTCATAAACGCTTTTTTCGGATGCAGAGGC
NMB1624_F_XhoI	CTCGAGATGAAGTATGTCCGGTTATTTTTCC
aniA5′XhoI_R	TGAATGTGGAAGTACGGCCTCGAGCGGT
aniA_R	TAAACGCTTTTTTCGGATGCAG
aniA5′XhoI_F	ACCGCTCGAGGCCGTACTTCCACATTCA
aniA_R500	CCACATTCAGCTTCAAAGCCCTGCA
aniA_upstm	AACTACCCTGCCTTTGCCTGATT
aniA_downstm	CCGAGGAAAAATAACCGGACATAC

for 16 h, and centrifugation at 18,000 rcf for 10 min. Protein was dried, resuspended in 50 μL 50 mM NH_4HCO_3 with 1 μg trypsin (proteomics grade, Sigma), and digested at 37 °C for 16 h. Peptides and glycopeptides were analysed by LC–ESI–MS/MS with an API QSTAR Pulsar i LC/MS/MS system (Applied Biosystems). Samples were separated on a ZORBAX SB-C18 5 μm, 150 × 0.5 mm column equilibrated with 5% acetonitrile, 0.1% formic acid in water, and eluted using a gradient to 90% acetonitrile, 0.1% formic acid over 45 min. For AniA-FLAG, Analyst QS 1.1 software was used to manually examine LC–MS and MS/MS data for the presence of predicted peptides and glycopeptides.

Results and discussion

AniA is an O-glycoprotein

Analysis of outer membrane fractions from *N. meningitidis* by western blot with anti-trisaccharide sera [1] revealed a ~68 kDa band in strain C311 that was not present in the mutant strains such as C311*pglA* that are unable to synthesize the trisaccharide (Fig. 1B). As the pilin glycoprotein migrates at 17 kDa, this additional ~68 kDa band was a putative novel glycoprotein. The ~68 kDa band was still present in a C311*pilE* mutant strain in which the gene that encodes the pilin protein, *pilE*, has been inactivated, confirming that the glycoprotein was not caused by aberrantly migrating pilin (Fig. 1B). To identify the novel glycoprotein, anti-trisaccharide sera reactivity was followed through ion exchange chromatography and gel electrophoresis to enrich for the immunoreactive band. Proteins present in this enriched fraction were digested with trypsin and identified with mass spectrometry. Three candidate glycoproteins were thereby identified: PorA, the major outer membrane porin of *N. meningitidis* [24], FrpB, an outer membrane protein involved in iron uptake [25], and AniA an outer membrane protein with nitrite reductase activity [26–29] (Supplementary Table 3).

Mutations were made in each candidate gene and these mutant alleles were transferred to the chromosome using established methods [8]. The additional anti-trisaccharide sera-reactive protein was still present in the C311*porA* and C311*frpB* mutant strains (Fig. 1C and D, respectively), but was lost in the C311*aniA* mutant, suggesting that AniA was the additional glycoprotein (Fig. 1E). Using anti-AniA mAb [16] it was confirmed that the C311*aniA* mutant did not express AniA (Fig. 1F). Western blot analysis using anti-trisaccharide sera in a series of strains deficient in various steps in the pilin glycosylation pathway (pglE, pglA and pglB: see Fig. 1A) showed that AniA is glycosylated by the same pathway as pilin (Fig. 1G). Increased migration of AniA was seen in western blot analysis of *N. gonorrhoeae* wild-type (disaccharide, strain 1291) and mutant strains (monosaccharide, 1291*pglA*; and no glycan 1291*pglD*) (Fig. 1H and I). This indicates that AniA may also be a glycoprotein in *N. gonorrhoeae*.

AniA, a major anaerobically induced protein in *Neisseria*, is a nitrite reductase [27,29]. We tested if the *O*-linked trisaccharide was required for AniA nitrite reductase activity (conversion of nitrite to nitric oxide) in an *in vitro* nitrite utilisation assay. This analysis showed that the glycan was not required for AniA function (Fig. 4C).

AniA is glycosylated in the carboxy-terminal region

AniA purified from C311*pglA* was digested with trypsin and the resulting peptides were analysed by LC–ESI–MS/MS. Ions corresponding to peptides from throughout the AniA protein sequence were detected (Supplementary Table 4). C311*pglA* lacks the PglA galactosyltransferase which adds the second galactose to the *N. meningitidis* glycan, and so glycosylates with a single 2,4-diacetamido-2,4,6-trideoxyhexose (DATDH) monosaccharide [7] (Fig. 1A). The detected peptides included several containing predicted

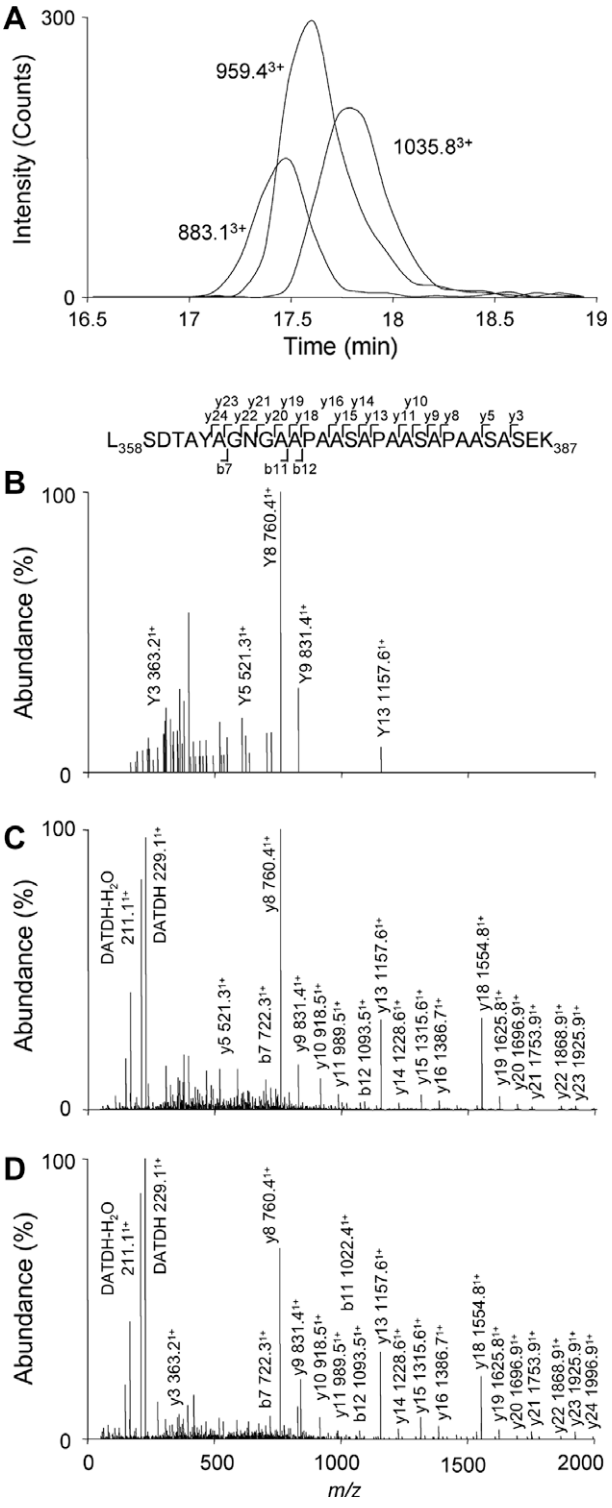

Fig. 2. Tryptic peptides and glycopeptides from FLAG-tagged AniA purified from *N. meningitidis* strain C311*pglA* analysed by LC–ESI–MS/MS. (A) Extracted ion chromatograms of ions corresponding to the tryptic peptide L_{358}-K_{387} (with N_{66} deamidated) and the same peptide with one or two DATDH monosaccharides (triple charged ions at *m/z* of 883.1, 959.4 and 1035.8, respectively). MS/MS spectra of (B) non-glycosylated, (C) mono- and (D) di-glycosylated L_{358}-K_{387} tryptic peptide.

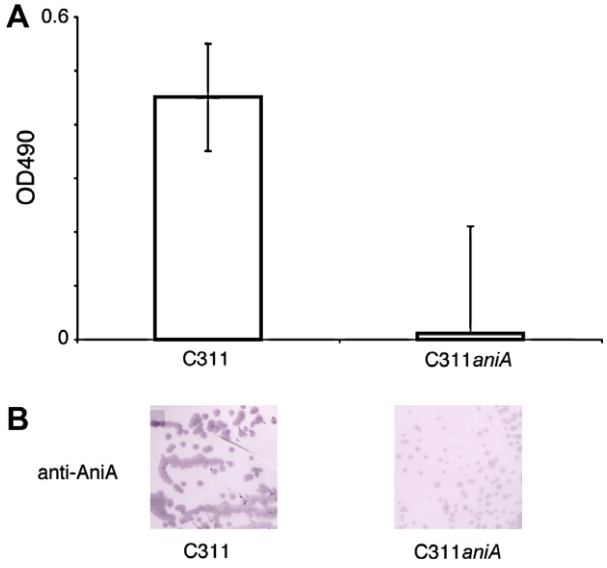

Fig. 3. Analysis of the surface expression of AniA by whole cell ELISA and immuno-colony blot. (A) Whole cell ELISA on AniA from wild-type C311 and mutant C311*aniA* using anti-AniA mAb. The values are the mean of triplicates. Error bar indicates ±1 standard deviation from the mean. (B) Immuno-colony blot using anti-AniA mAb with wild-type C311 and mutant C311*aniA*.

surface exposed serines and threonines, and hence potential substrates of the PglL *O*-oligosaccharyltransferase.

Ions corresponding to singly and doubly glycan-modified peptide were detected for the peptide L_{358}SDTAYAGNGAAPAAS APAASAPAASASEK$_{387}$, as well as unmodified peptide. MS/MS of these ions confirmed the expected peptide identity (Fig. 2B), and for the glycopeptides also confirmed the presence of DATDH modification (Fig. 2C and D). However, it was not possible to assign the modified serines or threonines within this sequence, due to the lability of the DATDH-Ser/Thr glycosidic bond. Nonetheless, the presence of these glycopeptides proves that AniA has covalently linked glycans.

The sequence of the glycosylated regions of PilE and AniA are not homologous. In PilE, the glycosylated S_{63} is located in a surface exposed but structured region of the protein in the sequence N_{60}NTS$_{63}$AGVA$_{67}$. In AniA, the glycosylated C-terminal region (L_{358}SDTAYAGNGAAPAASAPAASAPAASASEK$_{387}$) is outside the core nitrite reductase domain, and is not predicted to form secondary structural elements. Interestingly, a mutant strain not expressing the pilin glycoprotein shows additional glycosylation of AniA, resulting in an increase in apparent MW and reactivity to trisaccharide antisera (see Fig. 1B). This additional glycosylation could be due to increased occupancy of partially used glycosylation sites in the C-terminal peptide, or modification of further sites. The fact that AniA is glycosylated at all in a PilE expressing strain is clear evidence that it is a *bona fide* glycoprotein, and not an accidental substrate that is modified only when PilE is not being expressed. The additional glycosylation observed in the *pilE* mutant background also provides further evidence that these proteins are competing substrates for the same glycosylation pathway. The precise nature of the acceptor substrate specificity of the key component of this system, the PglL *O*-oligosaccharyltransferase [12], and other factors that influence competition between alternative substrates remains to be elucidated.

AniA is surface exposed and may be subject to immune selection

We identified the AniA glycoprotein in an outer membrane protein fraction (Supplementary Table 3). Whole cell ELISA (Fig. 3A) and colony immunoblotting (Fig. 3B) demonstrated that AniA is surface exposed, consistent with previous studies [26–28]), and is potentially subject to immune selection [30]. Sequence analysis revealed that 34% of clinical isolates surveyed contained a frame-shift mutation that results in premature termination of the protein. Typical examples are shown in Fig. 4A. Western blot analysis of two of the *N. meningitidis* strains containing the frame-shift mutation, 1000 and NGP20, revealed that neither express AniA (Fig. 4B) or reduce nitrite (Fig. 4C) suggesting that strains with the frame-shift mutation allele lack AniA expression. We measured the AniA phase variation rate to be less than 1:400,000 (data not shown). Together, this indicates that AniA expression has been lost in a significant minority of *N. meningitidis* isolates.

Multi locus sequence typing has been used to define the *N. meningitidis* population structure, which is comprised of distinct sequence types (STs) and clonal complexes [31]. The observed silencing frame-shift mutation (Supplementary Figs. 1 and 2; Fig. 4A) is present in a wide variety of clonal complexes (Fig. 4D), suggesting that *N. meningitidis* strains that do not express AniA are widespread, and that there may be selection against expression of AniA in *N. meningitides*. Clearly, AniA is not essential for colonisation or disease in this organism. During the preparation for the manuscript, Stefanelli et al. [32] also noted the same inactivating mutation in AniA (see Fig. 4A) in a survey of a large collection of *N. meningitidis* clinical isolates. In contrast, all of the 20 *N. gonorrhoeae* strains surveyed here have the wild-type allele, suggesting that AniA has a key role in *N. gonorrhoeae* biology.

Comparison of the deduced amino acid sequence of the 41 *N. meningitidis* strains revealed a number of sequence polymorphisms between strains (Fig. 4E and F; Supplementary Fig. 2). Examination of the position of these differences based on the structure of *N. gonorrheae* AniA [33] (red; Fig. 4G–I), showed that they are all surface exposed in the AniA trimer. Furthermore, theoretical translations of AniA from strains containing the frame-shifted allele revealed additional variant residues also on the surface of the protein (orange; Fig. 4G–I). The glycosylated C-terminal region is not included in the reported *N. gonorrhoeae* AniA crystal structure [33]. This glycosylated region would leave the core protein fold from a shallow cleft between AniA monomers (cyan C-$_{123}$ in Fig. 4G–I). This surface region lacked variable residues, and may bind the protein substrate of AniA [33]. The glycosylated C-terminal extension could shield this surface, protecting it from immune exposure.

In most other bacteria AniA is located in the periplasmic space and thus is not subject to immune selection. In the pathogenic *Neisseria* this protein appears to be surface exposed. Taken together the data presented here suggested that the protein may be under immune selective pressure. The silencing of the gene in many of *N. meningitidis* strains is consistent with immune selection against its expression, as are the amino acid sequence polymorphisms observed in key, surface exposed regions of the AniA protein.

The precise role that the glycosylation of pilin plays in the biology of pathogenic *Neisseria* has not been determined. However, pilin is under intense immune selection and is the archetypal example for antigenic variation. The addition of a phase variable glycan to this protein may serve as an additional immune evasion strategy. Therefore, the decoration of the surface exposed, flexible, C-terminal domain of AniA with the same phase variable *O*-linked glycan modification may also be an immune evasion strategy. Immune selection acting on the surface proteins of this host-adapted pathogen may have been the driving force for the evolution of this general *O*-glycosylation pathway.

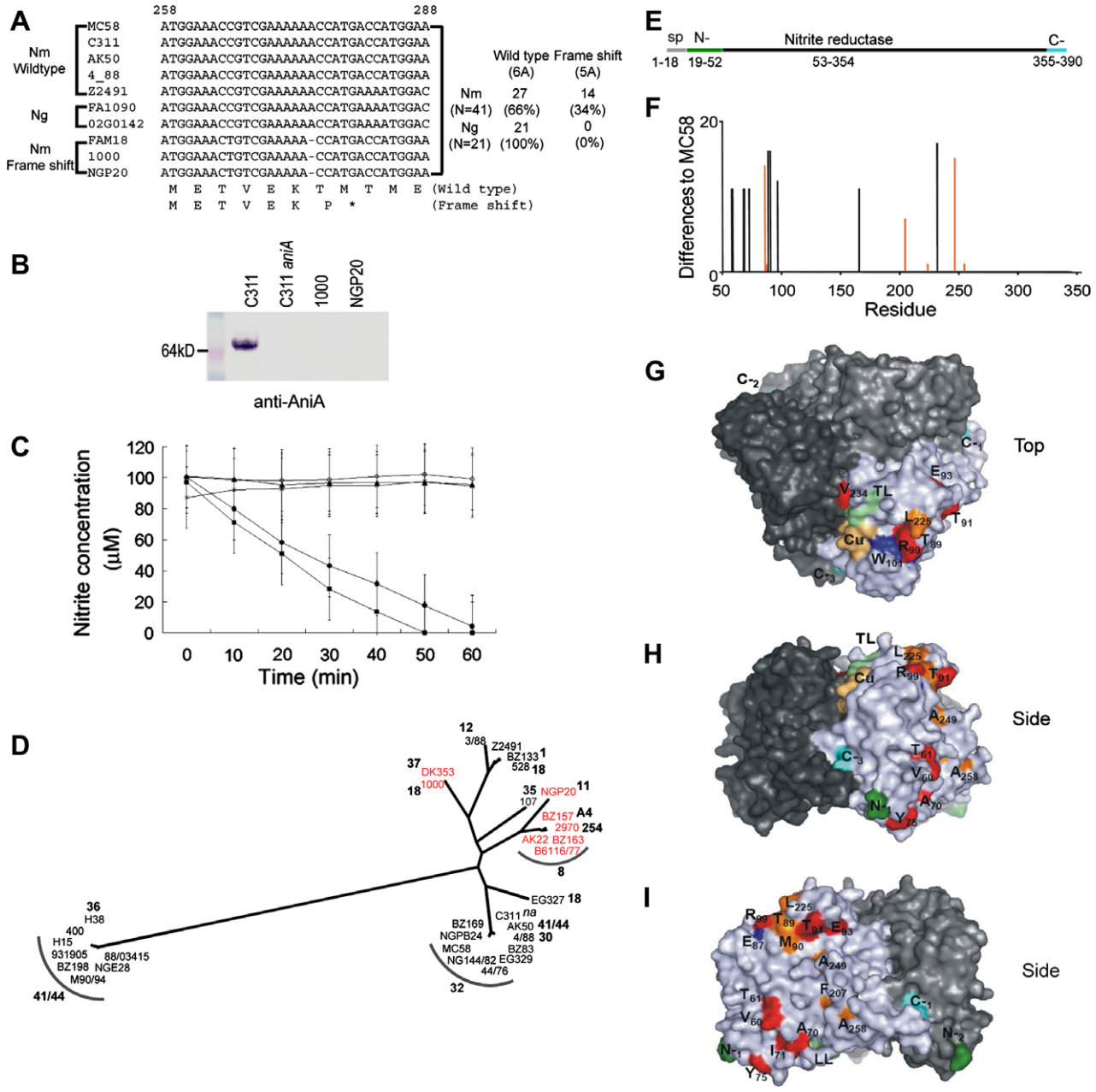

Fig. 4. Survey of sequence and expression of AniA. (A) Nucleotide sequence alignment of *aniA* from *N. meningitidis* and *N. gonorrhoeae* showing frame-shift mutation, and results of the survey of the frame-shift mutation allele in 41 *N. meningitidis* and 20 *N. gonorrhoeae* strains. (B) Western blot analysis of *N. meningitidis* strains C311, C311*aniA* and frame-shift strains 1000 and NGP20 with anti-AniA mAb. (C) Nitrite utilisation vs time for wild-type C311 (●), C311*pglA* (■), C311*aniA* (▲), and *N. meningitidis* frame-shift strains 1000 (×) and NGP20 (○). Results are the mean of triplicate independent biological samples. Error bars show ±1 standard deviation. (D) Phylogram of *aniA* nucleotide sequences from strains surveyed based on multiple sequence alignment (Supplementary Fig. 1). Red, strains with the silencing frame-shift mutation; Bold, ST complex type. (E) Variation in amino acid for each residue of *N. meningitidis* AniA (Supplementary Fig. 2). The coloured bar represents the full-length AniA protein; sp, cleaved signal peptide with palmitoylated cysteine (1–18, grey); N-,N-terminal repeat region (19–52, green); Nitrite reductase region included in the crystal structure of *N. gonorrhoeae* AniA [33] (53–354, black); C-, glycosylated C-terminal region (cyan). (F) Number of variants at each residue in the AniA core domain (53–354) comparing MC58 AniA and 40 *N. meningitidis* strains. Black, full-length proteins; orange, strains with frame-shift allele. (G–I) Surface representation of MC58 AniA trimer modelled on *N. gonorrhoeae* AniA [33]. Each monomer is shown in a different shade of grey. (G) Top view; (H) side view, rotated 90 from (G); (I) side view, rotated 90 from (H). Variable residues in sequenced *N. meningitidis* strains (red, wild-type; orange, frame-shift); green $N_{-1,2,3}$, N-terminal modelled residue, end of variable N-terminal repeat region; cyan $C_{-1,2,3}$, C-terminal modelled residue, start of glycosylated C-terminal region; yellow Cu, Copper centre (buried); pale green TL and LL, tower loop and Linker loop (deleted regions in *Neisseria* AniA); blue, proposed surface electron transport routes.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2008.11.025.

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